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EVALUACIÓN OBJETIVA DE LA MORFOMETRÍA DE LOS ESPERMATOZOIDES DE CIERVO. RELACIONES CON CONGELABILIDAD Y CALIDAD DEL SEMEN

por

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INTRODUCCIÓN GENERAL

El ciervo Ibérico

La especie *Cervus elaphus,* cuya amplia área de distribución abarca Europa, Asia y el Norte de África, se divide taxonómicamente en trece subespecies, alguna de las cuales, como el *C. elaphus hanglu*, se encuentra en grave peligro de extinción y el *C. elaphus wallichi* está extinguido. El ciervo Ibérico (*Cervus elaphus hispanicus*) está considerado en la actualidad como una única subespecie (Erxleben 1975). Se trata de una de las de menor tamaño de Europa, debido probablemente a su distribución en zonas de más baja latitud, con temperaturas más elevadas, lo que sin duda representa comportamientos bien diferentes del resto de las subespecies conocidas. Sin embargo, según Cabrera (1914) antiguamente se distinguían dos subespecies: *Cervus elaphus hispanicus*, que ocupaba el oeste del bajo Guadalquivir, y *Cervus elaphus bolivari*, de mayor tamaño, y que habitaba en las sierras del resto del cuadrante suroccidental de la Península Ibérica. Ambas compartían rasgos que las diferenciaban de las restantes subespecies europeas.

La cría del venado en nuestro país se desarrolla en libertad, siendo el aprovechamiento que se obtiene de él, el derivado del desarrollo de las actividades cinegéticas, que se fundamentan en la calidad de los trofeos de los machos. Actualmente, la caza del ciervo en España es practicada preferentemente en cotos que se encuentran delimitados por vallados. Esta práctica supone una fragmentación de la población original en poblaciones aisladas que contienen un número relativamente pequeño de individuos. Este hecho ha originado un incremento de las tasas de endogamia (Martínez *et al.* 2002), las cuales parecen ser, al menos parcialmente, las responsables de la disminución de la calidad de dichos trofeos. Por tanto, uno de los principales riesgos que conlleva la existencia de pequeñas poblaciones entre las que no existe flujo de animales nuevos es el aumento de la consanguinidad (Wildt *et al.* 1983). Estos efectos negativos de los vallados sobre la variabilidad genética de las poblaciones de animales han sido puestos de manifiesto también en ungulados africanos (Golber y Van Der Bank 1987). Los resultados obtenidos trabajando en tres especies de gacelas representan una importante aportación al estudio de los efectos de la consanguinidad sobre la calidad espermática, pues demuestran que la especie con niveles más elevados (*G. cuvieri*) experimenta una disminución en la proporción de espermatozoides normales y en la

motilidad de los mismos (Roldán *et al.* 1998), que son precisamente aquellas características del eyaculado más importantes de cara a mantener la capacidad fecundante (Malo *et al*. 2005). Esta relación no existe en las especies con niveles intermedios (*G. dama mohr*) y bajos (*G. dorcas*) de consanguinidad (Gomendio *et al.* 2000).

Además, la utilización de vallados cinegéticos en las fincas de caza ha hecho que las especies de caza mayor, consideradas como un bien ambiental, se hayan transformado en un bien económico ya que esta actividad ha tomado una orientación totalmente empresarial. Debido al intenso comercio que existe de animales para repoblación, ya sean granjas especializadas, cotos comerciales o capturaderos, una gran cantidad de ciervos se crían de forma controlada y pueden ser susceptibles de manipulaciones con objeto de aumentar la calidad de sus producciones.

Aplicación de las técnicas de Reproducción Asistida en las especies de cérvidos

La gran expansión que ha experimentado el aprovechamiento industrial de las distintas especies de cérvidos en los últimos veinte años ha venido acompañada por un rápido desarrollo y utilización de las tecnologías de Reproducción Asistida (Asher 1998). Estas tecnologías no solo han facilitado la difusión genética de los animales más productivos, sino que también han permitido de una forma relativamente sencilla, el movimiento de material genético alrededor del Mundo, han contribuido a la conservación de especies y subespecies en peligro de extinción (Wildt 2000; Berg y Asher 2003) y han permitido a los productores la obtención de híbridos altamente interesantes desde el punto de vista productivo (Willard *et al.* 1996).

La utilidad de las técnicas de Reproducción Asistida en el contexto de especies en peligro de extinción reside en el hecho de poder facilitar el manejo genético de poblaciones naturales y de los programas de cría en cautividad. Las especies amenazadas constituyen con frecuencia poblaciones pequeñas y aisladas, en las que hay falta de flujo genético. Por otra parte, los programas de cría en cautividad de especies en peligro de extinción cuentan, en general, con pocos individuos fundadores. En ambas situaciones son más frecuentes los cruces entre individuos emparentados, produciéndose un incremento de las tasas de consanguinidad.

Los animales consanguíneos son más vulnerables frente a parásitos y enfermedades y además sufren problemas reproductivos (Roldán *et al.* 1998) tal y como se ha señalado anteriormente.

En los cérvidos, de todas las técnicas de Reproducción Asistida existentes, la más ampliamente difundida ha sido la Inseminación Artificial (IA) (Asher 1998), seguida de la Transferencia de Embriones (TE) y de la Fecundación *in vitro* (FIV).

Actualmente, la IA se tiende a realizar mediante la utilización de semen congelado, teniendo una importancia mucho menor el uso del semen fresco o refrigerado, esto es debido a las grandes ventajas que presenta la criopreservación del material seminal sobre los demás sistemas de conservación seminal, como son:

- Conservación prácticamente indefinida en el tiempo
- Eliminación de riesgos sanitarios
- Racionalización económica del eyaculado
- Recogida de semen sólo en épocas reproductivas favorables
- Facilitar el testaje de los venados
- Permite el comercio internacional de dosis

Mantener los gametos masculinos a -196 ºC hace que disminuya el metabolismo celular, provocando un estado de inactividad total en las células espermáticas, del cual sólo se recuperan cuando son descongeladas y llevadas a temperatura ambiente (Watson y Morris 1987). Por ello, es un sistema ideal para conservar el semen, ya que su capacidad fecundante se mantiene en el tiempo de forma ilimitada. El mayor problema está en que algunos espermatozoides mueren durante el proceso (Watson y Morris 1987). Por ello ha habido que desarrollar nuevos y mejores protocolos para cada especie.

El ciervo Ibérico no está en peligro de extinción, pero existen algunos factores que pueden amenazarlo y por tanto que justifican la creación de una reserva de germoplasma en la subespecie:

• Es una forma de preservar la genética autóctona ante la creciente ola de hibridaciones con otras subespecies.

• En algunas fincas, y como consecuencia de las elevadas densidades de animales, existen importantes proporciones de animales afectados por enfermedades de relevancia en sanidad animal y para la salud pública, como la tuberculosis (Gortázar *et al*. 2005). Ante esta circunstancia, los bancos de semen pueden ayudar a minimizar los riesgos sanitarios de los traslados, o servir para conservar la riqueza genética ante operaciones de descaste selectivo.

• El interés principal de la explotación de los ciervos en nuestro país es el cinegético, el cual a su vez viene determinado por la calidad de los trofeos de los machos. Los bancos de germoplasma se han utilizado como una aplicación en la mejora genética de esta característica. El desarrollo de la criopreservación de material seminal procedente de machos con una cuerna altamente valorada permite, mediante la utilización de técnicas de Reproducción Asistida, la obtención de machos con un mejor trofeo. Esta última afirmación se basa en la alta heredabilidad encontrada para las características de la cuerna en este tipo de especies, siendo por tanto un carácter fácilmente transmisible de padres a hijos (Ball 1991).

Congelación del material seminal

Los medios de congelación empleados para el semen de ciervo poseen los mismos componentes que los usados en el resto de rumiantes. Así, Krzywinski y Jaczewski (1978) desarrollaron un medio de congelación constituido por glicerol, fructosa, citrato y leche descremada. Por otro lado, Jacobson *et al.* (1989) emplearon un medio compuesto por citrato, fructosa, Tris, yema de huevo y glicerol. Los niveles de glicerol utilizados más frecuentemente por los diferentes autores para la formulación de medios crioprotectores para el semen de ciervo están entre el 6 y el 8%.

En cuanto a la técnica de congelación, mencionar que se sigue la misma desarrollada para la congelación del semen de otros rumiantes. Así, para el envasado de las dosis en pajuelas, se realiza una dilución con la primera fracción del diluyente (la cual no suele llevar glicerol) a temperatura ambiente, depositándose el semen parcialmente diluido en

una cámara de refrigeración a 5 ºC, para que alcance dicha temperatura en dos horas. A continuación, y una vez que el semen ha alcanzado los 5 ºC, se añade la segunda fracción del diluyente (glicerolada) de forma fraccionada. Después de equilibrarse la muestra seminal a 5 ºC durante unas dos horas, se procede al llenado de las pajuelas que a continuación son congeladas sobre vapores de nitrógeno líquido, lo cual permite una congelación suave y controlada hasta los -100 ºC. Posteriormente, las dosis se almacenan definitivamente en nitrógeno líquido hasta su empleo (Ortiz 1999).

La descongelación de las dosis se realiza con frecuencia en un baño térmico a 37 ºC. Una vez descongeladas las dosis y antes de su utilización definitiva para la IA, es necesario evaluar la calidad de las mismas con objeto de evitar el empleo de dosis de mala calidad y/o bajo poder fecundante a la post-descongelación. Así, normalmente se suelen evaluar el porcentaje de espermatozoides móviles y el porcentaje de células espermáticas con acrosomas intactos, ya que ambos parámetros aportan datos importantes sobre la capacidad fecundante de las células espermáticas (Foote 1988).

Recientemente nuestro grupo de trabajo ha incorporado varias modificaciones a la técnica, que han permitido mejorar la calidad a la descongelación de las muestras espermáticas epididimarias de ciervo Ibérico (Fernández-Santos *et al.* 2006a; Fernández-Santos *et al.* 2006b; Fernández-Santos *et al*. 2006c; Fernández-Santos *et al*. 2006d).

Evaluación de la calidad seminal

El desarrollo de pruebas de laboratorio que fuesen capaces de predecir la fertilidad de las muestras espermáticas empleadas en inseminación artificial ha sido un tema de máximo interés para un gran número de estudios (Saacke 1982; Wood *et al.* 1986; Amann 1989; Amann y Hammerstedt 1993). Algunos de los cuales fueron llevados a cabo hace más de 60 años (Langerlof 1936). La evaluación de la motilidad espermática ha sido una de las pruebas más utilizadas para dicho fin (Budworth *et al.* 1988), sin embargo su relación con la fertilidad ha presentado siempre mucha controversia (Söderquist *et al.* 1991). Otros parámetros seminales como la viabilidad, el estado del acrosoma o la presencia de formas anormales, han sido usados también para este fin (Carrell *et al.* 1994). No obstante tampoco se ha encontrado, en general, ninguna relación clara entre éstas y la fertilidad de las dosis seminales. Para caracterizar adecuadamente

las muestras espermáticas con objeto de conocer su capacidad fecundante, debe recurrirse por un lado, al empleo de pruebas funcionales que evalúen los aspectos responsables de la interacción entre espermatozoides y ovocitos (o que las semejen, como la inducción de la reacción acrosómica por el A23187); y por otro a la utilización de métodos que sean capaces de determinar de forma objetiva las características de las células espermáticas.

En relación con el primer tipo de pruebas, recientemente se ha demostrado en la especie bovina la existencia de una buena relación entre las mismas y la fertilidad (Januskauskas *et al.* 2000); y en la especie canina (Szász *et al.* 2000) el buen valor predictivo de la reacción acrosómica inducida por el A23187 de la criosensibilidad de las dosis seminales.

En lo que se refiere al segundo grupo de pruebas, el reciente desarrollo de los sistemas automáticos para la evaluación objetiva de la morfometría (ASMA o CASA) de las cabezas de los espermatozoides ha proporcionado un método objetivo de análisis del semen que facilita la contrastación del mismo. Además, estos sistemas proporcionan una serie de parámetros de los espermatozoides que pueden ayudar a establecer los criterios necesarios para clasificar una muestra espermática de un macho, de una determinada especie como "normal" en base a las medidas de sus espermatozoides y a predecir la resistencia a la congelación y la fertilidad de las dosis seminales de la especie referida (Gravance *et al.* 1998b; Peña *et al.* 2005; Peña *et al.* 2006; Marco-Jiménez *et al.* 2006).

Morfometría espermática

Los primeros analizadores computerizados de morfología espermática (CASMA) aparecieron en el mercado en los años noventa del pasado siglo (Soler *et al.* 2003b). Uno de ellos, el Sperm-Class Analyser® (SCA), resultó útil en el análisis morfológico de las muestras de semen humano, tanto de la cabeza como de la pieza intermedia (Soler *et al.* 2003b). Los sistemas ASMA analizan de forma objetiva una serie de parámetros morfométricos que pueden ser usados para definir un espermatozoide con morfología "normal" de forma equivalente a como lo haría un técnico especializado. No obstante, la mayor ventaja de estos instrumentos no es la sustitución de los técnicos, sino el hecho de que aportan parámetros métricos objetivos que pueden reflejar de forma mucho más

interesante la realidad de la muestra seminal que la valoración subjetiva de lo que consideramos un espermatozoide normal (Katz *et al.* 1986; Soler *et al.* 2000).

Anteriormente al desarrollo de estos sistemas, la morfología espermática se determinaba mediante la observación al microscopio de las muestras previamente teñidas o fijadas. Un incremento de las anormalidades morfológicas de los espermatozoides de un eyaculado está asociado en humanos (Kruger *et al.* 1988), caballos (Jasko *et al.* 1990), machos cabríos (Chandler *et al.* 1988), toros (Saake 1970) y ciervos (Malo *et al.* 2005) con una disminución de las tasas de fertilidad. Además en los toros, se relaciona con variaciones estacionales de la fertilidad (Sekoni y Gustafsson 1987) y con problemas hereditarios de infertilidad (Hafez 1987). La morfología defectuosa de los espermatozoides también se ha asociado con pérdida embrionaria temprana (DeJarnette *et al.* 1992) y reducida capacidad para encontrar el ovocito (Kolt y Handel 1987). Sin embargo, en numerosas ocasiones no se ha encontrado una relación clara entre "normalidad" morfológica de la muestra espermática y fertilidad. Esta falta de relación entre ambos parámetros se ha atribuido a los resultados tan variables que se obtienen de la evaluación subjetiva empleada para la clasificación de la morfología espermática (Baker y Clarke 1987; Davis *et al.* 1995). Con objeto de reducir la subjetividad de este tipo de evaluaciones se desarrollaron, como ha quedado referido anteriormente, los sistemas CASMA, ASMA o CASA. Estos sistemas han sido aplicados para analizar de manera eficaz la morfometría de la cabeza espermática en hombre (Davis y Gravance 1993; Kruger *et al.* 1996), caballo (Davis *et al.* 1993), macho cabrío (Gravance *et al.* 1995), conejo (Gravance y Davis 1995), rata (Davis *et al.* 1994), toro (Gravance *et al.* 1996; Gravance *et al.* 1998b), morueco (Gravance *et al.* 1998a; Sancho *et al.* 1998) y cerdo (Hirai *et al.* 2001). Para ello, se han tenido que estandarizar previamente las condiciones de uso de los sistemas para las referidas especies. Además, en algunas de ellas, como la humana (Kruger *et al.* 1993), la equina (Casey *et al.* 1997) y la porcina (Hirai *et al.* 2001), se han encontrado relaciones significativas entre los valores de las dimensiones espermáticas, determinados por el sistema ASMA y las tasas de fertilidad. Esto ha permitido en estas especies establecer las medidas de los espermatozoides "normales" y "anormales" en base a la fertilidad de los donantes de los mismos. Por tanto, los sistemas ASMA son capaces de diferenciar las medidas espermáticas normales de los espermatozoides procedentes de machos fértiles, de las procedentes de machos subfértiles o estériles. Esto les ha permitido convertirse en una metodología de

evaluación muy interesante de cara a poder predecir la capacidad fecundante *in vivo* de las dosis seminales. Por último, mencionar que en las especies caprina (Gravance *et al.* 1997), bovina (Gravance *et al.* 1998b) y porcina (Thurston *et al.* 2001) se han encontrado variaciones de las medidas de las cabezas espermáticas determinadas por CASA entre el semen fresco y el semen descongelado, aunque no se ha evidenciado que estas variaciones de la morfometría puedan ser indicativas de la resistencia a la congelación en diferentes individuos de una misma especie.

Por todo ello, y en base a las ventajas que estos sistemas presentan de cara a la evaluación objetiva del semen, nuestro laboratorio incorporó en 1999 el sistema automático de análisis de la morfometría espermática Sperm-Class Analyzer® (SCA) para la evaluación rutinaria de las dosis seminales de las diferentes especies que procesamos.

Este sistema incorpora de forma comercial los parámetros necesarios para evaluar los espermatozoides de hombre, toro y caballo. Sin embargo, hasta el momento no se ha utilizado para evaluar la morfometría de las células espermáticas de ciervo. En esta especie, además los estudios sobre evaluación seminal han sido muy reducidos y se han llevado a cabo en su mayor parte por nuestro grupo de trabajo (Garde *et al.* 1997; Garde *et al.* 1998; Malo *et al.* 2005) y por el grupo de Reproducción y Obstetricia de la Universidad de León (Martínez-Pastor *et al.* 2005; Martínez-Pastor *et al.* 2006).

Efectos de la congelación sobre la morfometría de la cabeza de los espermatozoides

La criopreservación implica daños en el espermatozoide (Mazur 1984). Estudios previos que han utilizado ASMA han demostrado que este proceso afecta a la morfometría de la cabeza espermática de hombre (Thompson *et al.* 1994), toro (Gravance *et al.* 1998b), caballo (Arruda *et al.* 2002) y perro (Rijsselaere *et al.* 2004). En estos trabajos, las cabezas espermáticas fueron significativamente más pequeñas en las muestras congeladas que en las frescas. Las diferencias en las dimensiones morfométricas entre semen fresco y congelado se han explicado mediante distintos mecanismos, incluyendo la progresiva deshidratación de las células durante el proceso de criopreservación (England 1993) y la formación y eliminación de cristales de hielo (Peña

et al. 2005), el daño producido en el acrosoma y la posterior pérdida del contenido acrosomal (Gravance *et al.* 1998b), los cambios en la estructura de la cromatina (Royere *et al.* 1988) y la pérdida de la funcionalidad y de la integridad de la membrana. Todo ello da lugar a muerte celular y a la modificación de la capacidad de respuesta frente al estrés o al mantenimiento del volumen isotónico por parte de la célula espermática (Petrunkina *et al.* 2004). Además, los espermatozoides que han muerto presentan un tamaño de cabeza menor que los vivos (Marco-Jiménez *et al.* 2006), debido probablemente a que han perdido parte del contenido intracelular. En un primer momento, durante la fase de equilibración, la célula se comprime como consecuencia de la salida de agua al medio extracelular y posteriormente se hincha como respuesta al paso del agua y de los crioprotectores al interior (Agca *et al.* 2002). Sin embargo, en la congelación la célula vuelve a reducirse, debido a la salida de agua al exterior. Estas observaciones sugieren que las dimensiones de la cabeza espermática de muestras individuales de semen pueden ser un indicador de la criosupervivencia espermática.

Utilidad de la morfometría espermática para predecir la congelabilidad de una muestra.

Las diferencias en la resistencia al proceso de congelación-descongelación de los espermatozoides entre distintos individuos han sido observadas en varias especies, tanto domésticas (Curry 2000) como silvestres (Leibo y Songsasen 2002) basadas principalmente en la naturaleza de la membrana plasmática. En este contexto, los donantes de semen se han clasificado de forma rutinaria como “buenos” o “malos” congeladores de forma individual. Los mecanismos que clasifican a los individuos según la sensibilidad a la congelación de sus espermatozoides todavía no han sido desvelados, pero parece que existen diferencias fisiológicas entre los espermatozoides de diferentes machos dentro de una misma especie. Recientemente se ha demostrado que estas constantes variaciones inter-individuales en la congelabilidad espermática están determinadas genéticamente y puede relacionarse con el proceso de formación del espermatozoide que ocurre durante la espermatogénesis (Thurston *et al.* 2002). Los machos no pueden clasificarse como “malos” congeladores basándonos sólo en que los parámetros convencionales de calidad seminal se han visto muy perjudicados debido a la congelación. Pruebas recientes sugieren que las dimensiones de la cabeza espermática de una muestra individual en semen de cerdo pueden ser indicadoras de la criosupervivencia

espermática (Thurston *et al.* 2001; Peña *et al.* 2005). El eyaculado con la mayor proporción de espermatozoides con la cabeza ligeramente afilada presenta mayor resistencia al proceso de criopreservación. Es posible que el tamaño de la cabeza espermática influya sobre el volumen total de la célula dando lugar a alteraciones en el intercambio de agua, iones y crioprotectores. Hasta el momento no existen datos relativos a la morfometría espermática y su eventual relación con la congelabilidad del semen en las distintas especies de ciervo, por ello, en este trabajo trataremos de abordar esta cuestión en muestras epididimarias de ciervo Ibérico.

Identificación de subpoblaciones espermáticas

Conociendo la gran variabilidad entre machos que existe, parece razonable plantear la existencia de diferentes subpoblaciones espermáticas dentro del semen de especies tanto domésticas como silvestres. La existencia de subpoblaciones espermáticas según la motilidad dentro del eyaculado de mamíferos está ampliamente aceptada en la actualidad por la comunidad científica (Quintero-Moreno *et al.* 2003; Martínez-Pastor *et al.* 2006; Núñez-Martínez *et al.* 2006). Su origen no está del todo claro, aunque algunos autores piensan que es debido a los procesos de ensamblado de los espermatozoides durante la espermatogénesis, su diferente grado de maduración y el tiempo que han permanecido en la cola del epidídimo hasta el momento de la eyaculación (Abaigar *et al.* 1999).

