DEPARTAMENTO DE MEDICINA, CIRUGÍA Y ANATOMÍA VETERINARIA

ESTUDIO DEL FACTOR TÉRMICO EN LOS PROTOCOLOS DE CRIOCONSERVACIÓN DEL SEMEN DE OSO PARDO (*Ursus arctos*)

Evaluation of the temperature handling during cryopreservation of brown bear (*Ursus arctos*) semen

Memoria presentada para la obtención del grado de Doctor por la Universidad de León por la Licenciada en Veterinaria

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INFORME DE LOS DIRECTORES DE TESIS

El Dr. Luis Anel Rodríguez, la Dra. Mercedes Álvarez García y el Dr. Paulino de Paz Cabello, como Directores de la Tesis Doctoral titulada "ESTUDIO DEL FACTOR TÉRMICO EN LOS PROTOCOLOS DE CRIOCONSERVACIÓN DEL SEMEN DE OSO PARDO (*Ursus arctos*)" realizada por Dña. Elena López Urueña, en el programa de Sanidad Animal y Reproducción, informan favorablemente el depósito de la misma, dado que reúne las condiciones necesarias para su defensa.

Lo que firman, en León a 26 de noviembre de 2015.

Los Directores de la Tesis Doctoral

Fdo: Luis Anel **Fdo: Mercedes Álvarez** **Fdo: Paulino de Paz**

A mis padres

A mi hermano

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TABLA DE CONTENIDOS

RESUMEN

Las limitaciones que impone la recogida en condiciones de campo de los eyaculados de osos pardo, justifica la necesidad de redefinir los protocolos clásicos de crioconservación espermática. Una de las necesidades más perentorias podría residir en la generación de tiempos muertos en el periodo de precongelación de los eyaculados, lo que propiciaría la posibilidad de transportar las muestras a centros tecnológicos de referencia para la aplicación de biotecnologías de mejoramiento seminal (por ejemplo el sex-sorting) antes de la congelación, incrementando de este modo la efectividad de los Bancos de Recursos Genéticos.

En esta Tesis Doctoral, se han evaluado diferentes pautas de manejo precongelación del semen de oso pardo obtenido mediante electroeyaculación: 1) la aplicación de diferentes rampas de refrigeración y la simplificación de los protocolos (eliminación del tiempo de equilibrado sólo o con el periodo de refrigeración); 2) el almacenamiento a corto o largo plazo a 5 °C de las muestras espermáticas; 3) el control de la temperatura, el porcentaje de glicerol o la tasa de dilución, en el almacenamiento a largo plazo; 4) el uso de medio sólido para mejorar la calidad durante el almacenamiento a largo plazo.

En la evaluación del efecto de la velocidad de refrigeración y tiempo de equilibrado sobre la calidad postdescongelación, se han testado tres rampas de refrigeración (0,25, 1 y 4 °C/min), dos tiempos de equilibrado (0 y 1 h) y la eliminación de los periodos de refrigeración y tiempo de equilibrado (congelación directa) frente al protocolo estándar (refrigeración a 0,25 °C/min y 1 h de equilibrado, control). En la primera experiencia, los resultados indican que los valores postdescongelación disminuyen al incrementar la velocidad de refrigeración, obteniendo la rampa de 4 °C/min peor calidad respecto a la rampa lenta (VIAB, dACR y movilidades), y ambas similares a la velocidad intermedia (1 °C/min). Las muestras seminales equilibradas a 5 °C durante 1 h presentaron mayores valores de calidad postdescongelación (% acrosomas dañados -dACR-) y postincubación (% viability -VIAB-, movilidad total y progresiva -MT and MP-) que las no equilibradas. Además, se observa un daño en los espermatozoides conservados mediante congelación directa comparada con el control, daño que se hace más evidente tras la incubación (test de estrés térmico -ThS test-) para viabilidad, estado acrosomal y movilidad. Tras el análisis global de los datos, se recomiendan las rampas de refrigeración lentas (hasta 1 °C/min) y al menos 1 hora de equilibrado para una crioconservación efectiva de los espermatozoides de oso pardo.