Los estudios de subpoblaciones espermáticas a partir de la morfometría son escasos. Se recogen algunos de ellos en las especies porcina (Hirai *et al.* 2001; Thurston *et al.* 2001; Peña *et al.* 2005) y canina (Núñez-Martínez *et al.* 2006) basados en la relación que existe entre la aparición estas subpoblaciones y la resistencia a la criopreservación del semen, aunque no hay datos en otras especies.

En estudios anteriores, utilizando CASMA, se ha demostrado que la proporción de subpoblaciones espermáticas morfológicamente distintas dentro de un eyaculado está relacionada con la calidad del semen valorada después de la criopreservación (Thurston *et al.* 2001). Así, en esta Tesis Doctoral se planteará la identificación de subpoblaciones espermáticas según su morfometría, su evolución y su distribución tras el proceso de criopreservación en muestras epididimarias de ciervo Ibérico.

OBJETIVOS

- Determinar los efectos de la criopreservación sobre la morfometría de la cabeza de los espermatozoides epididimarios de ciervo Ibérico, utilizando un sistema ASMA.

- Evaluar la criosupervivencia espermática analizando la motilidad individual y la integridad de membranas tras la descongelación a partir de muestras recogidas de epidídimo de ciervo Ibérico.

- Determinar si el área y el perfil de la cabeza espermática son medidas útiles para predecir la congelabilidad de las muestras espermáticas epididimarias de ciervo Ibérico.

- Identificar subpoblaciones espermáticas morfométricamente diferentes, analizar su comportamiento tras el proceso de criopreservación y su relación con la congelabilidad en muestras epididimarias de ciervo Ibérico.

- Evaluar los efectos de dos protocolos diferentes de descongelación sobre el tamaño de la cabeza de los espermatozoides epididimarios de ciervo Ibérico y su distribución en distintas subpoblaciones espermáticas en base a las referidas dimensiones de la cabeza espermática.

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CAPÍTULO 2

Efectos de la congelación sobre la morfometría de la cabeza de los espermatozoides epididimarios de ciervo Ibérico

RESUMEN

En este estudio se utilizó el sistema Sperm-Class Analyzer® (SCA) para determinar el efecto del proceso de congelación-descongelación sobre la morfometría de la cabeza de los espermatozoides epididimarios de ciervo Ibérico. Las muestras espermáticas que se recogieron de 40 machos abatidos en monterías, fueron diluidas a temperatura ambiente en un medio Tris-citrato-20% yema de huevo-6% glicerol. Se realizó una preparación para microscopía antes de la congelación a partir de las muestras individuales y el resto fue congelado en vapores de nitrógeno. Tras la descongelación, también se tomaron muestras para morfometría espermática. Todos los frotis se secaron al aire y se tiñeron con Hemacolor. Se determinaron las dimensiones de la cabeza espermática para longitud, anchura, área, perímetro y perfil (long/anch) de al menos 145 espermatozoides en cada macho, mediante el SCA. En primer lugar, nuestros resultados revelan que la congelación reduce de forma sustancial ($p < 0,001$) la motilidad individual y la integridad de las membranas, tanto plasmática como acrosomal. Las cabezas de los espermatozoides de ciervo fueron significativamente más pequeñas en las muestras congeladas que en las frescas para el área (32,05 μm^2 *vs.* 32,56 μm^2; $p < 0,05$), la longitud (8,46 µm *vs.* 8,53 µm; $p < 0,0001$) y el perfil (1,833 *vs.* 1,849; $p < 0,0001$) en la mayoría de los machos analizados. Sin embargo, en el 25% de ellos no se produjeron estos cambios. En estos últimos machos, el tamaño de los espermatozoides en lasmuestras sin congelar fue significativamente menor y los parámetros de calidad seminal (SMI y viabilidad) a la descongelación tendieron a ser más altos, que en el resto de los machos que sí cambiaron sus dimensiones espermáticas con el proceso de congelación-descongelación. La variabilidad individual (CV) de las medidas de las cabezas espermáticas en las muestras frescas está correlacionada de forma negativa ($p < 0,005$) con el porcentaje de cambio en las medidas después de la congelación para el área ($r = -0,465$), la anchura ($r = -0,483$) y el perímetro ($r = -0,375$). Por ello, los resultados de este estudio sugieren que la variabilidad (heterogeneidad) en la morfometría de la cabeza espermática en el ciervo Ibérico de forma individual puede ser un buen indicador de la congelabilidad de su semen.

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The Effects of Cryopreservation on the Morphometric Dimensions of Iberian Red Deer (*Cervus elaphus hispanicus*) Epididymal Sperm Heads

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Contents

Computer-automated sperm-head morphometry was used in this study to determine the effects of cryopreservation on red deer sperm-head morphometry. Epididymal sperm samples were collected from 40 mature stags and were divided. One portion was diluted at room temperature in a Tris-citrate egg yolk medium, containing 6% glycerol. A microscope slide was prepared from single extended sperm samples prior to freezing. The remainder of each sample was frozen in nitrogen vapours. After thawing, sperm smears were prepared as described above. All slides were air dried and stained with Hemacolor. The sperm-head dimensions for length, width, area, perimeter and shape factor (length/width), for a minimum of 135 spermatozoa were determined for each slide by means of the Sperm-Class Analyser® (SCA). Firstly, our results show that cryopreservation substantially reduced ($p < 0.001$) sperm motility and plasma membrane and acrosome integrities. In addition, sperm heads were significantly smaller in cryopreserved spermatozoa than in the companion extended samples for area (32.05 μm^2 vs 32.56 μm^2; $p < 0.05$), length (8.46 μm vs 8.53 μm; $p < 0.0001$) and shape factor (1.833 vs 1.849; $p < 0.0001$) for all stags. These differences were found within 29 of 40 stags (75%) for at least three of the morphometric parameters. The individual variability (CV) of sperm head measurements from extended samples was negatively correlated ($p < 0.005$) with the per cent of change in sperm head measurements after cryopreservation for area ($r = -0.465$), width ($r = -0.483$) and perimeter ($r = -0.375$). Thus, the lower the sperm head variability in the extended samples, the greater the sperm change as a consequence of the cryopreservation. These results suggest that the variability (heterogeneity) in sperm head dimensions of individual stags may be a good indicator of sperm freezability.

Introduction

The routine evaluation of semen, including normal sperm morphology assessment, has long been employed to evaluate the effects of freezing-thawing procedures on sperm cryosurvival. Poor semen morphology is an important indicator of decreased fertility in men (Kruger et al. 1993); stallions (Jasko et al. 1990) and bulls (Sekoni and Gustafsson 1987). Sperm head abnormalities have been associated with early embryonic loss, lowered fertility and embryo quality (DeJarnette et al. 1992) and reduced capacity to bind to the ovum (Kolt and Handel 1987). Although normal sperm morphology may be an indicator of the fertility potential of a given male, correlations have been based on subjectively performed analyses. However, large variations between technicians and laboratories in the subjective evaluation of semen characteristics are known to exist (Saacke 1982), making accurate interpretation of the resulting data difficult.

The need for accurate objective assessment of sperm morphology has led to the development of computer-assisted sperm morphometry analysis (ASMA) (Katz et al. 1986; Pérez-Sánchez et al. 1994). The precision of the ASMA system has been utilized to detect morphometric differences in sperm head dimensions of fertile and subfertile males (Casey et al. 1997) as well as subtle changes in human sperm head morphometry because of toxicant exposure when no morphological differences were detected by manual assessment (Davis et al. 1993). Previous studies utilizing ASMA have also demonstrated that cryopreservation affects head morphometry of bull (Gravance et al. 1998), human (Thompson et al. 1994), stallion (Arruda et al. 2002) and dog (Rijsselaere et al. 2004) cryopreserved spermatozoa. In these studies, sperm heads were significantly smaller in cryopreserved spermatozoa than in fresh-extended sperm. The differences in morphometric dimensions between fresh and cryopreserved spermatozoa have been explained by several possible mechanisms including osmotic changes, acrosome damage and alterations in chromatin condensation (Gravance et al. 1998). These observations, suggest that sperm head dimensions of individual sperm samples, may be an indicator of sperm cryosurvival.

Thus, adopting methods previously utilized in other species (Sancho et al. 1998) and in red deer by us (Esteso et al. 2003), our objective was to determine the effects of cryopreservation on red deer sperm-head morphometry. To achieve this goal, we firstly evaluated the effects of cryopreservation on sperm motility and on acrosome and membrane integrities. Secondly, we evaluated the effects of cryopreservation on epididymal red deer sperm head size. In relation with this second general aim, we also carried out this study: (i) to determine whether the effects vary between individual stags and (ii) to determine which sperm head morphometric measurements, if any, are associated with changes that occur to the sperm during freezing and thawing.

Material and Methods

Materials

With the exception of DPX (Fluka, Madrid, Spain), all other chemicals were of reagent grade and were purchased from Sigma or Merck (both of Madrid, Spain).

Preparation of testes and collection of epididymal spermatozoa

For this study, we used spermatozoa recovered from the epididymides of 40 mature stags (age >4 years, weight >140 kg) that were legally culled and hunted in their natural habitat during the rutting season (September to November). The hunting of stags was performed in accordance with the harvest plan of each game reserve. The harvest plans were made following Spanish Harvest Regulation, Law 2/93 of Castilla-La Mancha, which conforms to European Union Regulation. Immediately upon removal, the testes with attached epididymides were placed into plastic bags and transported to the laboratory at room temperature (approximately 20°C) within 2 h after being removed. Samples were processed as soon as they arrived at the laboratory. Epididymal spermatozoa were collected as described by Soler et al. (2005). Epididymal contents from both testicles of an individual stag were pooled for processing, because an ejaculate contains a mixture of spermatozoa from both testicles.

Semen processing and cryopreservation

The sperm mass was diluted at room temperature to a final sperm concentration approximately 400×10^6 sperm/ml with a Tris-citrate-20% egg yolk medium, containing 6% glycerol (Fernández-Santos et al. 2005). At this point, sub-samples were taken for sperm head morphometric dimensions evaluation. Then, the sperm diluted was placed in a 15-ml centrifuge tube (Iwaki, Tokyo, Japan) and was slowly cooled to 5°C. For it, the tubes were placed in a beaker with water (75 ml at room temperature) and transferred to a refrigerator at 5°C. Cooling down to 5°C lasted for approximately 1 h and then extended samples were held for equilibration at that temperature for 2 h more. After the equilibration of the diluted sperm samples, the extended sperm was loaded into 0.25 ml plastic straws. Immediately, the straws were frozen in nitrogen vapours, 4 cm above the surface of the liquid nitrogen, for 10 min and then plunged into liquid nitrogen. The straws remained for a minimum period of 1 month in liquid nitrogen before thawing was carried out.

Frozen semen was thawed in a water bath (37°C) for 20 s and the content of the straws poured into a glass tube. Samples were evaluated for motility, viability, and acrosome and membrane integrities after 5 min of incubation at 37°C, using the methods described below. At thawing, subsamples were also taken for sperm head morphometric evaluation.

Semen evaluation

Sperm concentration and subjective scores of motility were assessed shortly after collection. Sperm concentrations of the original suspensions were determined using a haematocytometer. Percentage of individual motile sperm (motility) was noted and quality of motility was assessed using a scale of 0, lowest, to 5, highest. A Sperm Motility Index (SMI) was calculated = [% individual motility + (quality of motility × 20)] × 0.5. The sperm suspension was also used to assess acrosome integrity and viability. Acrosomal integrity was evaluated after a 1 : 1 dilution in 2% glutaraldehyde in 0.165 M cacodylate/HCl buffer (pH 7.3). The percentage of spermatozoa with intact acrosomes [% normal apical ridge (NAR)] was assessed by phase-contrast microscopy. Only samples with an initial sperm motility and NAR >65–70% were used for freezing.

In addition, samples were taken to assess the membrane integrity by means of the hypo-osmotic swelling (HOS) test. Plasma membrane functionality was assessed using a HOS test as described by Garde et al. (1998). The sperm membrane was considered functional in cases where the sperm tail was coiled and the result was expressed as HOS (%).

Membrane integrity (viability) was also evaluated by using a nigrosin-eosin (NE) stain. The NE stain was prepared according to the method by Soler et al. (2005). Live spermatozoa remained unstained, while dead cells were dull pink. The percentage of live spermatozoa was expressed as viability (%).

Two hundred sperm cells were assessed in each sample and for each sperm evaluation technique. Additionally, slides of extended and thawed semen were prepared from each sample for sperm head morphometric characterization.

Morphometric analysis of sperm heads

Microscopic slides were prepared from each extended (upon dilution) and cryopreserved sample by placing 5 μl of the sperm samples on the clear end of a frosted slide and dragging the drop across the slide. Semen smears were air dried and stained using a Hemacolor (Merck) procedure, originally described for staining of ram (Sancho et al. 1998) sperm heads; and recently adapted by our group to red deer spermatozoa (Esteso et al. 2003). Stained sperm samples were permanently mounted to the slide with a coverslip and dibutyl phthalate xylene (DPX).

Stained slides were used to perform ASMA using the morphometry module of a commercially available system (Sperm-Class Analyzer®; Microptic, Barcelona, Spain). The machine was equipped with a Nykon (Labophot-2, Tokyo, Japan) microscope with a ×60 bright-field objective and a Sony video camera (CCD AVC-D7CE; Sony Corporation, Tokyo, Japan) connected to a Pentium 950 MHz processor. The illumination source was centred and the intensity of the bulb and the gain and offset of the camera were standardized for all samples. The configuration of the computer system included a PIP-1024 B video digitizer board (Matrox Electronic Systems Ltd., Quebec, Canada), the sperm image analysis software and a high-resolution assistant monitor Sony Triniton PVM-1443MD (Sony Corporation). The array size of the video frame recorder was 512 × 512 × 8 bits, digitized images were made up of 262 144 pixels (picture elements) and 256 grey levels. Resolution of images was 0.15 and 0.11 μm per pixel in the horizontal and vertical axes, respectively.

The morphometric dimensions for area (A), perimeter (P), length (L), width (W), and shape factor (L/W) were

acquired for 140–150 images. Acquiring 140–150 images assures that a minimum of 135 properly measured sperm heads are analysed after improperly measured sperm heads are deleted from the analysis. The sperm cells were randomly selected for the morphometric analysis. The measurements of each individual sperm head from each stag and sperm treatment were saved in an Excel® (Microsoft® Corporation, Redmond, Washington, USA) – compatible database by the software for further analysis.

Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 11.0 (SPSS Inc, Chicago, IL, USA). The effects of cryopreservation on sperm motility, viability, acrosome and membrane integrities and sperm head morphometric dimensions were compared within and across stags by General Linear Model analysis of variance (GLM-ANOVA) using a split plot design. Stags served as the main plot and sperm cryopreservation step (pre-freezing or post-thawing) served as the subplot. Group differences were compared by Fisher's Least Significant Differences test. Effects were considered significant at $p < 0.05$. The effects of sperm treatment on sperm-head morphometry within stags, where effects were observed, were compared by Student's *t*-test. The within analysis variation (CV) and between stags variation within morphometric measures were compared between sperm treatments by the Mann–Whitney two-sample test. Correlations between sperm head measurements (values and CVs) and the standard semen parameters (motility, viability, HOS and NAR) after cryopreservation were performed by Spearman's rank correlation coefficients. Data that did not follow a normal distribution were transformed.

Results

In this work, epididymal spermatozoa from 40 stags were frozen and thawed. Because we used epididymal spermatozoa for this experiment, it was only possible to make single observations for each stag. After freezing and thawing, a decrease ($p < 0.001$) in all routine sperm parameters was observed. Thus, SMI decreased from 74.51 ± 2.2% in extended fresh samples to 63.88 ± 1.7% in thawed samples. Spermatozoa with normal acrosomes decreased ($p < 0.001$) from 82.15 ± 2.3% in extended samples to 62.79 ± 2.1% in thawed samples. The results found for the other parameters (HOS test and viability) were similar if not identical (data not shown).

The results of the comparison of sperm heads dimensions between extended and cryopreserved samples are summarized in Table 1. This table shows the mean sperm head measurements and the CVs between stags for extended and cryopreserved samples. A total of 5,451 property digitized spermatozoa for extended samples, and 5,416 for cryopreserved samples were analysed. There were no differences in the number of properly analysed sperm heads between extended and cryopreserved samples (data not shown). The values for all measures of sperm heads dimensions were determined to be normally distributed by Kolmogorov–Smirnov normality test. Analysis of variance showed a significant ($p < 0.001$) within-stag effect on sperm head morphometric dimensions in the extended and cryopreserved samples. Besides, significant effects ($p < 0.05$) of cryopreservation were found among stags on sperm head dimensions. In this sense, sperm heads were smaller in cryopreserved samples than in the companion extended samples for area (32.05 μm^2 vs 32.56 μm^2, $p < 0.05$), length (8.46 μm vs 8.53 μm, $p < 0.0001$) and shape factor (1.833 vs 1.849, $p < 0.0001$) between all males. The variation (per cent CV) of the mean dimensions across all stags ranged from 6.6% (L) to 9.6% (A) for extended samples, and 6.8% (L) to 9.6% (A) for cryopreserved samples (Table 1).

Table 1. Mean sperm head measurements of area (A), perimeter (P), length (L), width (W) and shape factor (L/W) for extended and cryopreserved sperm samples from 40 stags

	Parameters					
Sample	A (μm^2)	P (μm)	L (μm)	W (μm)	Shape factor	N
EXT	32.56 (9.6)[a]	30.05 (7.9)[a]	8.53 (6.6)[c]	4.63 (7.2)[a]	1.849 (8.4)[c]	5,451
CRYO	32.05 (9.6)[b]	30.00 (7.7)[a]	8.46 (6.8)[d]	4.63 (7.2)[a]	1.833 (8.8)[d]	5,416

EXT, extended sperm sample; CRYO, cryopreserved sperm sample; N, total number of sperm counted per treatment.
Coefficients of variation (% CV) between stags are shown in parentheses.
Values with different superscript letters in the same column were significantly different. [a, b] $p < 0.05$, [c, d] $p < 0.0001$.

Our results also revealed that all sperm head measurements were significantly ($p < 0.001$) affected by the interaction individual factor-sperm treatment. In this sense, no significant cryopreservation effect was found between the extended samples and the thawed ones for sperm head dimensions within stags for all males. Thus, in 29 of the 40 stags (75%), differences ($p < 0.05$) were observed in at least three of the five morphometric parameters between extended and cryopreserved samples. The per cent change in sperm head measurements from extended and cryopreserved samples of the eleven stags that showed no differences (ND) and the 29 males where differences (DIF) occurred, were different ($p < 0.0001$) for area (−5.6 vs 3.53%), length (−2.1 vs 2.5%) and width (−3.7 vs 1.8%). No significant differences in any sperm head dimensions were detected in cryopreserved samples when the ND group of stags was compared with the DIF group. Contrary, significant differences were found in sperm head dimensions in extended samples when the ND group was compared with the DIF group for area (ND = 31.43 μm^2 vs DIF = 33.13 μm^2; $p = 0.026$) and length (ND = 8.41 μm vs DIF = 8.66 μm; $p = 0.024$). No significant differences in the variability of any sperm head dimensions were detected in extended or cryopreserved samples when the ND group of stags was compared with the DIF group. Finally, at thawing SMI (ND: 68.87 ± 3.1% vs DIF: 61.59 ± 2.0%, $p = 0.06$) and sperm viability (ND: 70.25 ± 3.1% vs DIF: 63.22 ± 2.1%, $p = 0.06$) tended to be higher for the stags that did not incur changes in the morphometric parameters of the sperm head than for those did it, but differences were not statistically significant.

Table 2. Mean within analysis coefficients of variation (%) for area (A), perimeter (P), length (L), width (W), and shape factor of sperm heads of extended and cryopreserved sperm samples from 40 stags

	Parameters					
Sample	A	P	L	W	Shape factor	N
EXT	6.20[a]	6.20[a]	5.12[c]	5.41[a]	6.85[a]	5,451
CRYO	7.02[b]	6.48[a]	5.50[d]	5.50[a]	6.80[a]	5,416

EXT, extended sperm sample; CRYO, cryopreserved sperm sample; N, total number of sperm counted per treatment.
Values with different superscript letters in the same column were significantly different. [a, b]$p < 0.005$, [c, d]$p < 0.05$.

Besides, significant differences ($p < 0.05$) in the means within analysis CVs were found between the extended and cryopreserved samples for area and length among stags (Table 2). In this sense, the variability of sperm heads measurements was lower in extended semen samples than in cryopreserved ones for area (6.20% vs 7.02%, $p < 0.005$) and length (5.12% vs 5.50%, $p < 0.05$) when all samples were analysed. Finally, the results of the ANOVA procedure revealed that within analysis CVs were significantly ($p < 0.001$) affected by the interaction between individual factor and sperm treatment. Overall, the results from the 40 stags showed a similar pattern of response, with higher ($p < 0.05$) variability (per cent CV) in cryopreserved samples than in the extended ones. However, when we analyses only the 11 stags of the ND group, no significant differences ($p > 0.05$) in the means within analysis CVs were found between the extended and cryopreserved samples for any sperm head measurements.

The per cent difference in individual parameters of head measurements of spermatozoa from extended and cryopreserved samples for all stags was negatively correlated ($p < 0.005$) with the CVs of the corresponding measurements for area ($r = -0.465$), width ($r = -0.483$) and perimeter ($r = -0.375$) of the initial extended sample. These results are showed in Fig. 1 for sperm head area. These relations were not found with the CVs of the sperm heads dimensions for the cryopreserved samples.