Tras esta primera aproximación sobre el tiempo de equilibrado, en el segundo trabajo se aborda, más en profundidad, el estudio de este paso previo a la congelación; para ello, se evalúan diferentes tiempos de equilibrado (0, 0,5, 1 -

control-, 4-5, 7-8 y 10-12 h) y tres tiempos de almacenamiento a largo plazo (24, 48 y 72 h) frente al control (1 h), a 5 °C. En la primera experiencia, no se encontraron diferencias significativas entre los tiempos testados, salvo el grupo de 0 h que mostró valores de acrosomas intactos (iACR), MT y MP más bajos. En el estudio del almacenamiento a largo plazo, se observa una caída de la calidad postdescongelación del semen de oso pardo marcada y progresiva desde las 48 hasta las 72 h. Consecuentemente, el periodo de refrigeración precongelación se puede alargar hasta las 24 h lo que permite no congelar las muestras seminales inmediatamente después de la recogida.

En un tercer paso para redefinir los protocolos clásicos de semen de oso pardo, se evalúan las condiciones durante el almacenamiento a largo plazo, para ello, analizamos el efecto de tres temperaturas (temperatura ambiente -TA-, 15 y 5 °C), tres porcentajes de glicerol (0, 3 y 6 %) y dos tasas de dilución (1:1 -1782×10^6 spz/mL de concentración media- y final -a 100×10^6 spz/mL), hasta las 48 h. Con la temperatura de 5 °C se alcanzaron los mayores resultados en VIAB, MP e iACR a las 24 h, y en MT, MP y YOPRO- (viables y no apoptóticos) tras las 48 horas, en comparación con las otras temperaturas. Para el porcentaje de glicerol, en las primeras 24 h, la calidad post-descongelación se incrementó con el porcentaje utilizado; después de las 48 horas, el 6 % de glicerol presentó efectos beneficiosos en la crioconservación. Además, este porcentaje tuvo una congelabilidad claramente superior (viabilidad y movilidad). Ambas tasas de dilución obtuvieron similares datos dentro de las primeras 24 h, pero la dilución final mantuvo la mejor calidad post-descongelación a las 48 h. Ambas tasas de dilución presentaron similar congelabilidad, excepto tras las 48 h, donde la dilución final logró una mayor tasa de recuperación para la viabilidad. Estos hallazgos sugieren que las mejores condiciones para el almacenamiento a largo plazo (hasta las 48 h) de los electroeyaculados de oso pardo son: una temperatura de 5 °C, un porcentaje de glicerol del 6 % y a una concentración de 100×10^6 spz/mL.

Por último, para mejorar la calidad post-descongelación de las muestras seminales almacenadas a largo plazo (hasta 48 h), se estudia el efecto del estado sólido (gelatina 1,5%) y su "particular" manejo, por lo que se testan cuatro grupos experimentales: 1) dilución 1:1 con el diluyente estándar a temperatura ambiente, refrigeración en tubo, dilución final a 5 °C (Control 1, 24 and 48 h); 2) dilución final con el diluyente estándar a temperatura ambiente, refrigeración en tubo ("FD-Tube"); 3) dilución final con el diluyente estándar a temperatura ambiente, refrigeración en pajuela ("FD-Straw"); y 4) dilución final con el diluyente estándar suplementado con un 1,5 % de gelatina, refrigeración en pajuela ("Gel"). En la valoración precongelación tras las 48 h de almacenamiento, Gel logró mayor viabilidad, y progresividad aunque menor velocidad (VAP). Al análisis postdescongelación, Gel alcanzó mayor VIAB, YOPRO-, iACR y LIN, pero menor VAP

y ALH, independientemente del tiempo de almacenamiento. No se observan diferencias entre el resto de los grupos experimentales (Control 24/48 h, FD-Tube y FD-Straw). Estos resultados muestran que el estado sólido proporcionado por la gelatina, podría ser una alternativa adecuada para conservar la viabilidad y progresividad de los espermatozoides de oso pardo almacenados a 5 °C.

SUMMARY

Brown bear ejaculates are usually collected under field conditions, so to redefine the classic cryopreservation protocol from this species sperm could be necessary to ship them to reference laboratory for cryopreservation or reproductive biotechnologies (e.g. sex-sorting) before freezing, which could improve the effectiveness of Genetic Resource Bank of brown bear spermatozoa.

Throughout this work, we assessed different cooling and freezing guidelines of brown bear sperm collected by electroejaculation: 1) cooling rates and simplification of protocols (the omission of equilibration time alone or with cooling period); 2) short or long term storage at 5° C of spermatic samples; 3) control of temperature, glycerol percentage or dilution rate in long-term storage; 4) the use of solid state to improve sperm quality during long-term storage.

For assessment the effect of cooling rates and equilibration time before freezing on post-thawing quality, we tested three cooling rates (0.25, 1 y 4 °C/min), two equilibration times (0 y 1 h) and the omission of cooling and equilibration periods ("direct freezing") versus standard freezing (cooling at 0.25 °C/min and equilibration for 1 h, control). In first experience, our results indicated post-thawing scores dropped upon the increase of the cooling rate, rapid cooling rate (4 °C/min) yielded the worst quality scores respect slow one, and both were similar to rate of 1°C/min. Seminal samples kept at 5 °C for 1 hour showed higher results compared with the nonequilibrated ones for both post-thawing (% dACR) and post-incubation (% VIAB, TM and PM). Moreover, we observed damage induced in preserved sperm by direct freezing compared with the control group, this damage was more evident after incubation (thermal stress test -ThS test-), for viability, acrosomal status and motility. In conclusion, our results suggest that slow cooling rates (up to 1°C/min) and at least 1 hour equilibration time are necessary for the effective cryopreservation of brown bear sperm.

After this approach about the study of equilibration period, for second esperiments, we addressed, in-depth, the evaluation of this step; thus, we evaluated different equilibration times (0, 0.5, 1 -control-, 4-5, 7-8 y 10-12 h) and three times for a long-term storage (24, 48 and 72 h) versus control (1 h), at 5 °C before freezing. In the first experience, no significant differences were found for the different periods of equilibration tested, except for nonequilibrated group (0 h) which showed lower values of iACR, TM and PM. For evaluation of long-term storage, we observed a decline markedly and progressively since 48 up to 72 h, in post-thawing quality from brown bear sperm. In view of these results, we suggest that the pre-freezing cooling period up to 24 h could be extended without freezing the spermatic samples.

For a third step to redefine the classic protocols of brown bear sperm, we analyzed the conditions for long-term storage, to enable the seminal samples to be transported to reference laboratory. For that, we assessed the effect of three temperatures (room temperature -RT-, 15 y 5 °C), three glycerol percentages (0, 3 y 6 %) and two dilution rate (1:1 -1782×10^6 sperm/mL de media- y final -a 100×10^6 sperm/mL), up to 48 h. At 5 °C, samples yielded higher results for VIAB, PM and iACR for 24 hours, and for TM, PM and YOPRO- (viable and non-apoptotic status) after 48 hours, compared with the other temperatures. For glycerol percentage, for 24 h, post-thawing quality increased progressively with the percentage; after 48 h, 6 % glycerol showed beneficial effects on sperm cryopreservation. Besides, this percentage had a clearly superior freezability (viability and motility). Both dilution rates obtained similar data within first 24 h, but final dilution supported better post-thawing quality after 48 h. Both dilution rates showed similar freezability, except after 48 h, when the final dilution reached a higher percentage of recovery rates of viability. These findings suggested that the best conditions for long-term storage (up to 48 h) of brown bear electroejaculates are: a temperature of 5 °C, a glycerol percentage of 6 % and a sperm concentration of 100×10^6 sperm/mL.

Finally, to improve post-thawing quality of stored samples for long-term (up to 48 h), we evaluated the effect of solid state and its handling, so we tested four experimental groups: 1) 1:1 dilution in standard extender at RT (room temperature), cooling in tube, final dilution at 5 °C (Control 1, 24 and 48 h); 2) final dilution in standard extender at RT, cooling in tube ("FD-Tube"); 3) final dilution in standard extender at RT, cooling in 0.25 mL plastic straw ("FD-Straw"), and 4) final dilution with standard extender supplemented with 1.5 % gelatine at RT, cooling in 0.25 mL plastic straw ("Gel"). At prefreezing after 48 h of storage, Gel reached higher viability, and progressiveness although lower velocity (VAP). For post-thawing analysis, Gel yielded higher VIAB, YOPRO-, iACR and LIN, but lower VAP and ALH, independently storage time. No differences were found among the rest of experimental groups (Control 24/48 h, FD-Tube and FD-Straw). We could conclude that solid state by the supplementation with gelatine, could be a suitable alternative to preserve viability and progressiveness of brown bear spermatozoa stored at 5 °C.