Finally, the per cent difference in individual parameters of head measurements of spermatozoa from extended and cryopreserved sperm samples for all stags was not correlated ($p > 0.05$) with any sperm routine parameters determined in extended or cryopreserved samples. Besides, the variations (CV) of sperm head dimensions of spermatozoa from extended samples were not related to standard sperm parameters determined in cryopreserved samples when all stags were analysed.

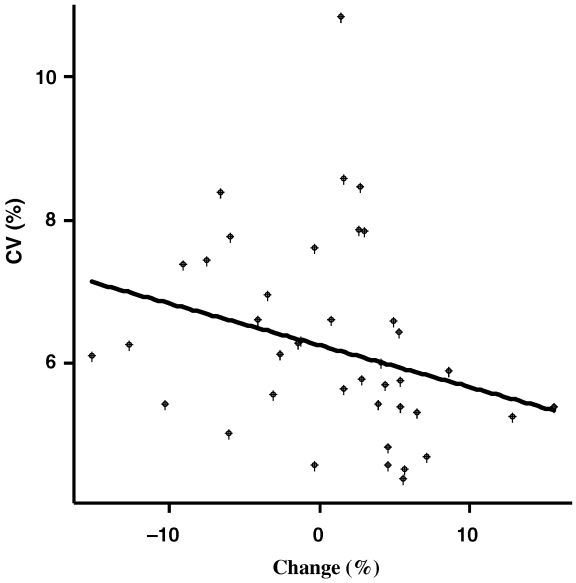

Fig. 1. The variability (% CV) of sperm head area in extended samples (unfrozen) is plotted as a function of the per cent difference in the corresponding measurement of head spermatozoa from extended and cryopreserved samples (solid points). The correlation coefficient is $r = -0.465$ ($n = 40$; $p < 0.005$)

Discussion

Firstly, we found a significant effect of cryopreservation on the head morphometry of red deer epididymal sperm, across a population of 40 stags. Sperm head measurements of cryopreserved samples were significantly lower than those of the extended samples for area, length and shape factor across all stags. Our results, thus, indicate that the extended spermatozoa, in addition to being the ones having higher sperm quality (determined by SMI, NAR, viability and HOS test), are also those with larger sperm heads, comparing with cryopreserved spermatozoa. Secondly, our results show too that, whereas significant freeze/thaw effects were found in the morphometric measurements across the population of 40 stags, significant differences were found only within some stags. Indeed, a low number of the stags (25%) in this study did not incur changes in the morphometric parameters of the sperm head. The detection of freeze/thaw effects within individual stags indicates that some individuals may be more sensitive to cryopreservation. Besides, sperm head dimensions from extended samples were significantly lower in those samples that showed no changes in measurements. Finally, at thawing, SMI and sperm viability tended to be higher for the stags that did not incur changes in the morphometric parameters of the sperm head than for those that did it, although the differences were not statistically significant. Therefore, our results indicate that extended sperm samples with lower initial dimensions of sperm heads, in addition to being the ones where morphometric changes in sperm heads did not occur after cryopreservation, are also those with a tendency to higher sperm quality at thawing, with the opposite being true for spermatozoa with larger initial sperm head dimensions.

In relation with the effects of cryopreservation on sperm head morphometric measurements, our results agree with earlier findings reported for bull (Gravance et al. 1998), human (Thompson et al. 1994), stallion (Arruda et al. 2002) and dog spermatozoa (Rijsselaere et al. 2004), in which sperm head dimensions (length, width, area and perimeter) were smaller in cryopreserved samples than in fresh extended samples. These differences in morphometric dimensions between fresh

and cryopreserved spermatozoa have been explained proposing several possible mechanisms, including osmotic changes, acrosome damage and alterations in chromatin condensation (Royere et al. 1988; Gravance et al. 1998; Love and Kenney 1998; Blottner et al. 2001). However, our results only partially agree with those earlier reported for the effects of cryopreservation on head morphometry of goat spermatozoa (Gravance et al. 1997). These authors found no overall effect of cryopreservation on goat sperm head morphometry, but they observed an effect within a limited number of bucks. Contrasting results may be because of a certain species specific sensitivity for the freezing process or to different cryopreservation protocols (i.e. glycerol levels, freezing and thawing rates), resulting in a different effect on the post-thaw sperm head characteristics (Thompson et al. 1994; Gravance et al. 1998).

Our results also show that the effect of cryopreservation on red deer sperm heads varies among individuals. However, in contrast to the previous findings of Gravance et al. (1997), the majority (75%) of the stags in this study incurred changes in the sperm head dimensions during the cryopreservation process. The average per cent change across all stags ranged from 0.5% for width to 12% for area. Although the average changes in dimensions were $<12\%$ for all measurements, they were still found to be significantly different, because of the precision of the SCA. This method of analysis was able to detect the very small changes in morphometric dimensions between extended and cryopreserved sperm heads.

The CVs (variation) of sperm head measurements within and across stags (Tables 1 and 2), indicate that sperm head measurements from different stags are extremely heterogeneous. In fact, at least as heterogeneous as the sperm population within a sample of a given stag. Besides, our results show that the variability of sperm heads measurements was lower in the extended semen samples than in the cryopreserved ones for area and length when all samples were analysed. However, when this effect was independently studied in the two groups of stags (ND and DIF), these differences were only significant for the DIF group. These results suggest that sperm head area and length from different stags are more heterogeneous in the cryopreserved samples than in the extended ones, especially in the samples from DIF stags. Thus, our results show that the variability of sperm head dimensions between cryopreserved and extended samples was only significantly different in the samples that showed changes in measurements during the cryopreservation.

Whereas cryopreserved sperm samples showed no significant differences in the sperm head morphometric dimensions of the ND and DIF populations, sperm head dimensions from extended samples were significantly lower in the samples that showed no changes in measurements. Taken together, our results indicate that extended sperm samples with lower initial dimensions of sperm heads, in addition to being the ones which less change their sperm head measurements between dilution and cryopreservation, are also those with lower variability of sperm head dimensions at thawing, with the opposite being true for spermatozoa with higher initial dimensions of sperm head. Thus, these two groups of males differ in the dimensions of their sperm heads, and it is possible that these differences have an impact on sperm water volume and membrane permeability to water and cryoprotectants and, in turn, on sperm freezability (Curry 2000). Therefore, the lower the sperm head dimensions in the extended samples, the greater the sperm cryoresistance. Our results also show that the change (%) in sperm head measurements from extended and cryopreserved samples for all stags was negatively correlated with the CVs of the corresponding measurements for area, width and perimeter of the extended sperm heads. These negative correlations indicate that, regardless of the male group (ND or DIF), as the variability of the extended samples decreased, the per cent difference in individual parameters of sperm head measurements from extended to cryopreserved samples increased in a correlation fashion. Indeed, our results show that the greater the sperm head area variability (CVs) in extended samples (Fig. 1), the lower the sperm cryodamage (as revealed by the per cent difference from extended to cryopreserved samples). Therefore, the sperm head morphometric variability of an extended sample can be a good indicator of the sperm cryosurvival of the same sample. To the best of our knowledge, this is the first report demonstrating that sperm head morphometric dimensions and heterogeneity of individual extended samples may be used as good indicators of sperm resistance to freezing-thawing process.

The examination of post-thaw spermatozoa with techniques such as partitioning in aqueous two phases systems to detect subtle differences in surface properties has demonstrated that heterogeneity is severely diminished after the freeze-thaw process (Ollero et al. 1998). Contrary, our results show that significant differences in the means within analysis CVs were found between the extended and cryopreserved samples for area and length among stags, being the variability (heterogeneity) of sperm head measurements lower in the extended samples than in the cryopreserved ones when all samples were analysed. These apparently conflicting findings could be accounted for in one of two ways. One of the explanations could be that the use of two different techniques to evaluate sperm heterogeneity might differentially affect results. Alternatively, when we analysed the effect of cryopreservation in the CVs separately for the two groups of males (ND and DIF), we only found significant differences ($p < 0.05$) in the means within analysis CVs between the extended and cryopreserved samples for DIF group. These results indicate that the variability of sperm head dimensions from cryopreserved samples was significantly higher than that from extended samples only in the samples that showed changes in measurements (i.e. samples that were more affected by cryopreservation). Therefore, in these stags the freeze-thaw process might affect an higher population of cells than in ND stags, originating a higher rate and extent of cellular damage. This higher degree of sperm lesion might be the responsible of the higher variability of sperm head dimensions from cryopreserved samples in this group of stags (DIF), giving the appearance of a more heterogeneous population.

In conclusion, sperm head morphometric dimensions were significantly smaller in the cryopreserved samples than in the extended ones, across a population of 40 males. The impact of the effects was variable across stags, with only 25% of the stags showing no significant change in morphometric dimensions. In addition, sperm freezability tended to be higher for the stags that did not incur changes in the morphometric parameters of the sperm head than for those did it, but the differences were not statistically significant. The variability (CV) of sperm head measurements from extended samples was negatively correlated with the per cent of change in sperm head measurements after cryopreservation. It appears that the variability of the sample prior to cryopreservation may be predictive of subsequent changes. Future work will utilize ASMA to identify possible relationships between sperm head dimensions and the fertility.

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CAPÍTULO 3

Significado funcional del tamaño y del perfil de la cabeza espermática en la determinación de la congelabilidad de las muestras epididimarias de ciervo Ibérico

RESUMEN

En este estudio, se utilizó el sistema Sperm-Class Analyzer® (SCA) para determinar si el área y el perfil de la cabeza de los espermatozoides epididimarios podrían usarse para diferenciar entre los machos de ciervo Ibérico clasificados como "buenos" o "malos" congeladores en base a la resistencia de sus espermatozoides a la congelación. Se prepararon muestras individuales con semen epididimario diluido, recogido de 38 venados abatidos en monterías. Se secaron al aire y se tiñeron con Hemacolor. El área y el perfil (long/anch) fueron determinados para unas 145 cabezas espermáticas de cada macho mediante SCA. El resto fue congelado en vapores de nitrógeno. Tras la descongelación, se evaluó la criosupervivencia espermática mediante microscopía, analizando la motilidad individual y la integridad de las membranas plasmática y acrosomal. Todos los parámetros espermáticos rutinarios evaluados a la descongelación fueron examinados mediante un análisis de cluster multivariante, originando dos grupos de machos en función de la congelabilidad de sus muestras espermáticas ("buenos" y "malos" congeladores). Nuestros resultados revelan que la calidad de las muestras a la descongelación, en todos los parámetros evaluados, es significativamente más alta ($p < 0,01$) en los "buenos" que en los "malos" congeladores (SMI = 67,4 ± 2,0 *vs.* 57,1 ± 2,8; NAR = 67,1 ± 2,5 *vs.* 54,5 ± 3,5%; viabilidad = 68,8 ± 2,0 *vs.* 60,1 ± 2,8%; HOST = 71,3 ± 2,2 *vs.* 63,1 ± 3,1%). Las dimensiones medias de las cabezas espermáticas en las muestras frescas de los dos grupos generados ("malos" y "buenos" congeladores) fueron comparadas con un ANOVA. Nuestros resultados revelan la aparición de diferencias en el área y en el perfil ($p < 0,01$) de las cabezas espermáticas entre los dos grupos de machos antes de la congelación, presentando los "buenos" congeladores las menores dimensiones (área = 32,04 *vs.* 34,42 μm^2). Así, cuanto menor es el área de la cabeza espermática en las muestras frescas, mayor es la criosupervivencia de la misma. Nuestros resultados revelan que los dos grupos de machos también difieren en el perfil de las cabezas espermáticas en las muestras frescas ("buenos": 1,96 *vs.* "malos": 1,72; $p < 0,01$). Es posible que el área y el perfil de la cabeza espermática influyan sobre el volumen total y/o la superficie de contacto del espermatozoide, causando diferencias en el intercambio de temperatura y en los movimientos de agua, iones y crioprotectores; y debido a ello, también sobre la congelabilidad del semen.

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Functional Significance of the Sperm Head Morphometric Size and Shape for Determining Freezability in Iberian Red Deer (*Cervus elaphus hispanicus*) Epididymal Sperm Samples

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ABSTRACT: In the present study, computer-automated sperm head morphometry of epididymal samples was used to determine if sperm head area and shape are useful measurements for separating "good" and "bad" Iberian red deer freezers. A microscope slide was prepared from single diluted sperm fresh samples collected from 38 mature stags. Slides were air-dried and stained with Hemacolor. The sperm head area and shape (length/width) for a minimum of 145 sperm heads were determined for each male by means of the Sperm-Class Analyser. The remainder of each sample was frozen. After thawing, sperm cryosurvival was judged in vitro by microscopic assessments of individual sperm motility and of plasma membrane and acrosome integrities. All sperm parameters evaluated at thawing were placed in a statistical database and a multivariate cluster analysis performed. Mean sperm parameters of the 2 clusters generated ("bad" and "good" freezers) were compared by ANOVA. Our results show that sperm quality at thawing for all sperm parameters evaluated was significantly higher ($P < .01$) for "good" freezers than for the "bad" ones (sperm motility index: 67.4±2.0 vs 57.1±2.8; NAR: 67.1±2.5 vs 54.5±3.5; viability: 68.8±2.0 vs 60.1±2.8; HOST: 71.3±2.2 vs 63.1±3.1). Additionally, differences ($P < .01$) in epididymal sperm head area and shape were found between "good" and "bad" freezers before freezing, with the smallest overall sperm head dimensions found in the "good" freezers group (area: 32.04 μm^2 vs 34.42 μm^2). Thus, the lower the sperm head area in the fresh samples, the greater the sperm cryoresistance. Our results show that the 2 groups of males also differ in sperm head shape in fresh samples (good: 1.96 vs poor: 1.72; $P < .01$). It is possible that sperm head area and shape influence total sperm volume, thus causing differences in heat exchange as well as in movements of water, ions, and cryoprotectants and, in turn, on sperm freezability.

Key words: cryopreservation, morphology, postmortem recovery.

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The interest in preserving germplasm of wild deer species has resulted in rather recent attention to the possible recovery, evaluation, and cryopreservation of sperm from the epididymides of dead animals (Zomborszky et al, 1999; Comizzoli et al, 2001a,b; Hishinuma et al, 2003; Soler and Garde, 2003; Soler et al, 2003, 2005). However, little information is published on freezing and thawing methods for epididymal spermatozoa of this species, although there have been many studies of freezing of ejaculated semen from stags (Asher et al, 2000). Most procedures used to cryopreserve epididymal spermatozoa from red deer have been modified from those developed for ejaculated semen. This approach would not seem to be very appropriate, since it is well known that there are important differences in the physiological characteristics of epididymal vs ejaculated spermatozoa, especially in their membrane properties, that affect the sperm cell survival after freezing.

Variation between individuals in the extent to which their spermatozoa are damaged by freeze-thawing has been widely reported in several species. These differences in the resistance to thawing of the spermatozoa of different individuals have been observed for spermatozoa of domestic (Curry, 2000) and wild (Leibo and Songsasen, 2002) species. Within this context, semen donors have routinely been categorized as "good" or "bad freezers." The mechanisms underlying differences in cryosensitivity between different individuals have yet to be elucidated, but there is some evidence for physiological differences between spermatozoa from individuals of the same species. Recently it has been

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demonstrated that these consistent interindividual variations in sperm freezability are genetically determined (Thurston et al, 2002).

Appropriate, sensitive, and rapid methods of assessment are necessary for adequate evaluation of sperm function. Conventional sperm parameters are not sufficient to identify animals known as "poor freezers," whose sperm quality is greatly impaired by cryopreservation. The routine evaluation of semen, including normal sperm morphology assessment, has long been employed to evaluate the effects of freezing-thawing procedures on sperm cryosurvival. Poor semen morphology is an important indicator of decreased fertility in men (Kruger et al, 1993); stallions (Jasko et al, 1990), bulls (Sekoni and Gustafsson, 1987), and goats (Chandler et al, 1988). Although normal sperm morphology may be an indicator of the fertility potential of a given male, correlations have been based on subjectively performed analyses. However, large variations between technicians and laboratories in the subjective evaluation of semen characteristics are known to exist (Saacke, 1982; Baker and Clarke, 1987), making accurate interpretation of the resulting data difficult. The need for accurate objective assessment of sperm morphology has led to the development of computer-assisted sperm head morphometry analysis, ASMA (Katz et al, 1986; Davis et al, 1992; Pérez-Sánchez et al, 1994). The precision of the ASMA system has been utilized to detect morphometric differences in sperm head dimensions of fertile and subfertile males (Casey et al, 1997) as well as subtle changes in human sperm head morphometry due to toxicant exposure when no morphological differences were detected by manual assessment (Davis et al, 1993).

Previous studies utilizing ASMA have also demonstrated that cryopreservation affects head morphometry of bull (Gravance et al, 1998), human (Thompson et al, 1994), stallion (Arruda et al, 2002), dog (Rijsselaere et al, 2004), and red deer (Esteso et al, 2006) spermatozoa. Recent evidence suggests that in boar semen, the sperm head dimensions of individual sperm samples may be an indicator of sperm cryosurvival (Thurston et al, 2001; Peña et al, 2005), while no data exist regarding sperm morphometry and freezability in deer species.

Thus, adopting methods previously utilized in other species (Sancho et al, 1998; Buendía et al, 2002) and in red deer by us (Esteso et al, 2003, 2006), our objective was to determine if sperm head area and shape are useful measurements for predicting freezability in Iberian red deer epididymal sperm samples. To achieve this goal, first, frozen-thawed spermatozoa from a single sample collected from 38 stags were evaluated for sperm motility and for acrosome (NAR) and membrane integrities (HOST and viability). All data generated were used for a multivariate cluster analysis which objectively classified all sperm samples (stags) within a data set into one of 2 groups, categorized as "good" or "bad" according with their freezability. Second, we retrospectively compared the routine sperm parameters and the sperm head size and shape in freshly epididymal sperm samples between the 2 defined groups.

Materials and Methods

With the exception of dibutyl phthalate xylene (Fluka, Madrid, Spain), all chemicals were of reagent grade and were purchased from Sigma or Merck (both of Madrid, Spain).

Preparation of Testes and Collection of Epididymal Spermatozoa

For this study, we used spermatozoa recovered from the epididymides of 38 mature stags (age > 4 years, weight > 140 kg) that were legally culled and hunted in their natural habitat during the rutting season (September–November). The hunting of stags was performed in accordance with the harvest plan of each game reserve. The harvest plans were made following Spanish Harvest Regulation, Law 2/93 of Castilla-La Mancha, which conforms to European Union Regulation. Immediately upon removal, the testes with attached epididymides were placed into plastic bags and transported to the laboratory at room temperature (approximately 20°C) within 2 hours after being removed. Samples were processed as soon as they arrived at the laboratory. For collection of epididymal spermatozoa, testes and epididymides were removed from the scrotal sac. Cauda epididymides, including about 5–10 cm of the proximal ductus deferens, were separated and transferred to 35-mm plastic dishes.

Spermatozoa were collected from the distal portion of the epididymis by repeated longitudinal and transverse cuts with a surgical scalpel and collecting the oozing sperm mass and placing it in 0.5 ml of Salamon modified solution (Fernandez-Santos et al, 2005); in particular, the base solution (Fraction A) containing Tris (2.70%, w/v), fructose (1%), citric acid (1.4%), glycerol (0%, v/v), and egg yolk (20%, v/v), with pH = 6.8 and osmolality of 300 mOsm. Epididymal contents from both testicles of an individual male were pooled for processing, because our previous observations showed that no differences appeared to exist in cell quality between testes belonging to the same individual (Garde et al, 1998).

Semen Processing and Cryopreservation

Then, the sperm mass was again diluted at room temperature to a final sperm concentration of ~400 × 10^6 sperm/ml with Fraction A of the extender. Sperm dilution was performed in a 2-step procedure at room temperature, first adding base extender up to 2 times the final desired sperm concentration and then a second extender (Fraction B) at a 1:1 ratio to achieve a final concentration ~200 × 10^6 spermatozoa/ml. Fraction B differed from the base diluent in the replacement of water (12%, v/v) with the same volume of glycerol (final concentration = 6%, v/v). This 2-step procedure was employed

for sperm dilution to obtain the same final concentration of glycerol for each stag. At this point, subsamples were taken for sperm head morphometric dimensions evaluation. Then the sperm diluted was placed in a 15-ml centrifuge tube (Iwaki, Japan) and was slowly cooled to 5°C. For this, the tubes were placed in a beaker with water (75 ml at room temperature) and transferred to a refrigerator at 5°C. Cooling down to 5°C lasted for about 1 hour, and extended samples were held for equilibration at that temperature for 2 hours more. After the equilibration of the diluted sperm samples, the extended sperm was loaded into 0.25-ml plastic straws. Immediately, they were frozen at 4 cm in nitrogen vapor (−120°C) for 10 minutes before being immersed into liquid nitrogen (−196°C) for storage. For freezing, straws were placed horizontally on a metal rack that was positioned into a freezing close container (Cryo Diffusion CD-45). The frozen straws remained for a minimum period of 1 month in liquid nitrogen before thawing was carried out.

Frozen semen was thawed in a water bath (37°C) for 20 seconds and the content of the straws poured into a glass tube. Samples were evaluated for motility, viability, and acrosome and membrane integrities after 5 minutes of incubation at 37°C, using the methods described below. At thawing, subsamples were also taken for sperm head morphometric dimensions evaluation.

Semen Evaluation

Sperm concentration and subjective scores of motility were assessed shortly after collection. Sperm concentrations of the original suspensions were determined using a hematocytometer. Percentage of individual motile sperm (motility) was noted, and quality of motility was assessed using a scale of 0 (lowest) to 5 (highest). A Sperm Motility Index was calculated = [% individual motility + (quality of motility × 20)] × 0.5. The sperm suspension was also used to assess acrosome integrity and viability. Acrosomal integrity was evaluated after a 1:1 dilution in 2% glutaraldehyde in 0.165 M cacodylate/HCl buffer (pH 7.3). The percentage of spermatozoa with intact acrosomes (% NAR) was assessed by phase-contrast microscopy.