INTRODUCCIÓN

La destrucción de distintos hábitats naturales como consecuencia de la actividad humana es sin duda el factor primario responsable de la reducción de la biodiversidad; en el caso del oso pardo de las montañas cantábricas, las "molestias" causadas por el hombre y la pérdida de hábitat son factores de amenaza de primer orden (Comisión Nacional de Protección de Naturaleza, 1999). El oso pardo (*Ursus arctos*) es el úrsido más ampliamente distribuido, y en la Lista Roja de IUCN de las Especies Amenazadas (2008), a nivel mundial, aparece en la categoría de "Baja Preocupación" (LC). Sin embargo, en España, está considerado en peligro de extinción (Real Decreto 439/1990 de la Ley Española, regulada en el Catálogo Nacional de las Especies Amenazadas), limitándose su distribución a la Cordillera Cantábrica. Los datos más recientes de censo (Fundación Oso Pardo, 2014), estiman que existen unos 230 osos divididos entre las dos subpoblaciones, la zona occidental con unos 200 ejemplares y un área de presencia de 2800 km^2, y la zona oriental con unos 30 osos distribuidos en un área de 2100 km^2. Ambos núcleos cumplen el criterio D (menos de 50 ejemplares maduros) de la categoría "En Peligro Crítico" (IUCN 2006). La zona oriental, dentro de la Cordillera Cantábrica, constituye el núcleo de recuperación más difícil puesto que un estudio genético (Doadrio et al., 2000) realizado sobre 20 ejemplares (14 machos y 6 hembras) indicó una grave pérdida de variabilidad genética (alta consanguinidad) y un importante desequilibrio poblacional entre sexos. El oso pardo cantábrico, posee un gran interés tanto desde un punto de vista sociocultural (especie autóctona) como interés genético al ser el único núcleo de *Ursus arctos* en pureza genética, debido a su situación geográfica. Con el fin de mejorar esta problemática se ha instaurado una Estrategia para la Conservación del Oso Pardo Cantábrico (Comisión Nacional de Protección de la Naturaleza, 2001).

Para la mejora de esta situación son necesarias las actuaciones en la recuperación del hábitat junto con la creación de un banco de recursos genéticos (BRG). Los BRG son una herramienta importante en la conservación de especies amenazadas (Holt & Pickard, 1999; Yoshida, 2000), ya que salvaguardan adecuadamente la variabilidad genética. La creación de un BRG permite la conservación de forma indefinida de biomateriales (gametos, embriones, células somáticas, etc.) pero necesita de diversas técnicas de reproducción asistida que permitan la entrada adecuada o en condiciones óptimas de dichos especímenes y la posibilidad de generar nuevos individuos con éxito. La aplicación de la inseminación artificial usando semen refrigerado o preferentemente crioconservado (obtenido mediante electroeyaculación o del epidídimo de ejemplares muertos -Anel et al., 2011-) facilitaría el movimiento de germoplasma entre los dos núcleos de la Cordillera Cantábrica como posible estrategia de conservación ex-situ.

La mayoría de los trabajos publicados en el ámbito de la criopreservación espermática como herramienta fundamental de los BRG, se centran casi exclusivamente en las especies domésticas. Por ello es necesario desarrollar y optimizar un protocolo de conservación espermática adaptado a las características de los espermatozoides de especies o poblaciones amenazadas, como el oso pardo (*Ursus arctos*) en España (Anel et al., 1999), para crear un BRG eficiente. En cada uno de los pasos de la crioconservación (obtención seminal, dilución espermática -composición del diluyente-, refrigeración -rampas de refrigeración-, equilibrado -crioprotector, tiempo-, congelación y descongelación), los espermatozoides pueden mermar o perder su funcionalidad, dado que estos procesos (refrigeración o congelación) pueden ser muy "estresantes" para los gametos (Watson, 1995).

Entre la escasa bibliografía referente a la congelación de semen de oso encontramos estudios que se centran principalmente en el método de obtención y los diluyentes utilizados (Oso negro japonés: Kojima et al., 2001; Okano et al., 2004a, 2006a, 2009; oso pardo de Hokkaido: Ishikawa et al., 2002) y en el manejo precongelación (Oso negro japonés: Okano et al., 2006a,b; panda gigante: Olson et al., 2003). En el oso pardo, nuestro grupo de investigación ha evaluado, entre otros, diferentes aspectos de la crioconservación espermática como: diluyentes de congelación adaptados (Anel et al., 2010; Alvarez-Rodríguez et al., 2013a) y comerciales (Gomes-Alves et al., 2014) en la congelación espermática del oso pardo, el efecto de la centrifugación-tasas de dilución en el manejo precongelación (Nicolas et al., 2011), el momento de adición del glicerol (Alvarez-Rodríguez et al., 2011), la concentración de glicerol y las rampas de congelación (de Paz et al., 2012) y la incubación post-descongelación como un test de resistencia/estrés térmico –ThStest- (López-Urueña et al., 2010 a,b). Teniendo en cuenta los resultados obtenidos por los distintos grupos de investigación en diferentes especies de úrsidos, parece que podemos englobar a los úrsidos dentro de las especies llamadas "buenas congeladoras". No obstante, al tratarse de muestras muy valiosas y difíciles de obtener, se debe profundizar en la especificidad y mejora de los protocolos de crioconservación, tratando de lograr la máxima rentabilidad en términos de recuperación funcional a la descongelación. Dicha mejora no se obtiene solo en base a características de calidad (movilidad, viabilidad, etc.) de las muestras conservadas, sino también a través de determinadas características intrínsecas (como por ej. el sexaje espermático), lo que dota de una mayor efectividad al BRG de la especie ante situaciones límite de la especie como puede ser el desequilibrio del sexratio de una población. En este contexto, cobra especial relevancia la selección de espermatozoides con mediante citometría de flujo que permite, a tiempo real, la separación física de las células en base a propiedades específicas de interés.

En las especies silvestres, la recogida y procesado seminal se lleva a cabo en condiciones de campo, que poco tienen que ver con los requerimientos estándar de los centros de producción de dosis seminales (Elefante: Graham et al., 2004; guepardo: Crosier et al., 2006; búfalo: Herold et al., 2006; oso pardo: de Paz et al., 2012). Este hecho, ligado a la idoneidad de la aplicación de técnicas de mejora espermática y a la escasa disponibilidad de centros tecnológicos de referencia para llevar a cabo dichas técnicas, hace que los protocolos clásicos de crioconservación espermática sean en principio poco útiles dentro del ámbito de la criopreservación de espermatozoides de especies silvestres. En consecuencia, es necesario modificar los protocolos estándar de congelación espermática; para garantizar una calidad óptima tras aplicar pautas de manejo que permitan un tiempo suplementario para el transporte de las muestras desde el lugar de recogida (medio silvestre) a los centros tecnológicos de referencia, y su posterior procesado precongelación. Para el transporte, el semen puede estar diluido, refrigerado y almacenado a 5 °C o bien, congelado a -196 °C (England & Ponzio, 1996; Alvarez-Rodriguez et al., 2013b). Durante la última década, el uso de semen refrigerado resulta una alternativa interesante a la congelación, cuando se trata de conservación de muestras espermáticas a corto-medio plazo (Verstegen et al., 2005). Este manejo del semen permitiría la aplicación de biotecnologías reproductivas, en centros distantes, antes de su congelación, tales como la técnica de sex-sorting, la fecundación in vitro u otros usos más inmediatos como la inseminación artificial.

Esta adaptación de los protocolos de conservación estándar de semen de especies en peligro, en ocasiones pasa por una simplificación de los pasos del procesado y una reducción en los tiempos de los mismos para la ejecución de protocolos a nivel campo, o por un aumento de dichos tiempos que permita su envío a centros biotecnológicos. Por esta razón, es necesario conocer la "permisividad" de los espermatozoides a la criopreservación mediante protocolos "no estandarizados" (Herold et al., 2006). En consecuencia, parece necesario estudiar la flexibilidad del periodo de refrigeración (5 °C) precongelación, con el fin de mejorar la eficiencia de los protocolos de conservación de eyaculados de oso pardo recogidos bajo condiciones de campo.