In addition, samples were taken to assess the membrane integrity by means of the hypo-osmotic swelling (HOS) test. Plasma membrane functionality was assessed using an HOS test, as described by Garde et al (1998). The osmotic swelling technique consisted of mixing 0.01 ml of diluted sperm samples with 0.1 ml of hypo-osmotic solution (100 mOsmol/kg) and incubating the mixture at room temperature for 30 minutes. The samples were then fixed in 2% glutaraldehyde buffered solution and evaluated by phase-contrast microscopy at 400×. The sperm membrane was considered functional in cases where the sperm tail was coiled, and the result was expressed as HOS (%).

Membrane integrity (viability) was also evaluated by using a nigrosin-eosin stain (NE). The NE stain was prepared according to the method of Soler et al (2005). Sperm (5 µl) was mixed with the NE stain (10 µl) at 37°C, incubated for 30 seconds, and smeared and dried on a warm plate at 37°C. The samples were evaluated using bright field microscopy at 400×. Live spermatozoa remained unstained, while dead cells were dull pink. The percentage of live spermatozoa was expressed as viability (%).

For sperm assessments, 100–200 spermatozoa were counted in each preparation. Additionally, slides of extended sperm samples were prepared from each sample for sperm head morphometric characterization. For each sperm parameter evaluated, a ratio was calculated to assess the cryoprotective abilities of the different individual samples, as follows:

$$\text{Cryoresistance ratio} = (\text{Value after thawing}/\text{value in fresh sperm}) \times 100.$$

Morphometric Analysis of Sperm Heads

Microscopic slides were prepared from each fresh (upon dilution) sample by placing 5 µl of the sperm samples on the clear end of a frosted slide and dragging the drop across the slide. Semen smears were air-dried and stained using a Hemacolor (Merck) procedure, originally described for staining of ram (Sancho et al, 1998) and alpaca (Buendía et al, 2002) sperm heads and recently adapted by our group to red deer spermatozoa (Esteso et al, 2003). Stained sperm samples were permanently mounted to the slide with a coverslip and dibutyl phthalate xylene.

Stained slides were used to perform ASMA using the morphometry module of a commercially available system (Sperm-Class Analyzer, version 99 CASMA system, Microptic, Barcelona, Spain). The machine was equipped with a Nikon (Labophot-2, Tokyo, Japan) microscope with a 60× bright-field objective and a Sony video camera (CCD AVC-D7CE, Sony Corporation, Tokyo, Japan) connected to a Pentium 950 MHz processor. The illumination source was centered, and the intensity of the bulb and the gain and offset of the camera were standardized for all samples. The configuration of the computer system included a PIP-1024 B video digitizer board (Matrox Electronic Systems Ltd., Quebec, Canada), the sperm image analysis software, and a high-resolution assistant monitor Sony Trinitron PVM-1443MD (Sony). The array size of the video frame recorder was 512 × 512 × 8 bits; digitized images were made up of 262 144 pixels (picture elements) and 256 grey levels. Resolution of images was 0.15 and 0.11 µm per pixel in the horizontal and vertical axes, respectively.

The morphometric dimensions for area (A) and shape factor (length/width) were acquired for 150–160 images. Acquiring 150–160 images assures that a minimum of 145 properly measured sperm heads are analyzed after improperly measured sperm heads are deleted from the analysis. The measurements of each individual sperm head from each stag and sperm treatment were saved in an Excel (Microsoft Corporation, Redmond, Wash)-compatible database by the software for further analysis.

Statistical Analysis

Statistical analyses were performed using SPSS for Windows, version 11.5 (SPSS Inc, Chicago, Ill). The effects of cryopres-

Table 1. *Effects of cryopreservation on characteristics of red deer epididymal spermatozoa* †*

	Assessed Parameters‡			
Sample	SMI§ (%)	NAR§ (%)	HOS§ (%)	Viability§ (%)
Fresh	83.16±1.7[a]	94.59±1.6[a]	89.61±1.6[a]	89.43±1.4[a]
Cryo	63.91±1.7[b]	62.97±1.6[b]	68.69±1.6[b]	66.15±1.4[b]

* Fresh indicates unfrozen sperm sample; Cryo, cryopreserved sperm sample.
† Values are least-squares means ± SEM.
‡ Values with different superscripts in the same column were significantly different ($P < .0001$).
§ See "Materials and Methods" for details of spermatozoa assessment.

ervation on sperm motility, viability, and acrosome and membrane integrities were compared across stags by a general linear model analysis of variance (GLM-ANOVA) using a split plot design. Stags served as the main plot, and sperm cryopreservation step (prefreezing or postthawing) served as the subplot. Group differences were compared by Fisher's least significant differences test. Effects were considered significant at $P < .05$.

Data of postthaw sperm motility, sperm viability, and acrosome and sperm membrane integrities obtained from each of the 38 epididymal sperm samples were used to build a single data set. The data set was subjected to a multivariate analysis as described by Esteso et al. (2003) for head sperm morphometric patterns. This procedure uses the cluster analysis to classify the sperm samples, using all measured sperm variables within the data set, on a small number of groups. Two groups were finally obtained from the non-hierarchical clustering (K-means clustering) of the 38 stag sperm samples tested. The statistics of each sperm variable of the 2 groups of males were calculated, and compared using GLM-ANOVA. Percentage data of sperm fresh parameters and sperm head area and shape in fresh samples from sperm samples clustered following the multivariate analysis were also compared using ANOVA. Data that did not follow a normal distribution were transformed. A probability of $P < .05$ was considered to be statistically significant.

Results

Effects of Cryopreservation

In this work, epididymal spermatozoa from 38 stags were frozen and thawed. Because we used epididymal spermatozoa for this experiment, it was only possible to make single observations for each stag. After freezing and thawing, a decrease ($P < .0001$) in all routine sperm parameters was observed (Table 1). Thus, sperm motility index decreased from 83.16±1.7% in extended fresh samples to 63.91±1.7% in thawed samples. The results found for the other seminal parameters evaluated are similar if not identical (Table 1). Our results also showed differences among males for most of the seminal parameters evaluated immediately after thawing (Figures 1 and 2).

Male Subpopulations Analysis

After the cluster analysis of frozen-thawed sperm quality, 2 groups of sperm samples (stags) were clearly identified. Those samples with the best frozen-thawed sperm characteristics were identified as "good," whereas the others represented samples showing considerably reduced frozen-thawed sperm characteristics and were considered as "bad" following a standard freezing protocol. Twenty-five samples were identified as "good" and 13 as "bad." Summary statistics for these 2 groups are shown in Table 2. Note the significant differences ($P < .01$) for all sperm parameters evaluated.

Sperm Quality Before Freezing

There was no significant variation between groups before freezing for all the sperm parameter evaluated (Table 3). Sperm samples from the 2 groups consisted of spermatozoa with high quality and would be expected to maintain this quality after cryopreservation.

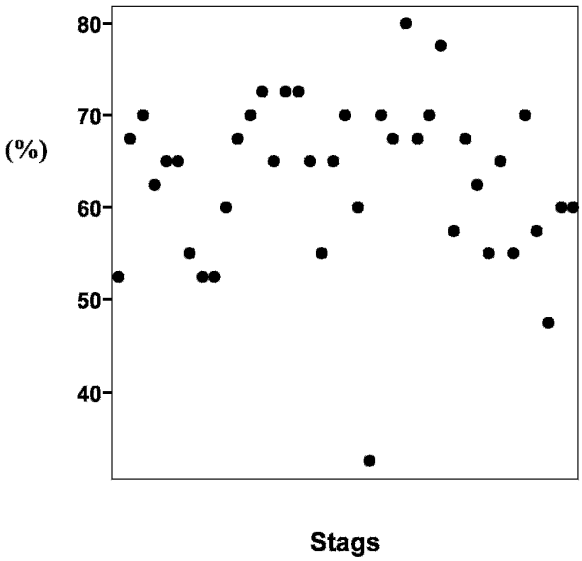

Figure 1. Sperm motility index of thawed red deer spermatozoa collected postmortem from epididymides of different stags.

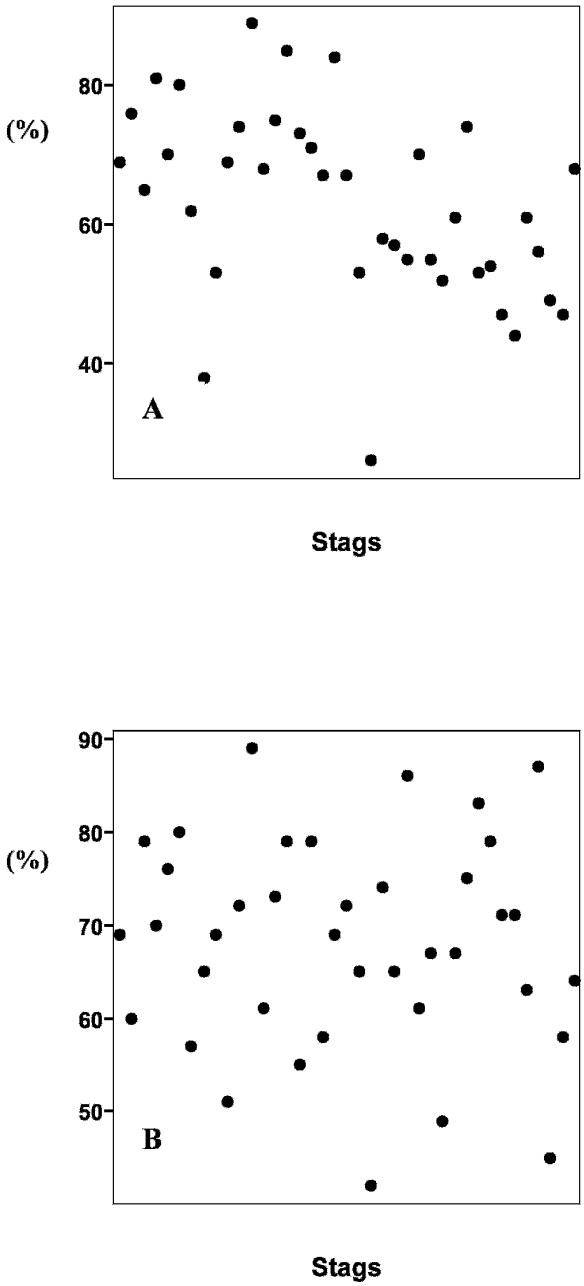

Figure 2. Individual characteristics of thawed red deer spermatozoa collected postmortem from epididymides of different stags: **(A)** percentage of sperm cells with intact acrosomes (NAR), and **(B)** percentage of spermatozoa with intact plasma membrane (HOS).

Sperm Cryoresistance

As a consequence of the fact that differences in fresh sperm quality between stags exist, we also determined the cryoresistance ratios for all the sperm parameters evaluated (see "Materials and Methods"). The use of rates rather than absolute values allows for a direct comparison between males that differ in fresh semen parameters (Table 4). Note the significant differences present between groups ($P < .05$ to $P < .0001$) for all the cryosurvival ratios. These results demonstrate that the resistance to the cryopreservation was different between "good" and "bad" freezers. Good freezers had the highest ratios (range: 70%–83%) in all the parameters evaluated (sperm motility index, % endosmosis, and % intact acrosomes), with results that in all cases were significantly different from those seen in the "bad" group (range: 58%–70%).

Sperm Head Size and Shape in Fresh Samples

Data of sperm head area and shape from stags classified as "good" and "bad" freezers, evaluating 3406 and 1821 properly digitized spermatozoa respectively, revealed that the frozen-thawed sperm characteristics (base for the discrimination into 2 groups) were significantly affected by the sperm head size and shape (Table 5). There were no differences in the percentage of properly analyzed sperm heads between "good" and "bad" freezers (data not shown). Our results showed that the smallest overall sperm head dimensions in fresh samples were found in the "good" freezers group (area: 32.04 μm^2 vs 34.42 μm^2). Therefore, the lower the sperm head area in the fresh samples, the greater the sperm cryoresistance. Our results also show that the 2 groups of males differ in sperm head shape (good: 1.96 vs bad: 1.72; $P < .01$). Thus, the sperm heads in the fresh samples from the "good" freezers were more elongated and narrow than those from the "bad" group. In this sense, the sperm head length in the fresh samples from "good" freezers was approximately 2 times higher than the width. Figure 3 shows the sperm head area for each stag categorized as "good" or "bad" freezers.

Discussion

Our previous studies utilizing ASMA have reported the normal sperm head dimensions for fresh and thawed red deer epididymal spermatozoa (Esteso et al, 2003; 2006). In the present study, the ASMA protocol used was useful to detect differences in sperm head area and shape between "good" and "bad" freezers before freezing. Thus, the lower the sperm head area in the fresh samples, the greater the sperm cryoresistance. Differences in the resistance to thawing of the spermatozoa of different individuals have been observed for spermatozoa of other domestic (Curry, 2000) and wild (Leibo and Songsasen, 2002) species. Within this context, semen

Table 2. *Descriptors of frozen-thawed sperm characteristics in the two groups ("good" vs "bad") of single stag sperm samples defined after multivariate cluster analysis**

		Assessed Parameters†			
Stag Group	Stags	SMI‡ (%)	NAR‡ (%)	HOS‡ (%)	Viability‡ (%)
Good	25	67.40±2.0[a]	67.08±2.5[a]	71.28±2.2[a]	68.80±2.0[a]
Bad	13	57.11±2.8[b]	54.54±3.5[b]	63.15±3.1[b]	60.15±2.8[b]

* Values are least-squares means ± SEM.
† Values with different superscripts in the same column were significantly different ($P < .01$).
‡ See "Materials and Methods" for details of spermatozoa assessment.

Table 3. *Descriptors of fresh sperm characteristics in the two groups ("good" vs "bad") of single stag sperm samples defined after multivariate cluster analysis**

		Assessed Parameters†			
Stag Group	Stags	SMI‡ (%)	NAR‡ (%)	HOS‡ (%)	Viability‡ (%)
Good	25	81.40±2.0[a]	95.52±1.6[a]	88.88±1.7[a]	90.24±1.7[a]
Bad	13	86.23±2.7[a]	93.00±1.9[a]	90.61±2.4[a]	87.61±1.2[a]

* Values are least-squares means ± SEM.
† Values with different superscripts in the same column were significantly different ($P < .05$).
‡ See "Materials and Methods" for details of spermatozoa assessment.

donors have routinely been categorized as "good" or "bad freezers." Although similar experiences have been reported for several species, no explanations for these differences have been substantiated. The mechanisms underlying differences in cryosensitivity between different individuals have yet to be elucidated, but there is some evidence for physiological differences between spermatozoa from individuals of the same species (Leibo and Bradley, 1999).

On the other hand, authors have been very interested in determining the relationship between single fresh semen quality characteristics such as motility, viability, morphology, or acrosome status and freezability. In any case, a single fresh semen characteristic could show a positive relationship with freezability, but the relationship was either of low significance or measurable in some males but not in others. Inclusion of several sperm variables, measured either by functional methods or by the combination of significant outcomes on a multivariate regression analysis, has been regarded as more discriminative, and in some cases even of predictive value. For more details see Rodriguez-Martinez (2003). Bearing this in mind, the present work aimed to group a series of postthaw sperm variables defining the survival status of red deer spermatozoa subjected to a conventional freezing protocol and, by using a multivariate cluster analysis as described by Esteso et al (2003) and used already by other authors to classify subpopulations of spermatozoa (Quintero-Moreno et al, 2003), defined 2 groups of stags as "good" and "bad" according to their freezability.

In this study, out of the 38 males, 25 were identified as "good" and 13 as "bad." To explore the differences between the 2 groups, conventional ANOVA analysis was undertaken, and significant differences were found for all parameters. The differences between stag groups were found for all quality parameters of thawed spermatozoa as well as the degree of declined cellular integrity by cryopreservation. However, there were no significant differences in routine semen quality between groups before freezing. On the other hand, differences in epididymal sperm head area and shape were found

Table 4. *Sperm cryosurvival in the two groups ("good" vs "bad") of single stag sperm samples defined after multivariate cluster analysis**

		Assessed Parameters†			
Stag Group	Stags	SMI‡ (%)	NAR‡ (%)	HOS‡ (%)	Viability‡ (%)
Good	25	83.58±2.3[a]	70.17±2.5[c]	80.69±2.5[c]	76.42±2.3[e]
Bad	13	66.00±3.2[b]	58.55±3.5[d]	70.04±3.5[d]	68.41±3.2[f]

* Values are least-squares means ± SEM.
† Values with different superscripts in the same column were significantly different: [a,b]($P < .0001$); [c,d]($P < .01$); [e,f] ($P < .05$).
‡ See "Materials and Methods" for details of cryoresistance rates determination.

Table 5. *Sperm head area and shape factor for fresh sperm samples in the two groups ("good" vs "bad") of single stags defined after multivariate cluster analysis* †*

Stag group	Stags	Spermatozoa	‡Area (μm²)	‡Shape Factor (length/width)
Good	25	3,406	32.04 (9.8)[a]	1.96 (8.65)[a]
Bad	13	1,821	34.42 (9.2)[b]	1.72 (7.72)[b]

* Values are least-squares means. Coefficients of variation (% CV) between stags are shown in parentheses.
† Values with different superscripts in the same column were significantly different ($P < 0.01$).
‡ See "Materials and Methods" for details of spermatozoa assessment.

between "good" and "bad" freezers before freezing, with the smallest overall sperm head dimensions found in the "good" freezers group. Besides, the sperm heads in the fresh samples from the "good" freezers were more elongated and narrow than those from the "bad" group. In this sense, the sperm head length in the fresh samples from "good" freezers was approximately 2 times higher than the width. It is noteworthy that when comparisons are made among species for their ability to sustain cold shock, clear sperm differences are evident; the spermatozoa of those species less sensitive to cryopreservation are smaller (Garde et al, 2003). Obviously, many other factors are involved in cryoresistance, but we hypothesized that sperm head area and shape cause differences in heat exchange as well as in movements of water and ions. It is therefore plausible to think that spermatozoa may vary in their physical properties depending on their area and shape and that these variations are at least partially responsible for the interindividual resistance to the cryopreservation process.

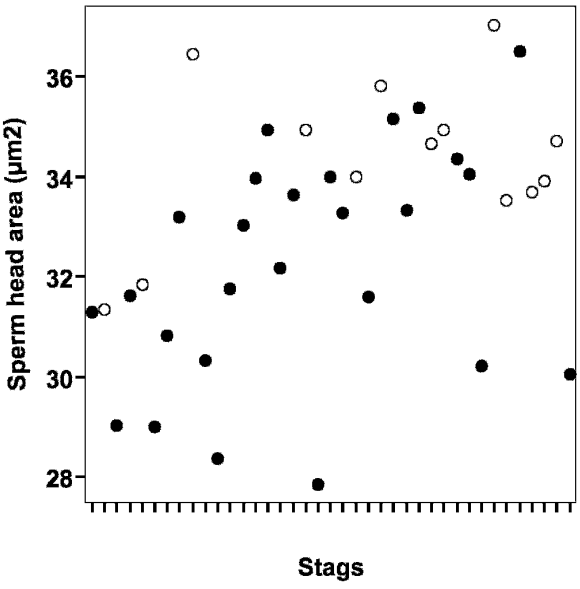

Figure 3. Sperm head area (μm²) for individual "good" (●) and "bad" freezers (○).

This relationship between sperm head dimensions in fresh ejaculates and sperm freezability has been previously supported for boar spermatozoa (Thurston et al, 2001; Peña et al, 2005). The study of Thurston et al (2001) reported that the percentage of spermatozoa in the fresh ejaculates with slightly tapering heads was positively correlated with sperm quality after thawing. Thurston et al (2001) support the hypothesis that the interindividual variations in sperm morphology are genetically determined, and therefore those differences in sperm morphology in fresh samples that affect sperm freezability are indicative of the genetic variations responsible for the relative ability of spermatozoa to withstand freezing procedures. Here we present the hypothesis that these variations in sperm morphology among stags can influence per se biophysical characteristics of the spermatozoa that are essential for successful cryopreservation. Taken together, our results revealed that fresh spermatozoa from the "good" freezers had small and elongated (higher shape) sperm heads, whereas spermatozoa from "bad" freezers had large and wide (lower shape) sperm heads. However, there was no significant variation between stag groups before freezing for the routine semen parameters evaluated. Therefore, these 2 groups of males only differed in the dimensions of their fresh sperm heads, and it might be assumed that sperm head size and shape in spermatozoa of fresh samples were good indicators of freezability. However, these results must be carefully interpreted, since there were sperm samples classified as "good" showing large sperm heads (>34 μm²) and sperm samples categorized as "bad" with small sperm heads in the fresh samples (Figure 3). It seems reasonable, then, based on our results, that sperm head area and shape of fresh spermatozoa discriminate between 2 clear-cut populations whose sperm freezability is different. However, such a relationship, though it exists, is not enough to accurately estimate, much less predict, the sperm freezability of individual samples. More studies are needed in order to develop procedures for prospectively selecting sperm samples for cryopreservation.

The observation that sperm head size and shape are highly indicatives of stag sperm survival after cryopres-

ervation is important for 2 reasons. First, it is possible that sperm head area and shape influence total sperm volume, thus causing differences in heat exchange as well as in movements of water, ions, and cryoprotectants and, in turn, in sperm freezability (Curry, 2000). Second, it suggests that sperm survival from individuals considered as "bad freezers" (with large and wide sperm heads) can be optimized by modifying either the cryoprotectant concentration or the cooling rates. Thus, previous works have reported that sperm characteristics like surface area and volume have important implications for determining optimum cooling and warming procedures for sperm cryopreservation (Curry et al, 1996). In this sense, improvements in boar sperm cryosurvival have been reported when spermatozoa were frozen at higher rates (Fiser and Fairfull, 1990). When cell suspensions are frozen, they are cooled at finite rates, often referred as slow or fast. A rate that is slow for one cell type may be rapid for a second type. The optimum cooling rate is the one at which maximum survival is observed. Thus, the optimum cooling rates for freezing human, boar, and ram spermatozoa are different (see Leibo and Bradley, 1999). Besides, sperm area and volume have important implications for determining optimum cooling procedures for cryopreservation (Curry et al, 1996). These sperm characteristics influence the rate at which the cell can lose water; and the rate at which a cell can lose water is a principal determinant of its optimum cooling rate. For reasons briefly described above, efforts to improve sperm freezing protocols have increased substantially during the past years. In this sense, we propose that sperm samples from the different stags require different cooling rates for optimal cryosurvival. Thus the results of the present study suggest that the freezability of the spermatozoa from individuals considered as "bad freezers" (with large and wide sperm heads) could be increased in the future using different cooling rates than those used in this work (20°C/min). We base this hypothesis on the fact that cellular area and volume have important implications for determining optimum cooling rates for cryopreservation (Curry et al, 1996; Leibo and Bradley, 1999).

In summary, the results of the present study show that although a multivariate pattern analysis based on frozen-thawed red deer sperm quality attributes was able to separate "good" and "bad" freezers, and further that these populations could relate to sperm head area and shape in fresh samples, there were sperm samples outside this pattern. This situation confirms the need for caution when aiming at estimating the potential freezability of a given sperm sample by assessing only phenotypic variables of the spermatozoa. Future work will utilize ASMA to identify sperm morphometric subpopulations in epididymal fresh sperm samples and their possible relationships with freezability.

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CAPÍTULO 4

Identificación de subpoblaciones espermáticas con características morfométricas diferentes en muestras epididimarias de ciervo Ibérico

RESUMEN

En este estudio se utilizó el Sperm-Class Analyzer® (SCA) para identificar subpoblaciones espermáticas de acuerdo a su morfometría en muestras epididimarias de ciervo Ibérico. Estas muestras fueron recogidas de 37 machos abatidos durante la época de monterías y diluidas a temperatura ambiente en un medio Tris-citrato-20% yema de huevo-6% glicerol. Se realizó una preparación para microscopía antes de la congelación a partir de las muestras individuales y el resto fue congelado en vapores de nitrógeno. Tras la descongelación, se tomaron muestras para morfometría espermática de nuevo. Todos los frotis se secaron al aire y se tiñeron con Hemacolor. Se determinaron las dimensiones de la cabeza espermática para cuatro parámetros de tamaño y cinco parámetros de forma, de al menos 145 espermatozoides en cada muestra, mediante SCA. Todos los parámetros evaluados sobre 10.867 espermatozoides, fueron examinados mediante un análisis de cluster multivariante, originando tres subpoblaciones de espermatozoides (SP_1, SP_2, SP_3) con diferentes medidas morfométricas ($p < 0,001$). El porcentaje relativo de estas subpoblaciones fue variable entre los machos analizados ($p < 0,001$). Sin embargo, la proporción de espermatozoides presente en cualquiera de las 3 subpoblaciones permaneció constante ($p > 0,05$) tras el proceso de criopreservación. Además, nuestros resultados revelan que el cambio en la distribución de los espermatozoides dentro de cada subpoblación durante la criopreservación, muestra notables diferencias entre machos. Se evaluó la calidad espermática *in vitro* mediante examen microscópico antes de la congelación y tras la descongelación, tanto de la motilidad individual como de la integridad de la membrana plasmática y del acrosoma. El cambio en los porcentajes de la distribución de los espermatozoides en algunas subpoblaciones (SP_1 y SP_3) durante la criopreservación afecta a la calidad espermática tras la descongelación. El protocolo CASMA utilizado en este estudio fue capaz de detectar diferencias morfométricas muy pequeñas entre espermatozoides. La combinación de este análisis con un método estadístico multivariante nos revela nueva información sobre el efecto de la criopreservación en las características biológicas de los espermatozoides de ciervo Ibérico, no encontradas mediante métodos de evaluación espermática convencionales.

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Identification of Sperm-Head Morphometric Subpopulations in Iberian Red Deer Epididymal Sperm Samples

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Contents

Computer-assisted sperm morphometry analysis (CASMA) was used in this study to identify sperm morphometric subpopulations in Iberian red deer epididymal sperm samples. Epididymal sperm samples were collected from 37 mature stags and were divided. One portion was diluted in a Tris–citrate–egg yolk medium. A microscope slide was prepared from single extended sperm samples prior to freezing. The remainder of each sample was frozen in nitrogen vapours using a conventional protocol. After thawing, sperm smears were prepared as described for extended samples. All slides were air-dried and stained with Hemacolor®. The sperm-head dimensions for a minimum of 145 sperm-heads were analyzed from each sample by means of the Sperm-Class Analyser®, and the mean measurements recorded. Each sperm-head was measured for four primary sperm-head parameters, and five parameters of head shape. All sperm morphometric parameters evaluated were placed in a statistical database and a multivariate cluster analysis was performed. The clustering analyses, based on 10 867 individual spermatozoa, revealed the existence of three subpopulations (SP_1, SP_2, SP_3) of spermatozoa with different morphometric characteristics ($p < 0.001$). The proportion of spermatozoa present in any of the three subpopulations remained constant ($p > 0.05$) through the cryopreservation process. Pre-freeze and post-thaw sperm quality was *in vitro* evaluated by microscopic assessments of individual sperm motility and of plasma membrane and acrosome integrities. In conclusion, our results show that applying the CASMA techniques and multivariate cluster analyses, it was possible to determine that three subtle subpopulations of spermatozoa with different morphometric characteristics coexist in red deer semen.

Introduction

The routine evaluation of sperm quality, including normal sperm morphology assessment, has long been employed to evaluate the effects of freezing–thawing procedures on sperm cryosurvival. Poor semen morphology is an important indicator of decreased fertility in men (Kruger et al. 1993), stallions (Jasko et al. 1990), bulls (Sekoni and Gustafsson 1987) and goats (Chandler et al. 1988; Skalet et al. 1988). Although normal sperm morphology may be an indicator of the fertility potential of a given sample, correlations have been based on subjectively performed analyses. However, large variations between technicians and laboratories in the subjective evaluation of semen characteristics are known to exist (Saacke 1982; Baker and Clarke 1987), making accurate interpretation of the resulting data difficult. The need for accurate objective assessment of sperm morphology has led to the development of computer-assisted sperm morphometry analysis (CASMA), which facilitates the rapid examination of a high number of mammalian spermatozoa (Gravance et al. 1995; Kondracki et al. 2005). In addition, applying the techniques of CASMA and multivariate cluster analyses, it was possible to determine discrete subpopulations of sperm-head dimensions in fertile and sub-fertile males accurately (Gravance et al. 1996). Previous studies utilizing CASMA have also demonstrated that the proportion of morphologically distinct sperm subpopulations within the ejaculates correlated with the semen quality assessments made following cryopreservation (Thurston et al. 2001).

Although sperm-head morphometry can be considered a good indicator of semen quality in domestic species, the investigation of morphometric sperm subpopulations in red deer semen has received little attention, there being no studies dealing with this issue. In addition, no studies that have the goal of examining differences on sperm subpopulations between extended and frozen-thawed semen exist in this species.

Thus, adopting methods previously utilized in other species (Thurston et al. 2001; Quintero-Moreno et al. 2003, 2004; Martínez-Pastor et al. 2005) and in red deer by us (Esteso et al. 2003, 2006a,b), our main objective was to evaluate the effects of cryopreservation on red deer spermatozoa distribution in morphometrically distinct subpopulations. To achieve this goal, we initially developed a simple procedure to identify sperm subpopulations within the red deer sperm samples based on data gathered with CASMA, using a commercially available statistical package (SPSS); then the influence of a conventional cryopreservation procedure on the frequency distribution of spermatozoa within these three subpopulations was evaluated.

Material and Methods

Materials

With the exception of DPX (Fluka, Madrid, Spain) all other chemicals were of reagent grade and were purchased from Sigma or Merck (both of Madrid, Spain).

Semen collection and processing

For this study, we used spermatozoa recovered from the epididymides of 37 mature stags (age > 4.5 years,

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weight > 150 kg) that were legally culled and hunted in their natural habitat during the rutting season (September–November). The hunting of stags was performed in accordance with the harvest plan of each game reserve. The harvest plans were made following Spanish Harvest Regulation, Law 2/93 of Castilla-La Mancha, which conforms to European Union Regulation. Immediately upon removal, the testes with attached epididymides were placed into plastic bags and transported to the laboratory at room temperature (approximately 20°C) within 2 h after being removed. Samples were processed as soon as they arrived at the laboratory. For collection of epididymal spermatozoa, testes and epididymides were removed from the scrotal sac.

Spermatozoa were collected from the distal portion of the epididymis by repeated longitudinal and transverse cuts with a surgical scalpel and collecting the oozing sperm mass and placing it in 0.5 ml of a Salomon's modified solution (Fernández-Santos et al. 2006). In particular, the base solution (Fraction A) containing Tris (223 mM), fructose (55 mM), citric acid (66 mM), glycerol (0%, v/v) and egg yolk (20%, v/v), with pH 6.8 and osmolality of 300 mOsm. Epididymal contents from both testicles of an individual male were pooled for processing.

Sperm cryopreservation

Then, the sperm mass was further diluted at room temperature to a final sperm concentration of $\sim 400 \times 10^6$ sperm/ml with the Fraction A of the extender. Sperm dilution was performed in a two-step procedure at room temperature, first adding base extender to two times the final desired sperm concentration and subsequently adding the second extender (Fraction B) at a 1 : 1 ration to achieve a final concentration $\sim 200 \times 10^6$ spermatozoa/ml. Fraction B differed from the base diluent in the replacement of water (12%, v/v) with the same volume of glycerol (final concentration = 6%, v/v). This two-step procedure was employed for sperm dilution to obtain the same final concentration of glycerol for each stag. At this point, sub-samples were taken for sperm-head morphometric evaluation. Then the diluted sperm was placed in a 15-ml centrifuge tube (Iwaki, Japan), which was placed in a beaker with water (75 ml at room temperature). This was transferred to a refrigerator at 5°C to slowly cool to 5°C over approximately for 1 h interval. Extended samples were held for equilibration at 5°C for 2 more hours. After equilibration of the diluted sperm samples, the extended sperm was loaded into 0.25 ml plastic straws. Straws were immediately frozen in nitrogen vapour 4 cm above the liquid (−120°C) for 10 min before being immersed into liquid nitrogen (−196°C) for storage. The frozen straws remained for a minimum period of 1 month in liquid nitrogen before thawing.

Frozen semen was thawed in a water bath (37°C) for 20 s and the content of the straws poured into a glass tube. Samples were evaluated for motility, and acrosome and membrane integrities after 120 min of incubation at 37°C, using the methods described below. At thawing, sub-samples were also taken for sperm-head morphometric dimensions evaluation.

Semen evaluation

Sperm concentration and subjective scores of motility were assessed shortly after collection. Sperm concentrations of the original suspensions were determined using a haematocytometer. Percentage of individual motile sperm (motility) was noted and quality of motility was assessed using a scale of 0, lowest, to 5, highest. A Sperm Motility Index (SMI) was calculated according to method attributed to Kikuchi et al. (1998) = [% individual motility + (quality of motility × 20)] × 0.5. The sperm suspension was also used to assess acrosomal integrity as per method described by Garde et al. (1998). Acrosomal integrity was evaluated after a 1 : 1 dilution in 2% glutaraldehyde in 0.165 M cacodylate/HCl buffer (pH 7.3). The percentage of spermatozoa with intact acrosomes (% NAR) was assessed by phase-contrast microscopy.

In addition, samples were taken to assess the membrane integrity by means of the hypo-osmotic swelling (HOS) test. Plasma membrane functionality was assessed using a HOS test as described by Garde et al. (1998). The sperm membrane was considered functional in cases where the sperm tail was coiled and the result was expressed as HOS (%).

For sperm assessments, 100–200 spermatozoa were counted in each preparation. Additionally, slides of extended sperm samples were prepared from each sample for sperm-head morphometric characterization.

Morphometric analysis of sperm-heads

Microscopic slides were prepared from each extended (upon dilution) and thawed sample by placing 5 μl of the sperm samples on the clear end of a frosted slide and dragging the drop across the slide. Semen smears were air-dried and stained using a Hemacolor® (Merck) procedure, originally described for staining of ram sperm-heads (Sancho et al. 1998); and recently adapted by our group to red deer spermatozoa (Esteso et al. 2003, 2006a,b). Stained sperm samples were permanently mounted to the slide with a coverslip and dibutyl phthalate xylene (DPX).

Stained slides were used to perform CASMA using the morphometry module of a commercially available system (Sperm-Class Analyzer®, SCA, version 99 CASMA system; Microptic, Barcelona, Spain). The machine was equipped with a Nykon (Labophot-2, Tokyo, Japan) microscope with a × 60 bright-field objective and a Sony video camera (CCD AVC-D7CE; Sony Corporation, Tokyo, Japan) connected to a Pentium 950 MHz processor. The illumination source was centred and the intensity of the bulb and the gain and offset of the camera were standardized for all samples. The configuration of the computer system included a PIP-1024 B video digitizer board (Matrox Electronic Systems Ltd, Quebec, Canada), the sperm image analysis software and a high-resolution assistant monitor Sony Triniton PVM-1443MD (Sony Corporation). The array size of

the video frame recorder was 512 × 512 × 8 bits, digitized images were made up of 262 144 pixels (picture elements) and 256 grey levels. Resolution of images was 0.15 and 0.11 μm per pixel in the horizontal and vertical axes, respectively.

The morphometric dimensions for four primary sperm-head parameters [area (A), perimeter (P), length (L), width (W)]; and five parameters of head shape (FUN1 [L/W], FUN2 [$4\pi A/P^2$], FUN3 [$(L-W)(L+W)$], FUN4 [$\pi LW/4A$], $p2a$ [$P^2/4\pi A$]) were acquired for 150–160 images. Acquiring 150–160 images assures that a minimum of 145 properly measured sperm-heads are analyzed after improperly measured sperm-heads are deleted from the analysis. The shape feature, $p2a$ compares the perimeter of an object to its area (Sailer et al., 1996). This parameter has a minimum value of 1.0 for a circle. As this ratio increases, the less like a circle is the object. The measurements of each individual sperm-head from each stag and sperm treatment were saved in an EXCEL® (Microsoft® Corporation, Redmond, WA, USA)-compatible database by the software for further analysis.

Statistical analysis

The data matrix consisted of 5451 observations for extended semen and 5416 for thawed samples for the morphometric analysis. Statistical analyses were performed using SPSS for Windows, version 14.0 (SPSS Inc., Chicago, IL, USA). All sperm-head measurements within each cryopreservation step (that is, extended or thawed) were then clustered by length, width, area, perimeter and shape factors using iterative k-means cluster analysis to classify the spermatozoa of the data set into a reduced number of subpopulations according to their sperm-head dimensions as previously described (Esteso et al. 2003). The separation of spermatozoa into clusters was performed over the 10 867 stained spermatozoa. The final number of clusters was set at three. The morphometric descriptors for the three subpopulations of spermatozoa were calculated, and compared using General Linear Models analysis of variance (GLM-ANOVA). The influence of each cryopreservation step on the percentages of spermatozoa belonging to each subpopulation was analyzed by chi-squared test. Correlations between the proportion of spermatozoa present in any of the three subpopulations and the standard semen parameters after cryopreservation were performed by Pearson's correlation coefficients. A probability of $p < 0.05$ was considered to be statistically significant.

Results

Effects of cryopreservation on post-thaw sperm quality

In this work, epididymal spermatozoa from 37 stags were frozen and thawed. Because we used the epididymal spermatozoa for this experiment, it was possible only to make single observations for each stag. After freezing and thawing, a decrease ($p < 0.0001$) in all routine sperm parameters was observed (Table 1).

Table 1. Effects of cryopreservation on characteristics of red deer epididymal spermatozoa

Sample	SMI (%)	NAR (%)	HOS (%)
Extended	79.47 ± 2.2[a]	89.79 ± 1.3[a]	85.16 ± 1.7[a]
Thawed	52.30 ± 2.2[b]	59.15 ± 1.3[b]	54.74 ± 1.7[b]

SMI, sperm motility index; NAR, acrosome integrity; HOS, positive endosmosis.
[a,b] Values with different superscripts in the same column were significantly different ($p < 0.0001$).
Values are least-squares means ± SEM.

Sperm subpopulations based on morphometric properties of the spermatozoa

The values for all measures of dimensions of sperm-heads were determined to be normally distributed by Kolmogorov–Smirnov normality test.

Three sperm subpopulations were identified after the cluster analysis of the 10 867 individual spermatozoa (observations). The disclosed subpopulations were characterized by different values ($p < 0.001$) of sperm-head dimensions. Summary statistics of these subpopulations are shown in Table 2, and the qualitative interpretation of them is outlined as follows.

- *Subpopulation 1 (SP_1)*. This subpopulation was characterized by spermatozoa with average sperm-head dimensions. This subpopulation was characterized by spermatozoa with the highest values of $p2a$. Thus, this is the subpopulation with the spermatozoa that looks less like a circle. In extended samples, more than 20% (21.5%) of the spermatozoa in the data set were assigned to this subpopulation. In the thawed samples, this SP increased until 24.22%.
- *Subpopulation 2 (SP_2)*. This subpopulation of the sperm was characterized by spermatozoa with the smallest sperm-head dimensions. In the extended samples, the percentage of spermatozoa which were included in this subpopulation was 29.92%, whereas in the thawed samples, the values go up to 32.8%.
- *Subpopulations 3 (SP_3)*. This subpopulation included those spermatozoa whose sperm-head is long and wide. The spermatozoa of this subpopulation are

Table 2. Morphometric descriptors of sperm heads for each sperm subpopulation found after the cluster analysis

	Sperm subpopulations		
Morphometric descriptors	SP_1	SP_2	SP_3
Length (μm)	8.56 ± 0.5[b]	8.08 ± 0.4[c]	8.79 ± 0.4[a]
Width (μm)	4.58 ± 0.3[b]	4.40 ± 0.3[c]	4.85 ± 0.3[a]
Area (μm^2)	31.72 ± 1.9[b]	29.25 ± 2.0[c]	34.81 ± 1.8[a]
Perimeter (μm)	32.80 ± 1.7[a]	28.74 ± 1.6[c]	29.32 ± 1.6[b]
FUN1	1.87 ± 0.1[a]	1.84 ± 0.2[b]	1.81 ± 0.1[c]
FUN2	0.36 ± 0.4[a]	0.44 ± 0.3[b]	0.50 ± 0.2[c]
FUN3	0.30 ± 0.0[a]	0.29 ± 0.0[b]	0.28 ± 0.0[c]
FUN4	0.97 ± 0.0[a]	0.95 ± 0.0[c]	0.96 ± 0.0[b]
$p2a$	2.72 ± 0.0[a]	2.27 ± 0.0[b]	1.97 ± 0.0[c]
n	2802	3745	4320

[a,b,c] Values with different superscripts in the same row were significantly different ($p < 0.0001$).
Values are least-squares means ± SEM. The total number of spermatozoa analyzed was 10 867.

the biggest. This subpopulation was characterized by spermatozoa with the smallest values of *p2a*. Thus, this is the subpopulation with the spermatozoa with the shape of the sperm-head more similar to a circle. This subpopulation contained the largest number of spermatozoa, as about 49% and 43% of the extended and thawed spermatozoa respectively were included in this subpopulation (~45% of total spermatozoa).

The proportions of spermatozoa in each of the three sperm subpopulations identified within the data set are shown in Table 3 both in extended and frozen-thawed samples. There were no significant ($p > 0.05$) differences in the distribution of these three sperm subpopulations between extended and thawed samples. However, our results revealed that the changes in the frequency distribution of spermatozoa within each subpopulation during cryopreservation were affected by the interaction of individual factor-cryopreservation step (Table 4). In this sense, no identical cryopreservation effect was found between the extended samples and the thawed ones for the frequency distribution of spermatozoa within each subpopulation within stags for all males. Thus, in 17 of the 37 stags (45%), the proportion of spermatozoa within SP_1 decreased between the extended and the thawed samples. Within the SP_2 and SP_3, the reduction of the percentage of spermatozoa from extended to thawed samples was respectively observed in 38% and 60% respectively of evaluated males (Table 4). Finally, there was a significant negative correlation ($r = -0.76$; $p < 0.0001$) between the changes in the percentages of distribution of SP_1 and SP_3 from extended to thawed samples. This result indicates that the changes in the percentages of these two subpopulations from extended to thawed step were inversely related. No significant correlations were found between the changes in the frequencies of the other subpopulations.

Table 3. Percentage of spermatozoa in each subpopulation found after the cluster analysis. The table shows pooled data, extended and thawed results

	Sperm subpopulations		
	SP_1	SP_2	SP_3
Total samples	22.80	31.40	45.70
Extended samples	21.50	29.92	48.56
Thawed samples	24.22	32.83	42.94
Change extended vs thawed (%)	+2.72	+2.91	−5.62

No differences were detected between extended and thawed samples ($p > 0.05$)

Table 4. Classification of the stags according to the effect of cryopreservation on the distribution of spermatozoa between sperm subpopulations. Increase (subpopulations 1 and 2) and decrease (subpopulation 3) indicate the kind of change induced by cryopreservation. Observe the heterogeneity of responses between males

Subpopulation	Effect of the cryopreservation process on the frequency distribution of spermatozoa within each subpopulation	Males (n)	Males (%)
1	Increase	20	55
	No increase	17	45
	Total	37	100
2	Increase	23	62
	No increase	14	38
	Total	37	100
3	No decrease	15	40
	Decrease	22	60
	Total	37	100

Table 5. Sperm characteristics (thawed samples) of each stag group, as defined in Table 4. Samples with increasing SP_1 and decreasing SP_3 after thawing showed lower quality on average

Subpopulation	Male's group	SMI (%)	NAR (%)	HOS (%)
1	Increase	46.47 ± 3.7^a	39.79 ± 2.9^a	61.60 ± 2.2^a
	No increase	58.30 ± 3.9^b	48.11 ± 3.2^b	69.94 ± 2.4^b
2	Increase	50.00 ± 3.6^a	42.00 ± 2.8^a	52.48 ± 2.6^a
	No increase	55.17 ± 4.6^a	46.34 ± 3.6^a	57.07 ± 3.4^a
3	No decrease	60.57 ± 4.1^a	46.79 ± 3.5^a	59.13 ± 3.2^a
	Decrease	46.10 ± 3.4^b	41.15 ± 2.9^a	50.80 ± 2.6^b

SMI, sperm motility index; NAR, acrosome integrity; HOS, positive endosmosis.
[a,b] Values with different superscripts in the same column within each sperm subpopulation were significantly different ($p < 0.05$).
Values are least-squares means ± SEM.

Relationships between sperm subpopulations morphology and post-thaw sperm viability

There were no significant correlations between the proportions of spermatozoa in each of the three sperm subpopulations in extended or thawed samples and post-thaw sperm viability (data not shown). However, the changes in the percentages of the frequency distribution of spermatozoa within some subpopulations (SP_1 and SP_3) during cryopreservation affected the semen quality assessments made following thawing. In that sense, at thawing SMI, NAR, and HOS were lower ($p < 0.05$) for the stags that exhibited an increase in the percentage of SP_1 during cryopreservation than for those did not it (Table 5). No significant effects on post-thaw sperm quality were found for the changes on SP_2. Finally, at thawing SMI, and HOS were lower ($p < 0.05$) for the stags that exhibited a decrease in the percentage of SP_3 during cryopreservation than for those did not it (Table 5).

Discussion

Our previous studies utilizing CASMA have reported the normal sperm-head dimensions for extended and thawed red deer epididymal spermatozoa (Esteso et al. 2006b). In addition, we have earlier reported that sperm-heads were significantly smaller in cryopreserved spermatozoa than in the companion extended samples (Esteso et al. 2006a). In the present study, over 10 800 spermatozoa from extended and thawed sperm samples were analyzed using a CASMA system (Sperm-Class Analyzer®, SCA), in an attempt to identify sperm morphometric subpopulations in Iberian red deer epididymal sperm samples. In addition, this study was carried out to assess the effects of cryopreservation on red deer spermatozoa distribution in morphometrically distinct subpopulations. To the authors' knowledge, this is the first description of subpopulations of spermatozoa with different morphometric characteristics in red deer

semen. We have also re-evaluated the effects of cryopreservation on red deer sperm parameters.

Our results showed that by applying the CASMA techniques and multivariate cluster analyses, it was possible to determine that three subtle subpopulations of spermatozoa with different morphometric characteristics coexist in red deer sperm samples (large, average and small spermatozoa). After this cluster analysis, it became evident that red deer sperm samples were not homogeneous populations of spermatozoa. The procedure employed in our study gave relevant information on the characteristics of stags sperm samples. The data derived from the morphometric analysis could be then successfully used to discriminate sperm morphometric subpopulations within extended or thawed sperm samples. Although there are other studies (Gravance et al. 1996; Thurston et al. 2001) mainly using Fourier shape descriptors to classify sperm-heads, this is, to the best of our knowledge, the first description of sperm subpopulations in red deer spermatozoa based on morphometric data directly derived from a CASMA analysis. The statistical procedure hereby used was simple and appeared useful to detect sperm subpopulations. In our study we have used the SPSS software and a *k*-means cluster procedure that is indicated when there is a large set of data, as was the case in our study (10 867 observations). This procedure has the relative inconvenience that the operator must set the number of cluster *a priori*. Such cluster numbers were determined in a series of preliminary tests, until the optimal number of clusters was found. On the other hand, a major advantage of the *k*-means cluster procedure is the easy detection of outliers (Martínez-Pastor et al. 2005; Peña et al. 2005). To explore the differences between the different sperm subpopulation, conventional GLM analysis was undertaken in our study and significant differences were found for all sperm-head dimensions among the three subpopulations.

The disclosed subpopulations were characterized by different values of sperm-head and shape factors. Subpopulation 1 was characterized by spermatozoa with average sperm-head dimensions. In extended samples, more than 20% (21.5%) of the spermatozoa in the data set were assigned to this subpopulation. In the thawed samples, about 24% of the spermatozoa were included in this SP. Subpopulation 2 included those spermatozoa with the smallest sperm-head dimensions. In the extended samples, the percentage of spermatozoa which were included in this subpopulation was 29.92%, whereas in the thawed samples, 32.8% of the spermatozoa were presented in this SP. Subpopulation 3 included those spermatozoa whose sperm-head is long and wide. The spermatozoa of this subpopulation are the biggest and containing the largest number of spermatozoa, since, about 49% and 43% of the extended and thawed spermatozoa respectively were included in this subpopulation (~45% of total spermatozoa). These data confirm the idea that sperm samples are not homogeneous, and that sperm subpopulations can be identified using different parameters (Martínez-Pastor et al. 2005).

Overall, the results of the 37 stags show that the proportion of spermatozoa presents in each of the three subpopulations remained constant ($p > 0.05$) through the cryopreservation process. Taken together, our results indicate that cryopreservation did not affect the overall proportions of spermatozoa within each of the three subpopulations. Our results also show that the effect of cryopreservation on red deer spermatozoa distribution within each subpopulation varies among individuals (Table 4). In this sense, we classified the stags according to the effect of the cryopreservation process on the frequency distribution of spermatozoa within each subpopulation. This classification was done following the general trend of every subpopulation in relation to the cryopreservation process (Table 3). Within subpopulation 1 and 2, stags were classified inside the *Increase group* when the percentage of spermatozoa in the respective subpopulation increased from the extended to the thawed step, with the opposite being true for *the No Increase group*. Within SP3, stags were classified inside the *Decrease group* when the percentage of spermatozoa in this subpopulation decreased from the extended to the thawed step, with the opposite being true for the *No Decrease group*.

The majority (60%) of the stags in this study reported a reduction in the percentage of SP_3 during the cryopreservation process. SP_2 showed an opposite trend to SP_3 (62% of stags showed an increase in the percentage of SP_2 during the cryopreservation process). SP_1 showed a similar trend to SP_2. In addition, the freezability was lower in stags that exhibited a reduction in the percentage of SP_3 during cryopreservation ($p < 0.05$) than for those did not it. The opposite results were found in relation with SP_1 (i.e. freezability was lower in stags that exhibited an increase in the percentage of SP_1 during cryopreservation). Accordingly, it can be assumed that the changes in these two subpopulations from extended to thawed samples are good indicators of sperm freezability. Knowledge of the changes in sperm morphometric patterns through cryopreservation can provide important information for understanding the physiological events that spermatozoa undergo during this procedure. Considering that SP_3 is characterized by spermatozoa whose sperm-heads are long and wide (The spermatozoa of this subpopulation are the biggest), leads to the speculation that when this subpopulation decreased at thawing, it would express a continuing process of sperm-head structure degeneration during freezing and thawing, ultimately leading to cell death. Clearly, this process of cell death can be indicative of the lower freezability found in these stags, the opposite being true for the males where SP_3 did not decrease. The differences in morphometric dimensions between extended and cryopreserved sperm-head have been explained by several possible mechanisms including osmotic changes, acrosomal damage and alterations in chromatin condensation (Gravance et al. 1998). Taken together, our results suggest that those sperm samples (stags) where diminution of SP_3 did not occur, were also those with higher sperm freezability, with the opposite being true for SP_1. It can be assumed that when the proportion of spermatozoa within SP3 decreased after thawing, these spermatozoa increased the percentage of SP_1 ($r = -0.76$; $p < 0.0001$). Thus, our results also show that those

stags where an increase of SP_1 occurred, were also those with lower sperm freezability. The increase of SP1 may be an indication of increased proportion of death in the spermatozoa (Marco-Jiménez et al. 2006).

In conclusion, our results show that by applying the CASMA techniques and multivariate cluster analyses, it was possible to determine that three subtle subpopulations of spermatozoa with different morphometric characteristics coexist in red deer semen. The disclosed subpopulations were characterized by different values of sperm-head dimensions. Our results also show that the proportion of spermatozoa within each of the three sperm subpopulations as well as the degree of change in the frequency distribution of spermatozoa within each subpopulation during cryopreservation, showed considerable differences among males. Further research using CASMA to identify possible relationships between sperm-head morphometric subpopulations and fertility in red deer is required.

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CAPÍTULO 5

Efecto del proceso de descongelación sobre las dimensiones de los espermatozoides descongelados de ciervo Ibérico

RESUMEN

El objetivo de este estudio fue el de evaluar los efectos del proceso de descongelación sobre la distribución de los espermatozoides epididimarios de ciervo Ibérico en distintas subpoblaciones en base a las medidas de las cabezas espermáticas. Para ello, se utilizaron dos protocolos de descongelación diferentes (I = 37 ºC durante 20 segundos; II = 70 ºC durante 5 segundos). Las muestras epididimarias se diluyeron en el medio Triladyl® con 20% de yema de huevo a temperatura ambiente y se congelaron en vapores de nitrógeno. La congelabilidad de los espermatozoides se evaluó por medio de microscopía, valorando la motilidad espermática. El proceso de descongelación influyó sobre el porcentaje de motilidad ($p < 0,05$), apareciendo los mejores valores utilizando el protocolo lento (76,8 ± 1,8 *vs.* 70,6 ± 1,8%). Además, se analizaron las dimensiones morfométricas de al menos 200 cabezas espermáticas para cada muestra por medio del Sperm-Class Analyzer® (SCA). Las medidas medias fueron significativamente más pequeñas ($p < 0,01$) cuando el protocolo de descongelación utilizado fue el rápido (área = 30,02 *vs.* 30,32 μm^2; anchura = 4,47 *vs.* 4,51µm; longitud = 8,05 *vs.* 8,11 µm). Todas las medidas fueron analizadas estadísticamente mediante un análisis multivariante y se generaron cinco subpoblaciones espermáticas dentro de cada uno de los dos protocolos de descongelación. Los parámetros medios para los grupos más importantes de los dos protocolos de descongelación fueron comparados mediante un ANOVA. Los valores medios de longitud, anchura, área, perímetro, forma y perfil del grupo principal fueron significativamente diferentes ($p < 0,001$) para los dos protocolos utilizados. Estas diferencias para los parámetros morfométricos, se encuentran en la totalidad de los machos analizados, apareciendo las menores dimensiones en los espermatozoides descongelados con el protocolo rápido. Además, la descongelación rápida produce una gran pérdida de heterogeneidad. Podemos afirmar, según nuestros resultados, que la mayor pérdida de heterogeneidad y el mayor grado de daño espermático debido a la descongelación se producen cuando se utiliza el protocolo de descongelación rápido frente al lento.

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HEAD DIMENSIONS OF CRYOPRESERVED RED DEER SPERMATOZOA ARE AFFECTED BY THAWING PROCEDURE

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Abstract

The objective of this study was to evaluate the effects of the thawing procedure on red deer spermatozoa distribution in morphologically distinct subpopulations after freezing and thawing. For this purpose, epididymal spermatozoa were thawed using two different thawing protocols (I = 37 °C for 20 s vs. II = 70 °C for 5 s). The spermatozoa, from 10 Iberian deer stags, were diluted at room temperature in a Triladyl® -20% egg yolk medium and frozen in nitrogen vapor. Standard sperm freezability was judged by microscopic assessments of sperm motility (%). The thawing procedure had an effect on sperm motility percentage ($P < 0.05$), with the best overall recovery rates found with the use of protocol I (76.8 ± 1.8 vs. 70.6 ± 1.8). Moreover, the morphometric dimensions for a minimum of 200 sperm heads were analyzed from each sample by means of the Sperm-Class Analysez® (SCA), and the mean measurements recorded. Deer sperm heads were significantly ($P < 0.01$) smaller when spermatozoa were thawed using protocol II than when using procedure I (area = 30.02 μm^2 vs. 30.32 μm^2; width = 4.47 μm vs. 4.51 μm; length = 8.05 μm vs. 8.11 μm), but not for all stags. All sperm head measurements were placed in a statistical database and a multivariate cluster analysis performed. Mean measurements for all parameters of the major clusters for the two different thawing procedures were compared by ANOVA. The mean values for length, width, area, perimeter, shape factor and width/length in the major cluster of sperm head dimensions for thawing protocol I were significantly different from those for protocol II ($P < 0.001$). In addition, differences were found within all stags for whole morphometric parameters ($P < 0.001$), with the smallest overall sperm head dimensions found with the use of protocol II. Additionally, the rapid thawing protocol produced a dramatic loss of heterogeneity. Finally, our results showed that the greater the loss of heterogeneity, the greater the degree of sperm cryoinjury.

Keywords: ASMA, cryopreservation, freezing, morphology, red deer, variation

INTRODUCTION

The routine evaluation of semen, including normal sperm morphology assessment, has long been employed to evaluate the effects of freezing-thawing procedures on sperm cryosurvival. Poor semen morphology is an important indicator of decreased fertility in men (10), stallions (8), bulls (15) and goats (4). Although normal sperm morphology may be an indicator of the fertility potential of a given sample, correlations have been based on

subjectively performed analyses. However, large variations between technicians and laboratories in the subjective evaluation of semen characteristics are known to exist (13), making accurate interpretation of the resulting data difficult. The need for accurate objective assessment of sperm morphology has led to the development of computer-assisted sperm head morphometry analysis, ASMA, (5,9,12). Previous studies utilizing ASMA have demonstrated that cryopreservation affect head morphometry of bull (7), human (19) and stallion (1) cryopreserved spermatozoa. In these studies, sperm heads measured after thawing were significantly smaller in cryopreserved spermatozoa than in fresh-extended sperm. The differences in morphometric dimensions between fresh and cryopreserved spermatozoa have been explained by several possible mechanisms including osmotic changes, acrosome damage and alterations in chromatin condensation (2,7,18). These observations, suggest that sperm head dimensions of individual thawed samples, may be an indicator of sperm cryosurvival. In addition, applying the techniques of ASMA and multivariate cluster analyses, it was possible to determine discrete subpopulations of sperm head dimensions in fertile and subfertile males accurately (6). Previous studies utilizing ASMA have also demonstrated that the proportion of morphologically distinct sperm subpopulations within the ejaculates correlated with the semen quality assessments made following cryopreservation (20).

Thus, adopting methods previously utilized in other species (3,14), our objective was to evaluate the effects of two different thawing procedures (37 °C for 20 s vs. 70 °C for 5 s) on epididymal red deer spermatozoa distribution in morphologically distinct subpopulations after cryopreservation. To achieve this goal, we firstly evaluated the effects of the thawing procedure on sperm motility of thawed samples. Secondly, we determined for the two thawing procedures different morphological classes using an analysis based on the clustering of variables and observations.

MATERIALS AND METHODS

Collection, freezing and thawing of epididymal spermatozoa

For this study, we used spermatozoa recovered from the epididymides of 10 mature stags (age > 4.5 years, weight > 130 kg) that were legally culled and hunted in their natural habitat during the rutting season (September-October). Spermatozoa were collected from the distal portion of the epididymis as described by Soler *et al.*, (17). Only samples with a sperm motility initially greater than 65-70% were used for freezing. The sperm mass was diluted at room temperature to a final sperm concentration ~ $400x10^6$ sperm/ml with Triladyl® (Minitüb, Tiefenbach, Germany)- 20% egg yolk medium, containing 6% glycerol (Minitüb, Tiefenbach, Germany). The diluted sperm suspensions were refrigerated slowly at 5°C for 1.5 h, equilibrated at that temperature for 2 h and loaded into 0.25 mL straws. The straws were frozen in nitrogen vapors, 4 cm above the surface of the liquid nitrogen, for 10 min and then plunged into liquid nitrogen.

Thawing was carried out using 2 different warming procedures: slow (I: 37 °C for 20 s) and rapid (II: 70 °C for 5 s), reaching the following thawing rates respectively: I) 1359 °C/min and II) 2715 °C/min. Thawing was carried out by placing the straws in a water bath containing water at different temperatures. After thawing, the straws were dried and the samples were poured into previously warmed test tubes (37 °C) and the sperm was allowed to equilibrate for 5 min before evaluation. Two straws from each epididymal specimen (i.e., stag) were used, one for each of the described thawing protocols. The entire experiment was repeated 3 times; thus, triplicate independent straws of spermatozoa from each stag for each thawing procedure were assessed for motility as described by Soler *et al.*, (17). Additionally in replicates 1 and 2, slides of thawed semen were prepared from each sample for sperm head morphometric characterization.

Morphometric analysis of sperm heads

Microscopic slides were prepared from each sample by placing 5 µl of the thawed semen on the clear end of a frosted slide and dragging the drop across the slide. Semen smears were air dried and stained using a Hemacolor (Merck) procedure, originally described for staining of ram (14) and alpaca (3) sperm heads. Stained sperm samples were permanently mounted to the slide with a coverslip and dibutyl phthalate xylene (DPX). Stained slides were used to perform ASMA using the morphometry module of a commercially available system (Sperm-Class Analyzer®, Microptic, Barcelona, Spain). The machine was equipped with a Nikon (Labophot-2, Tokyo, Japan) microscope with a x 60 bright-field objective and a Sony video camera (CCD AVC-D7CE, Sony Corporation, Tokyo, Japan) connected to a Pentium 950 MHz processor. The illumination source was centred and the intensity of the bulb and the gain and offset of the camera were standardized for all samples. The configuration of the computer system included a PIP-1024 B video digitiser board (Matrox Electronic Systems Ltd., Quebec, Canada), the sperm image analysis software and a high-resolution assistant monitor Sony Triniton PVM-1443MD (Sony Corporation, Tokyo, Japan). The array size of the video frame recorder was 512 x 512 x 8 bits, digitised images were made up of 262,144 pixels (picture elements) and 256 grey levels. Resolution of images was 0.15 and 0.11 µm per pixel in the horizontal and vertical axes, respectively. The morphometric dimensions for area (A), perimeter (P), length (L), width (W), width/length (W/L) and shape factor (attending the expression $4x\ \pi\ x\ Area/[Perimeter]^2$) were acquired for 225-230 images. Acquiring 225-230 images assures that a minimum of 200 properly measured sperm heads are analysed after improperly measured sperm heads are deleted from the analysis. The measurements of each individual sperm head from each stag and thawing procedure were saved in an Excel® (Microsoft® Corporation, Redmond, Washington, USA)-compatible database by the software for further analysis.

Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 11.0 (SPSS Inc, Chicago Il1). The effects of thawing procedure on sperm motility and on sperm head morphometric dimensions were compared within and across stags by General Linear Models analysis of variance (GLM-ANOVA). Group differences were compared by Fisher's Least Significant Differences (LSD) test. Effects were considered significant at $P < 0.05$. The effects of thawing procedure on sperm-head morphometry within stags, where effects were observed, were compared by Student's *t*-test.

All sperm head measurements within each group (that is, from thawing procedure I and II) were then clustered by length, width, area, perimeter and width/length using iterative *k*-means cluster analysis (6). The spermatozoa were divided into clusters such that every observation belonged to one and only one cluster. Sperm measurements that are very close to each other are usually assigned to the same cluster, while spermatozoa that are far apart are in different clusters. The number of clusters to create was determined when the explained variance between clustering steps was equal to or less than 5% for all parameters. The final number of clusters was set at five. The sperm dimension means, CV and number of sperm heads for each cluster were recorded for each stag and treatment. The effects of clusters within and between treatments for the measurements of length, width, area, perimeter, width/length and shape factor were analysed by GLM-ANOVA. The mean measurements of sperm heads for the major clusters were compared between thawing procedures by Fisher's LSD test. Linear regression analysis was used to investigate the relationship between the proportion of sperm heads within the major clusters and sperm motility in those same samples.

RESULTS

The results of the ANOVA showed significant differences ($P < 0.05$) between treatments for sperm motility, where the best overall recovery rate (76.8 ± 1.8 vs. 70.6 ± 1.8) was found with the use of protocol I (slow).

A total of 3998 properly digitised spermatozoa thawed using procedure I (37 °C for 20 s), and 3988 spermatozoa thawed using procedure II (70 °C for 5 s), were analysed. The values for all measures of sperm heads dimensions were determined to be normally distributed by Kolmogorov-Smirnov normality test. Analysis of variance showed a significant effect of stag ($P < 0.001$) and treatment ($P < 0.01$). The mean measurements for all sperm heads for all stags within each treatment was significantly higher for area (30.32 μm^2 vs. 30.02 μm^2; $P < 0.001$), width (4.51 μm vs. 4.47 μm; $P < 0.001$) and length (8.11 μm vs. 8.05 μm; $P < 0.01$) in the samples thawed at 37 °C for 20 s than in those thawed at 70 °C for 5 s. Additionally, spermatozoa were more tapered, lower width/length (0.555 vs. 0.558; $P < 0.001$), for thawing procedure II. Finally, the results of the ANOVA procedure revealed that sperm head measurements were significantly ($P < 0.001$) affected by the interaction between individual factor and thawing procedure, as can be seen in Figure 1A for head area. However, no significant thawing effect was found between the two protocols for sperm dimensions within stags for all males.

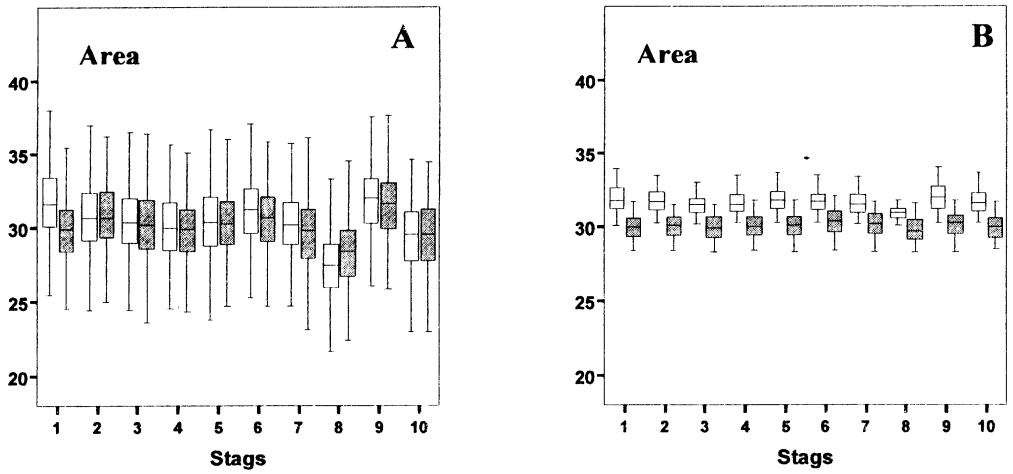

Figure 1. The effect of thawing procedure on deer sperm head area (μm^2). **(A)** Values from all sperm heads of spermatozoa thawed using two different procedures. **(B)** Values from sperm of the major clusters of spermatozoa thawed using two different procedures. Box-whisker plots show variations in sperm head morphometric values of epididymal samples collected from 10 stags after their spermatozoa were cooled, stored in LN_2, and then thawed either at 37 °C for 20 s (□) or at 70 °C for 5 s (▨). At least, 200 spermatozoa were analysed per slide. Each box encloses the 25th and 75th percentiles, the line in the middle is the median values and the whiskers extend to the 5th and 95th percentiles of the means values.

The percentages of sperm heads falling into the major clusters from thawing procedures I and II are respectively showed in Tables 1 and 2. The mean values for sperm head dimensions of the major cluster of spermatozoa thawed using procedure I, were significantly larger ($P < 0.001$) than those of the major cluster of the spermatozoa thawed with protocol II (Table 3). For all stags, significant ($P < 0.001$) differences were observed between the two different thawing

procedures for most of the sperm head dimensions of the major clusters of spermatozoa. These differences were specially marked for area (Figure 1B). Finally, the results of the ANOVA procedure revealed that thawed sperm heads dimensions were not significantly ($P > 0.05$) affected by the interaction between individual factor and thawing procedure. The results from the ten stags showed a similar pattern of response, with the smallest overall sperm head dimensions found with the use of protocol II.

Table 1. Mean values and number of sperm heads in each generated cluster for length, width, area, perimeter and width/length for spermatozoa thawed at 37 °C for 20 s.

	Cluster				
Parameter	1	2	3	4	5
Length (µm)	8.21	7.91	8.56	8.13	7.68
Width (µm)	4.68	4.44	4.84	4.39	4.04
Area (µm^2)	31.74	29.18	33.97	29.50	26.00
Perimeter (µm)	24.93	24.07	26.62	27.12	24.31
Width/length	0.57	0.56	0.57	0.54	0.53
N	1144	994	680	612	568
Percentage	28.6	24.8	17.0	15.3	14.1

Table 2. Mean values and number of sperm heads in each generated cluster for length, width, area, perimeter and width/length for spermatozoa thawed at 70 °C for 5 s.

	Cluster				
Parameter	1	2	3	4	5
Length (µm)	8.04	8.40	7.67	7.99	8.35
Width (µm)	4.50	4.73	4.17	4.23	4.61
Area (µm^2)	30.07	32.81	26.76	28.04	31.94
Perimeter (µm)	24.32	25.40	23.62	26.57	28.21
Width/length	0.56	0.57	0.55	0.53	0.55
N	1562	889	560	544	433
Percentage	39.2	22.3	14.0	13.6	10.8

Table 3. Mean values of area (A), perimeter (P), length (L), width (W), W/L and Shape factor for the major clusters of spermatozoa thawed using two different procedures.

Thawing	Assessed Parameters					
Procedure	A (µm^2)	P (µm)	L (µm)	W (µm)	W/L	Shape factor
37°C/20 s	31.74 (2.5)[a]	24.93 (3.2)[a]	8.21 (4.5)[a]	4.68 (4.7)[a]	0.571 (7.9)[a]	1.76 (7.7)[a]
70°C/5 s	30.07 (3.2)[a]	24.32 (3.8)[a]	8.04 (4.8)[a]	4.50 (5.1)[a]	0.562 (8.4)[a]	1.79 (8.3)[a]

Coefficients of variation (% CV) are shown in parentheses.
[a] Values within columns are significantly different ($P < 0.001$).

Regardless of the thawing protocol, our results showed that the percentage of spermatozoa within the major cluster for thawed samples was inversely proportional to the percentage of sperm motility in those same samples ($R = - 0.33$; $P = 0.039$; Figure 2).

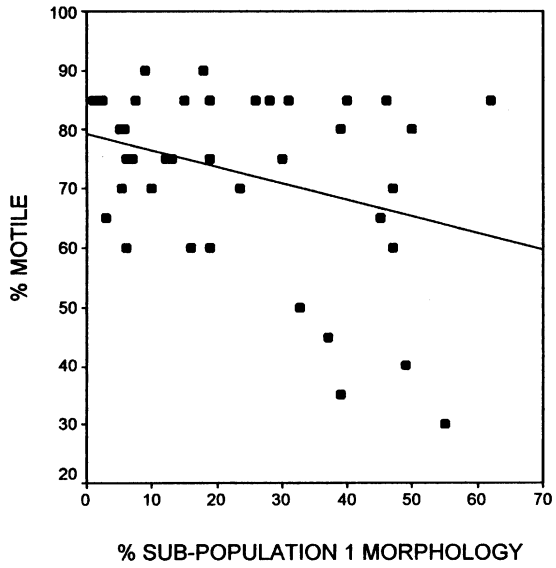

Figure 2. Correlation between the percentage of spermatozoa within major clusters in thawed samples and percentage of sperm motility in those same thawed samples (R = - 0.33; P = 0.039).

DISCUSSION

In this study, the results show that epididymal red deer spermatozoa exhibited the maximum percentage of sperm motility at thawing when they were slowly warmed ($P < 0.05$). Similar results have been observed earlier in ejaculated red deer spermatozoa (16).

Moreover, our results indicate that the thawing procedure have a significant effect on the morphometry of deer sperm head across a limited population of 10 stags. Sperm head dimensions of spermatozoa thawed using the rapid protocol (70 °C for 5 s) were significantly lower than those of spermatozoa thawed using the slow procedure (37 °C for 20 s) across all stags. Taken together, results indicated that spermatozoa thawed using the slow procedure, in addition to being the ones which better resist the freezing process, are also those with greater dimensions of sperm heads, with the opposite being true for spermatozoa thawed using the rapid protocol. Thus, the lower the sperm cryodamage, the greater the sperm head dimensions. Our results agree with earlier findings reported for bull (7), human (19) and stallion spermatozoa (1) in which sperm head dimensions (length, width, area and perimeter) were smaller in samples exposed to cryopreservation stress than in fresh-extended samples. The possible underlying mechanisms that may explain these differences in sperm head dimensions between thawing procedures have been given in previous papers (2,7,18). Because our research did not touch on the morphology of the nucleus and acrosome separately, we cannot provide evidence of the contribution of these two sperm compartments to the morphometric differences found between the two thawing procedures. One possible hypothesis for the differences in sperm head dimensions may be an increase in the number of spermatozoa in which acrosomal damage or loss occurred during cryopreservation and thawing (18). The difference in morphometric dimensions between the two thawing procedures observed in this study may also be associated with changes in the sperm chromatin structure (7). Studies investigating chromatin condensation and morphology of spermatozoa suggest that abnormal chromatin condensation may be reflected by or lead to morphological abnormalities. A variety

of spermatozoal injury, such as heat stress and cryopreservation can induce changes in sperm chromatin structure, resulting in acid-induced denaturation (2). However, in our study no significant thawing effect was found between the two protocols for sperm dimensions within stags for all males (Figure 1A). Sperm head dimensions were higher for thawing procedure I for most of stags, nearly the same in other ones and smaller for only two males (No. 8 and No.10).

The use of multivariate statistical methods, including cluster analysis, to define sperm morphological classes has been used in several species (6,20). This is a very useful and powerful statistical tool for determining which parameters and sperm classes should be considered in the morphometrical evaluation of the reproductive quality of species or samples with a high variation in the spermatozoa present in the semen. When multivariate analysis was applied to the groups of sperm head dimensions, discrete subpopulations (clusters) were generated based on the set number of five clusters. In the analysis of subpopulations of sperm head dimensions, significant differences in the values for length, width, area and perimeter were found between the major subpopulations of spermatozoa thawed using two different protocols. The mean values for sperm head dimensions of the major cluster of spermatozoa thawed using protocol I were significantly larger than those of the major cluster of the group thawed using protocol II. These findings emphasize our previous results related to the effect of the rapid thawing procedure (II) on sperm heads dimensions.

Additionally, thawing procedure II produced a dramatic loss of heterogeneity. In this sense, the percentage of sperm heads falling into the major cluster from thawing procedure II was 39.2% (Table 1) compared with 28.6% (Table 2) in the major cluster of sperm head dimensions of spermatozoa thawed using procedure I. Moreover, for thawing procedure I the greatest number of spermatozoa fell into cluster 1 for six stags while the largest membership for another three stags was found in cluster 2 (data not shown). However, for thawing procedure II the greatest number of spermatozoa fell into cluster 1 for eight stags (data not shown). The loss of heterogeneity in a thawed sample was inversely correlated to the percentage of sperm motility in those same samples (Figure 2). Therefore, the rapid thawing procedure produces a homogeneous sperm sample, which leads to a loss of functional versatility and capacity to respond to different situations. As can be seen in our study, results showed that the higher the loss of heterogeneity, the higher the sperm cryodamage. Similarly, it has been reported in ram spermatozoa that cryopreservation produces a dramatic reduction of heterogeneity (11). This loss of heterogeneity was lower when the extender used exhibited a significantly higher cryoprotection.

In conclusion, our results demonstrate that spermatozoa thawed using the slow procedure (optimum protocol), in addition to being the ones which better resist the freeze-thawing process, are also those with greater dimensions of sperm heads, with the opposite being true for spermatozoa thawed using the rapid protocol. Finally, our results also show that the sperm head morphometric heterogeneity of a thawed sample can be a good indicator of the sperm cryosurvival of the same sample. To the best of our knowledge, this is the first report demonstrating that the sperm head morphometric heterogeneity of individual thawed samples may be used as a good objective indicator of sperm quality at thawing.

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CAPÍTULO 6

Discusión

DISCUSIÓN GENERAL

Efectos de la congelación sobre la morfometría de la cabeza de los espermatozoides epididimarios de ciervo Ibérico

Nuestro estudio demuestra que existe un efecto significativo de la criopreservación sobre la morfometría de la cabeza de los espermatozoides epididimarios de ciervo Ibérico, estudiando una población de 40 machos (Capítulo 2). Las dimensiones medias de las cabezas espermáticas en las muestras congeladas fueron significativamente más pequeñas que las encontradas en las frescas para el área, la longitud y el perfil espermático. Además, la calidad seminal (determinada por SMI, NAR, viabilidad y HOST) también se ve reducida tras el proceso de la congelación. Estos mismos resultados se suman a los encontrados por otros autores que han demostrado este efecto de la congelación sobre la morfometría espermática en hombre (Thompson *et al.* 1994), toro (Gravance *et al.* 1998), caballo (Arruda *et al.* 2002) y perro (Rijsselaere *et al.* 2004), donde las dimensiones de las cabezas espermáticas (longitud, anchura, área y perímetro) fueron también menores en las muestras congeladas que en las frescas. Estas diferencias se han tratado de explicar mediante varios mecanismos, incluyendo cambios osmóticos, daño en el acrosoma y alteraciones en la condensación de la cromatina del espermatozoide (Gravance *et al.* 1998; Love *et al.* 1998; Royere *et al.* 1998; Blottner *et al.* 2001). Sin embargo, nuestro trabajo coincide sólo parcialmente con los resultados obtenidos para macho cabrío (Gravance *et al.* 1997) donde no se encontraron los efectos de la congelación sobre la morfometría de la cabeza espermática en la totalidad de los machos estudiados, pero sí dentro de un número limitado de ellos. Estas diferencias entre estudios pueden ser debidas a una sensibilidad específica de cada especie frente al proceso de congelación o a los diferentes protocolos utilizados para la congelación del semen en los distintos trabajos. Todo ello aparece reflejado en la motilidad espermática (Olar *et al.* 1989; Watson 1995) y en la integridad de la membrana (Garner *et al.* 1988) y del acrosoma (Thomas *et al.* 1998) y en los distintos efectos sobre las características de la cabeza de los espermatozoides evaluados tras la descongelación (Thompson *et al.* 1994; Gravance *et al.* 1998). En nuestra experiencia, únicamente no se produjeron cambios en las dimensiones morfométricas debidos a la congelación en el 25% de los individuos analizados (grupo ND), presentando además estos machos unos parámetros de calidad seminal a la descongelación (SMI y viabilidad) que tendieron a ser superiores a los de los

machos restantes en los que sí aparecieron variaciones en la morfometría espermática tras la congelación (grupo DIF). Esto puede ser indicativo de una mayor crioresistencia de las muestras espermáticas que pertenecen al grupo de machos ND.

Otros resultados que se desprenden de nuestro trabajo, indican que las muestras espermáticas de semen fresco que presentan un menor tamaño en la cabeza de sus espermatozoides, son los que a su vez tienen una tendencia a mostrar una mejor calidad espermática a la descongelación, ocurriendo lo contrario en las muestras que presentan espermatozoides inicialmente más grandes. Nuestros resultados se suman a los de Peña *et al*. (2005) para la especie porcina, los cuales indican que los espermatozoides menos sensibles al proceso de congelación son los que presentan un menor tamaño y una forma más redondeada en el eyaculado de cerdo.

Los coeficientes de variación (CV) de las medidas de la cabeza espermática intra y entre machos (Capítulo 2: tablas 2 y 1) indican que estos parámetros procedentes de diferentes individuos son muy heterogéneos, al menos tanto como lo son las poblaciones espermáticas dentro de un mismo macho (Peña *et al.* 2005; Núñez-Martínez *et al.* 2006). Los análisis del semen tras la descongelación llevados a cabo con técnicas como el sistema de división en dos fases acuosas para detectar diferencias mínimas en las propiedades superficiales, han demostrado que la heterogeneidad se ve muy disminuida tras el proceso de congelación-descongelación (Ollero *et al.* 1998). Al contrario, en nuestro estudio revelamos que existen diferencias significativas en las medias dentro del análisis de los CVs entre semen fresco y congelado para el área y la longitud entre machos, siendo la variabilidad de las medidas de las cabezas espermáticas más pequeña en las muestras frescas que en las congeladas cuando se analizaron todas las muestras. Una posible explicación para las diferencias encontradas en los dos trabajos podría ser que el uso de dos técnicas distintas para evaluar la heterogeneidad espermática puede afectar de forma diferente a los resultados. Alternativamente, cuando analizamos el efecto de la criopreservación sobre el CV de forma separada para los dos grupos de machos (ND y DIF), sólo encontramos diferencias significativas entre las muestras frescas y las congeladas en el grupo DIF. Estos datos indican que la variabilidad de las dimensiones de la cabeza espermática en las muestras congeladas fue significativamente más alta que en las frescas sólo en el grupo DIF (las más afectadas por la congelación). Por tanto, en estos machos, el proceso de congelación-descongelación debería afectar a una mayor población

de células que en el grupo ND, originando un valor más alto de daño celular. Este alto grado de lesión espermática podría ser el responsable de la alta variabilidad en la morfometría del semen congelado en esta población (DIF), dando lugar a la aparición de una mayor heterogeneidad.

En este capítulo se demuestra que la morfometría de la cabeza espermática y la heterogeneidad individual de una muestra fresca pueden usarse como buenos indicadores de la resistencia del semen al proceso de congelación-descongelación.

Significado funcional del tamaño y del perfil de la cabeza espermática en la determinación de la congelabilidad de muestras epididimarias de ciervo Ibérico

La existencia de diferencias individuales en la resistencia de los espermatozoides al proceso de congelación es un hecho perfectamente constatado en diferentes especies domésticas (Curry 2000) y silvestres (Leibo y Songsasen 2002). Dentro de este contexto, los machos se han clasificado de forma rutinaria como “buenos” o “malos” congeladores, en función de la resistencia de sus espermatozoides a este proceso.

Por otro lado, algunos autores han mostrado mucho interés en determinar la relación que existe entre las características individuales del semen fresco (motilidad, viabilidad, morfología y estado del acrosoma) y su congelabilidad (Curry 2000; Leibo *et al.* 2002). La calidad individual de las muestras frescas ha tenido relaciones positivas con la congelabilidad, pero con significación variable según los casos (Thurston *et al.* 2002).

En esta experiencia, se utilizó el sistema ASMA para detectar si existían diferencias en el área y el perfil de la cabeza de los espermatozoides epididimarios de ciervo Ibérico antes del proceso de criopreservación entre los venados clasificados como “buenos” o “malos” congeladores.

En una primera fase, se utilizó un análisis de cluster multivariante (Quintero-Moreno *et al.* 2003) para clasificar a los machos estudiados en dos grupos en función de la congelabilidad de sus muestras espermáticas: “buenos” o “malos” congeladores.

Este estudio fue desarrollado con 38 machos, 25 de los cuales fueron identificados como “buenos” y 13 como “malos” congeladores. Para analizar las diferencias entre los

dos grupos, se llevó a cabo un ANOVA convencional y se encontró que eran significativas para todos los parámetros de calidad en el semen descongelado, así como para el grado de pérdida de integridad celular debido a la criopreservación. Sin embargo, no existieron diferencias significativas en la calidad del semen entre los dos grupos antes de la congelación. Cuando se compararó el tamaño de la cabeza espermática en las muestras frescas de los dos grupos de machos, las menores dimensiones para el área de la cabeza espermática se presentaron en el grupo de los "buenos" congeladores. Además, las cabezas espermáticas en las muestras frescas de este grupo fueron más alargadas y estrechas que las del grupo de los "malos" congeladores. Nuestros datos se suman a los obtenidos por otros autores. Garde *et al.* (2003) compararon la resistencia del semen a la congelación en tres especies de gacelas, las cuales presentaban diferencias en el tamaño de sus espermatozoides. Los resultados que obtuvieron mostraron que el semen que mejor resistió el proceso de congelación fue el que pertenecía a la especie cuyas células espermáticas eran más pequeñas (Cassinello *et al.* 1998). Además, Thurston *et al.* (2001) sugirieron que el porcentaje de espermatozoides que presenta las cabezas ligeramente afiladas en un eyaculado de cerdo se correlaciona de forma positiva con la calidad del semen tras la descongelación. Estos mismos autores (Thurston *et al.* 2002), proponen la hipótesis de que las variaciones inter-individuales en la morfología espermática están genéticamente determinadas y por lo tanto, sus diferencias en el semen fresco, que afectan a su congelabilidad, son indicativas de las variaciones genéticas responsables de la capacidad relativa de los espermatozoides a resistir los procesos de congelación.

Los espermatozoides son capaces de modificar sus propiedades físicas dependiendo de su área y perfil y estos cambios son, al menos parcialmente, responsables de la resistencia inter-individual al proceso de criopreservación (Leibo y Bradley 1999). Nuestra hipótesis se basa en que el tamaño y la forma de la cabeza espermática influyen sobre el volumen total del espermatozoide hasta el punto de causar diferencias en el intercambio de temperatura y en los movimientos de agua, iones y crioprotectores y posteriormente sobre la congelabilidad espermática (Curry 2000). Además, en trabajos anteriores (Curry *et al.* 1996) se ha recogido que el área y el volumen espermáticos, tienen gran importancia en la determinación de los protocolos óptimos de enfriamiento en el proceso de criopreservación. Durante la congelación, el enfriamiento se produce a velocidades finitas (lentas o rápidas). La velocidad de congelación óptima es aquella en la cual la célula puede perder agua y al mismo tiempo se observa la máxima supervivencia

celular. Dicha velocidad es diferente si se trata de semen humano, porcino u ovino (Leibo y Bradley 1999). En este sentido, se han encontrado mejoras en la criosupervivencia del semen de cerdo cuando los espermatozoides son congelados a velocidades más rápidas (Fiser y Fairfull 1990).

Según lo anteriormente expuesto, parece razonable afirmar, basándonos en nuestros resultados, que el área y el perfil de las cabezas espermáticas en las muestras frescas son capaces de discriminar claramente entre dos poblaciones de machos en las que la congelabilidad espermática es diferente. Sin embargo, aunque esta relación existe, no es suficiente para asegurarlo con exactitud ya que aparecen machos que no siguen la pauta definida (Capítulo 3: figura 3). Por tanto, son necesarios más estudios para desarrollar métodos capaces de identificar las muestras seminales según su capacidad de criopreservación. Nosotros proponemos que las muestras espermáticas de distintos machos requieren diferentes velocidades de congelación para una óptima criosupervivencia. Así, los resultados de este estudio sugieren que la congelabilidad de los espermatozoides de individuos considerados como "malos" congeladores se podría incrementar en el futuro usando velocidades de congelación diferentes a la utilizada en este trabajo (20 ºC/min). Esta hipótesis se basa en la importancia que el área y el volumen celular tienen en la determinación de la velocidad de congelación óptima durante la congelación (Curry *et al.* 1996; Leibo y Bradley 1999).

Según los resultados que hemos obtenido hasta ahora, podemos asegurar que los espermatozoides con menores dimensiones en las muestras frescas tienden a no cambiar durante el proceso de congelación (Capítulo 2), siendo además los que presentan mejor congelabilidad (Capítulo 3). Por ello, podemos afirmar que la morfometría espermática de las muestras epididimarias de ciervo Ibérico puede revelar datos importantes que indiquen el potencial de congelabilidad de una muestra dada.

Identificación de subpoblaciones espermáticas con características morfométricas diferentes en muestras epididimarias de ciervo Ibérico

En el Capítulo 3 de esta Tesis Doctoral se han expuesto las dimensiones normales de la cabeza de los espermatozoides epididimarios de ciervo, tanto en muestras precongeladas como en descongeladas. Además, también se ha comprobado que el

proceso de criopreservación reduce el tamaño de los espermatozoides de ciervo (Capítulo 2). Por todo ello, en el Capítulo 4 se utilizó un sistema CASMA (SCA) para tratar de identificar subpoblaciones espermáticas morfométricamente diferentes, analizar su comportamiento tras el proceso de criopreservación y su relación con la congelabilidad de muestras epididimarias de ciervo Ibérico. Hasta el momento, no existen trabajos previos que aborden esta cuestión en machos de la especie referida.

Nuestros resultados han permitido identificar la existencia de tres subpoblaciones espermáticas según su morfometría (espermatozoides grandes, medianos y pequeños) utilizando el SCA junto a un análisis de cluster multivariante, demostrándose así la heterogeneidad que existe en este tipo de muestras. Estudios previos han permitido utilizar otros sistemas para clasificar las cabezas espermáticas (Gravance *et al.* 1996; Thurston *et al.* 2001). Sin embargo, esta es la primera descripción de subpoblaciones en espermatozoides de ciervo basadas en parámetros morfométricos derivados directamente de un análisis CASMA.

En esta experiencia se ha empleado el modelo k-means dentro de un análisis de cluster, que es eficaz cuando existe una gran cantidad de datos (10.867 observaciones). Presenta el inconveniente de ajustar el número de conglomerados antes de realizar el análisis. Por otro lado, una gran ventaja de este modelo es la sencilla detección de valores extremos (Peña *et al.* 2005).

Las tres subpoblaciones identificadas fueron diferentes en cuanto a tamaño y forma de los espermatozoides. Más del 20% (21,5%) de las células analizadas se incluyeron dentro de SP_1, presentando los parámetros morfométricos medianos. A la descongelación dicho grupo aumentó hasta un 24,22%. Por otro lado, SP_2 incluyó a los espermatozoides con las menores dimensiones. Esta subpoblación representaba el 29,92% a la precongelación, mientras que a la descongelación este valor aumentó hasta el 32,8%. Finalmente, la subpoblación más numerosa (49% en las muestras frescas y 43% en las descongeladas) fue SP_3, compuesta por los espermatozoides de mayor tamaño. Estos datos indican que las muestras son heterogéneas y que se pueden identificar subpoblaciones espermáticas utilizando diferentes parámetros (Martínez-Pastor *et al.* 2005).

Estos datos indican que las muestras son heterogéneas, y que las subpoblaciones espermáticas pueden ser identificadas utilizando diferentes parámetros (Martínez-Pastor *et al.* 2005).

Los resultados obtenidos en este estudio mostraron gran variabilidad entre machos en cuanto a la proporción de espermatozoides dentro de cada una de las tres subpoblaciones. El origen de estas subpoblaciones no está del todo claro. En este sentido, estudios previos en ratón han demostrado que existe un componente genético responsable de la variabilidad en la morfología de los espermatozoides (Beatty y Sharma 1960). Estas variaciones en la morfología espermática se producen durante la espermatogénesis, cuando el genotipo aún actúa sobre la estructura espermática. El fenotipo de la morfometría de los espermatozoides estaría controlado por genes transcritos durante la fase premeiotica del desarrollo (Burgoyne 1975). Además, los coeficientes de consanguinidad se han relacionado con la baja calidad del eyaculado, demostrando así el control genético de la morfología espermática (Roldán *et al.* 1998). Este hecho, junto a la fácil identificación de las diferencias que existen en las muestras espermáticas entre machos, nos permitiría identificar a los más adecuados para los diversos procedimientos biotecnológicos, tales como la criopreservación espermática. Sin embargo, no se encontraron relaciones entre la proporción de espermatozoides dentro de cada una de las tres subpoblaciones con la viabilidad de las muestras evaluadas antes y después de la criopreservación. Estos resultados son contradictorios a a los encontrados en otras especies (Thurston *et al.* 2001; Peña *et al.* 2005). El estudio de Thurston *et al.* (2001) reveló que la proporción de espermatozoides con cabezas ligeramente afiladas presentes en el eyaculado se relacionaba de forma positiva con la calidad seminal a la descongelación. Las diferencias en los resultados podrían deberse a varios factores, como son el origen de los gametos, diferentes protocolos de congelación o un posible factor especie-específico en la sensibilidad al proceso de criopreservación (Thompson *et al.* 1994; Gravance *et al.* 1998). Por otro lado, diferentes técnicas de evaluación de subpoblaciones espermáticas podrían dar lugar a resultados distintos.

Los resultados del análisis de los 37 machos muestran que la proporción de espermatozoides presentes en las tres subpoblaciones permanece constante ($p > 0,05$) tras el proceso de congelación. En general, los resultados obtenidos en este trabajo muestran que la criopreservación no afecta a la totalidad de las proporciones de espermatozoides

presentes en cada subpoblación. Con detalle, pudimos observar que la proporción de células que se encontraban en SP_1 y en SP_2 aumentaba en las muestras descongeladas con respecto a las frescas. Además, la proporción de SP_3 mostraba una tendencia opuesta a la de SP_1, disminuyendo tras la descongelación. También advertimos que el efecto de la criopreservación sobre la distribución en las subpoblaciones de los espermatozoides de ciervo variaba entre individuos. Por todo ello, clasificamos a los machos según el efecto de la congelación sobre la frecuencia de distribución de cada subpoblación. En cada una de ellas, los machos se clasificaron dentro del grupo denominado "de reducción" cuando el porcentaje de espermatozoides en esa subpoblación disminuía tras la descongelación; y dentro del grupo "de no reducción" cuando este hecho no se producía.

La mayoría de los machos analizados (60%) presentaba una disminución en el porcentaje de la subpoblación de los espermatozoides medianos (SP_1) durante el proceso de criopreservación, ocurriendo lo contrario con los pequeños (SP_2) y con los de mayor tamaño (SP_3). En general, los machos en los que se reducía la proporción SP_3 y en los que aumentaba el porcentaje de SP_1 la congelabilidad de las muestras fue peor. Por ello, podemos asumir que los cambios producidos en estas dos subpoblaciones tras el proceso de criopreservación son buenos indicadores de la congelabilidad de una muestra.

Una vez conocidos los cambios que se producen en la morfometría de los espermatozoides tras la congelación, pueden ayudarnos a comprender las circunstancias fisiológicas que rodean al proceso de criopreservación. SP_3 se caracteriza por presentar los espermatozoides con la cabeza más alargada y ancha (los de mayor tamaño). Cuando esta subpoblación disminuye, estaría expresando una degeneración en la estructura de la cabeza espermática debida a la criopreservación y asociada con procesos de muerte celular. Este proceso puede relacionarse con los bajos valores de congelabilidad encontrados en esos machos. Las diferencias en la morfometría de la cabeza espermática producida por el proceso de criopreservación, como ya se comentó anteriormente, se relacionan con cambios osmóticos, daño en el acrosoma y en las membranas y alteraciones en la condensación de la cromatina (Gravance *et al.* 1998).

La conclusión más relevante del Capítulo 4 de esta Tesis Doctoral, es que los machos que presentan mejor congelabilidad son aquellos en los que no disminuye la proporción de SP_3 (espermatozoides grandes), produciéndose lo contrario para SP_1 (espermatozoides

medianos). Puede decirse que cuando la proporción de espermatozoides en SP_3 disminuye tras la descongelación, estas mismas células incrementan el porcentaje de SP_1 ($R= -0,76$; $p < 0,0001$). Por ello, las muestras espermáticas donde ocurre un aumento de SP_1 presentan valores de congelabilidad menor, debido a un incremento en los procesos de muerte celular (Marco-Jiménez *et al.* 2006).

Efecto del proceso de descongelación sobre las dimensiones de los espermatozoides descongelados de ciervo Ibérico

Los resultados obtenidos en este estudio muestran que los espermatozoides epididimarios de ciervo Ibérico presentan un porcentaje máximo de motilidad a la descongelación cuando este proceso se produce lentamente, tal y como afirmaron también otros autores para semen eyaculado (Soler *et al.* 2003a) y de epidídimo de la misma especie (Soler *et al.* 2003b). Además, el procedimiento de descongelación tiene un efecto significativo sobre la morfometría de la cabeza espermática del ciervo en una población limitada de 10 animales. Así, las dimensiones de la cabeza espermática en muestras descongeladas usando un protocolo de descongelación lento (37 ºC durante 20 segundos) fueron significativamente mayores que las de los espermatozoides descongelados de forma rápida (70 ºC durante 5 segundos), y fueron también las que menor daño sufrieron durante el proceso de congelación-descongelación al asumir que la criopreservación reduce sustancialmente el tamaño de la cabeza espermática, como indican varios autores en estudios realizados en hombre (Thompson *et al.* 1994), toro (Gravance *et al.* 1998) y caballo (Arruda *et al.* 2002).

Una posible hipótesis para intentar explicar los resultados obtenidos, puede ser la existencia de un mayor número de espermatozoides que han sufrido daño en el acrosoma o lo han perdido durante el proceso de congelación-descongelación rápido (Thomas *et al.* 1998). Las diferencias observadas en las dimensiones espermáticas pueden estar asociadas también a cambios en la estructura de la cromatina (Gravance *et al.* 1998). Estudios previos sobre la condensación de la cromatina y morfología del espermatozoide sugieren que una condensación anormal puede asociarse a posibles anomalías morfológicas. Cualquier tipo de daño en el espermatozoide, como el estrés debido a la congelación, puede provocar cambios en la estructura de la cromatina, dando lugar a una desnaturalización ácido-inducida (Blottner *et al.* 2001).

En nuestro trabajo no estudiamos de forma independiente la morfología del núcleo y del acrosoma, por ello no podemos identificar la contribución de cada una de estas estructuras celulares a las diferencias morfométricas encontradas entre los dos procedimientos de descongelación.

En esta experiencia fue aplicado un análisis de cluster multivariante y se generaron 5 subpoblaciones espermáticas dentro de cada uno de los dos protocolos de descongelación (Capítulo 5: tablas 1 y 2). Estos métodos estadísticos han sido utilizados en varias especies para definir clases o subpoblaciones de espermatozoides en función de sus dimensiones (Gravance *et al.* 1996; Thurston *et al.* 2001). Estos métodos estadísticos son muy útiles y potentes a la hora de determinar qué parámetros y clases espermáticas podrían considerarse en la evaluación morfométrica de la calidad reproductiva de especies o muestras con una alta variación en los espermatozoides presentes en el semen (Peña *et al.* 2005; Núñez-Martínez *et al.* 2006).

Según nuestros resultados, los valores medios de longitud, anchura, área y perímetro espermáticos dentro de la subpoblación más numerosa de cada tratamiento fueron significativamente superiores en los descongelados de forma lenta (Capítulo 5: tabla 3).

La subpoblación principal en el protocolo de descongelación rápido estuvo compuesta por un mayor número de espermatozoides que la misma en el proceso de descongelación lento (Capítulo 5: tablas 1 y 2). Por tanto, podemos afirmar que la descongelación rápida dio lugar a una muestra espermática más homogénea que se acompañaba con un menor porcentaje de motilidad a la descongelación.

La criopreservación produce también una gran pérdida de heterogeneidad en los espermatozoides de morueco (Ollero *et al.* 1998), lo que indica que el daño sufrido durante el proceso de congelación-descongelación favorece la pérdida de versatilidad funcional y reduce la capacidad para responder a diferentes situaciones (Gravance *et al.* 1998).

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CAPÍTULO 7

Conclusiones

CONCLUSIONES

- La criopreservación produce una disminución en las dimensiones de la cabeza del espermatozoide epididimario de ciervo ibérico.

- Los espermatozoides de menor tamaño en las muestras frescas tienden a no cambiar sus dimensiones tras el proceso de criopreservación, siendo además los que presentan una mayor resistencia a la congelación.

- Los machos que mejor resisten el proceso de congelación espermática (buenos congeladores) se caracterizan por presentar en las muestras recién obtenidas, espermatozoides de menor tamaño y más alargados que los machos catalogados como malos congeladores.

- Es posible determinar la existencia de subpoblaciones con características morfométricas diferentes en las muestras espermáticas epididimarias de ciervo.

- La criopreservación reduce el porcentaje de la subpoblación formada por los espermatozoides que presentan cabezas más alargadas y anchas, por lo tanto los de mayor tamaño.

- Existe gran variabilidad entre machos en cuanto a la proporción de espermatozoides encontrada dentro de cada subpoblación y en el cambio en la distribución de las frecuencias que se produce debido a la criopreservación.

- Un protocolo de descongelación lento produce un menor daño sobre los espermatozoides epididimarios de ciervo Ibérico en el proceso de criopreservación, que se ve reflejado en una mejor motilidad y unas mayores medidas morfométricas de las células espermáticas, comparándolo con un protocolo de descongelación rápido.

- La variabilidad (heterogeneidad) de las medidas morfométricas de una muestra espermática puede ser predictiva de los cambios que se producen en la misma durante el proceso de congelación.

CAPÍTULO 8

Resumen General

RESUMEN GENERAL

Los sistemas de análisis morfométrico de las cabezas de los espermatozoides (ASMA o CASA) proporcionan una serie de parámetros que pueden ayudar a establecer los criterios necesarios para clasificar una muestra espermática de un macho, de una determinada especie como “normal” en base a las medidas de sus espermatozoides y a predecir la resistencia a la congelación y la fertilidad de las dosis seminales.

En esta Tesis Doctoral se ha empleado el sistema de análisis ASMA como herramienta para estudiar el efecto de la criopreservación sobre la morfometría de la cabeza de los espermatozoides y su posible utilidad en la determinación de la congelabilidad de las muestras epididimarias de ciervo Ibérico.

Así, en el Capítulo 2 de este trabajo se estudiaron los efectos de la congelación sobre la morfometría de la cabeza de los espermatozoides epididimarios de ciervo Ibérico. Según los resultados encontrados, las cabezas de los espermatozoides en las muestras congeladas eran más pequeñas que en las frescas en el 75% de los machos analizados. En el 25% de los machos restantes las dimensiones espermáticas no cambiaron con la criopreservación, presentando además un tamaño menor en fresco y mejores parámetros de calidad seminal a la descongelación en sus espermatozoides. La variabilidad individual en la morfometría de la cabeza espermática se correlacionó de forma negativa con el porcentaje de cambio en las medidas tras la congelación. Por lo tanto, podemos sugerir que la heterogeneidad en los parámetros morfométricos de la cabeza de los espermatozoides epididimarios de ciervo ibérico puede ser un buen indicador de la congelabilidad de su semen.

En el Capítulo 3 se utilizó este mismo sistema para determinar si el área y el perfil de la cabeza de los espermatozoides epididimarios de la especie referida podrían usarse para diferenciar entre los machos clasificados como “buenos” o “malos” congeladores. Se evaluó la supervivencia espermática tras la descongelación y se originaron estos dos grupos de individuos en función de la congelabilidad de sus muestras. Además, en general, los “buenos” congeladores presentaban las cabezas espermáticas más pequeñas y alargadas que las de los “malos” en las muestras frescas. Por ello, podemos afirmar que la

morfometría espermática de las muestras epididimarias de ciervo Ibérico puede revelar datos importantes que indiquen el potencial de congelabilidad de una muestra dada.

En el Capítulo 4 identificamos tres subpoblaciones espermáticas (SP_1, SP_2, SP_3) de acuerdo con las características morfométricas en muestras epididimarias de ciervo Ibérico (espermatozoides de tamaño mediano, pequeño y grande respectivamente), su comportamiento tras el proceso de criopreservación y su relación con la congelabilidad de la muestra. Para ello, evaluamos los parámetros de calidad espermática antes y después del proceso de congelación y observamos una disminución en ellos tras dicho proceso. Según nuestros resultados el porcentaje relativo de estas subpoblaciones era diferente entre machos, reflejando una gran variabilidad entre ellos. La proporción de espermatozoides presente en cualquiera de las tres subpoblaciones permaneció constante a través del proceso de criopreservación aunque mostraba una tendencia a ser positiva para SP_1 y SP_2 y a ser negativa para SP_3. El efecto producido por la congelación en la distribución de cada una de las subpoblaciones resultó ser diferente en las muestras frescas y descongeladas y variaba dentro de machos en la totalidad de ellos. Además, el cambio producido en la distribución de las células espermáticas dentro de SP_1 y SP_3 debido a la criopreservación afectó a los parámetros de calidad espermática tras la descongelación. Por tanto, concluimos que ASMA resulta útil para detectar diferencias morfométricas sutiles entre espermatozoides. Su combinación con un análisis multivariante revela nueva información sobre las características criobiológicas de los espermatozoides epididimarios de ciervo, no aportada hasta ahora por los estudios espermáticos convencionales.

En el Capítulo 5 evaluamos los efectos del proceso de descongelación sobre el tamaño de la cabeza espermática y la distribución en distintas subpoblaciones. Para ello, utilizamos dos protocolos de descongelación diferentes (lento y rápido). Según nuestros resultados la descongelación rápida resultó ser más agresiva para los espermatozoides, hecho que se reflejó en una mayor disminución de la motilidad y de las dimensiones espermáticas. Además, se generaron 5 subpoblaciones dependiendo de la morfometría espermática dentro de cada uno de los protocolos de descongelación empleados. La subpoblación principal (la más numerosa) dentro de la descongelación rápida estaba compuesta por un mayor número de espermatozoides que la misma dentro de la descongelación lenta. Por

tanto, este proceso de descongelación originó una muestra espermática más homogénea en cuanto a las características morfométricas. Para concluir, podemos afirmar que la mayor pérdida de heterogeneidad y el mayor grado de daño espermático debido a la descongelación se producen cuando se utiliza el protocolo de descongelación rápido frente al lento.

Por todo lo anteriormente expuesto, podemos decir que el SCA nos permite estudiar el efecto de la criopreservación sobre la morfometría de la cabeza de los espermatozoides y establecer su relación con la congelabilidad del semen.

GENERAL SUMMARY

Automated analysis systems for the assessment of sperm head morphometry (ASMA or CASA) provide many parameters that can be used to determine the normal morphometry in a species, therefore predicting the ability to survive cryopreservation and the fertility of the seminal doses of a given male from the morphometry of its spermatozoa.

In the present Doctoral Thesis, the ASMA has been used for studying the effect of cryopreservation on the sperm head morphometry, and its possible utility for the determination of the freezability of epididymal sperm from Iberian red deer.

Thus, Chapter 2 treat on the effects of freezing on the head morphometry of epididmal spermatozoa from Iberian red deer. According to the results, sperm heads in cryopreserved samples were smaller than in the fresh samples in 75% of the studied males. The remainder 25% did not show a change of sperm dimensions after cryopreservation, showing a smaller size in fresh samples and better quality parameters after thawing. Here, individual variability in the morphometry of the sperm head was negatively correlated with the percentage of change in the measures after thawing. Therefore, we suggest that the heterogeneity in the morphometric parameters of epididymal spermatozoa from Iberian red deer can be a good indicator of the freezability of the semen.

In Chapter 3 ASMA was used to determine if the area and shape of the sperm heads could be used to discriminate males among “good” and “bad” freezers. We evaluated sperm survival after thawing and separated males among these two groups, according to the freezability of their samples. In general, the sperm heads from “good” freezers in fresh samples were smaller and more elongated than those from “bad” freezers. Thus, we can assert that sperm morphometry of epididymal samples from Iberian red deer can reveal important data related tot the potential freezability of a given sample.

In the Chapter 4 we identified three sperm subpopulations (SP_1, SP_2, SP_3), according to the morphometric characteristics in epididymal samples of Iberian red deer (spermatozoa of medium, small and large size, respectively), their changes after the cryopreservation

process, and its relathionship with the freezability of the sample. To identify them, we evaluated the sperm quality parameters before and after cryopreservation, noticing that they decreased after the process. Our results indicated that the relative percentage of these subpopulations was different between males, reflecting a great variability among them. The proportion of spermatozoa in each of the three subpopulations remained constant along the cryopreservation process, showing a trend towards increasing for SP_1 and SP_2, and towards decreasig for SP_3, though. The distribution of each subpopulation was different for fresh and cryopreserved samples, and varied within males. Moreover, the change in the distribution of spermatozoa in SP_1 and SP_3 due to cryopreservation affected to the sperm quality parameters after thawing. Therefore, we conclude that ASMA is useful for detecting subtle morphometric differences between spermatozoa. The combination of this technique with multivariate analysis revealed new information on the cryobiology of epididymal spermatozoa from Iberian red deer, that has not been provided by conventional semen assessment.

In Chapter 5 we evaluated the effects of the thawing process on head sperm size and the distribution of different subpopulations in epididymal samples from Iberian red deer. Therefore, we used two thawing protocols (slow and rapid). According to our results, rapid thawing was more aggressive for spermatozoa, reflecting into a higher decrease of motility and sperm size. Besides, we found five sperm subpopulations depending on the sperm morphometry within each thawing protocol. The most prominent subpopulation (largest) within rapid thawing included more spermatozoa than the same one within the slow thawing protocol. Therefore, the fast thawing protocol rendered a sperm sample more homogeneous regarding the morphometric characteristics. In conclusion, we can state that a higher heterogeneity loss and a higher degree of sperm damage due to thawing were produced when the rapid thawing protocol was used against the slow one.

Summarizing, we can state that the ASMA allows us studying the effect of cryopreservation on the head morphometry of epididymal spermatozoa from Iberian red deer, and to relate it to sperm freezability.