El proceso de refrigeración es un punto crítico ya que en él se produce el shock térmico, que provoca, en mayor o menor medida, la pérdida de movilidad y de lípidos de membrana, aumento en la permeabilidad de la misma, cambios en la concentración de electrolitos, reducción de la actividad metabólica y daños en el acrosoma y otras estructuras, lo que deriva en una pérdida de la capacidad fecundante de las muestras espermáticas crioconservadas (Watson, 2000). Este fenómeno aparece en el rango de 15 a 5°C (Drobnis et al., 1993), aunque no todas las especies presentan la misma susceptibilidad térmica (Caballo: 18 a 8 C -Aurich et al., 2008-; cerdo: por debajo de 12 °C -Althouse et al., 1998-). Los

espermatozoides de ungulados parecen ser particularmente sensibles a la refrigeración, mientras que otras especies como el conejo, perro y humano muestran una mayor resistencia (Watson, 1995).

Bailey et al. (2008) en muestras de semen porcino, encontraron que las modificaciones en la membrana producidas durante la refrigeración, están asociadas con la pérdida del colesterol, observando que con la refrigeración a 5 °C hay una mayor proporción de espermatozoides capacitados y con acrosoma reactivo que cuando son refrigerados a 15 °C. Derivado del mantenimiento de las muestras espermáticas a temperaturas bajas, podría producirse la fragmentación de ADN con una reducción de la fertilidad en semen almacenado, dicho efecto se ha comprobado en espermatozoides de caballo aproximadamente a partir de las 48 h de almacenamiento en refrigeración (Aurich et al., 2008). López-Fernández et al. (2007) sugirieron que el almacenamiento en frío induce rutas de apoptosis (vía estrés oxidativo), lo cual deriva en la fragmentación del ADN. El sistema de movilidad de los espermatozoides requiere una alta tasa de actividad metabólica intracelular que genera radicales libres que podrían llevar a dañar la cromatina (Vishwanath and Shannon, 1997). Por otro lado, la enzima amino-ácido oxidasa (AAAO) se activa tras la muerte celular y su metabolito (peróxido) es el verdadero responsable del efecto tóxico de los espermatozoides muertos (Shannon and Curson, 1971; Upreti et al., 1998). Además de los posibles daños provocados por el almacenamiento a 5 °C, existen otros derivados del uso del glicerol más acentuados cuando se incrementa el tiempo de exposición. A pesar de sus ventajas como crioprotector gracias a sus propiedades coligativas (disminución del punto de congelación y de las concentraciones de electrolitos en la fracción no congelada), es conocido su efecto citotóxico sobre los espermatozoides (Holt, 2000; Li et al., 2005).

En estudios previos, se han observado diferentes resultados sobre la velocidad de refrigeración en la calidad del semen de mamíferos. Algunos autores consiguieron los mejores resultados con una rampa de refrigeración lenta (<0,5 °C/min) (Perro: Bouchard et al., 1990; camello asiático: Niasari-Naslaji et al., 2007; toro: Januskauskas et al., 1999; caballo: Varner et al., 1988); sin embargo, en otros estudios se obtuvieron mejores resultados con una rampa rápida (~4 °C/min) (Ciervo rojo: Fernández-Santos et al., 2006a,b). En otras especies no se encontraron diferencias entre las rampas evaluadas (Gato: Hermansson & Axner, 2007). Por tanto, se observa una alta variabilidad en la resistencia entre especies al shock térmico. Baudi et al. (2008) evaluaron el efecto de las rampas de refrigeración en semen de dos especies de *Leopardus spp* (ocelote: *L. pardalis*, y tigrina: *L. tigrinus*) y encuentran que la calidad postdescongelación de los espermatozoides de ocelote mejora con la rampa de 0,7 C/min, mientras que en los de tigrina lo fue con la de 0,16 °C/min.

Otro aspecto básico a tener en cuenta en la adaptación de los protocolos de crioconservación es el periodo de equilibrado precongelación; la reducción o incluso la omisión de este paso simplificarían enormemente los protocolos. Por otro lado, puede ser interesante disponer de un tiempo "extra" tras la recogida seminal para adaptar el protocolo de congelación a la infraestructura disponible ("estrategia de conservación precongelación a corto plazo", con tiempos de equilibrado hasta 12 horas). Esta estrategia nos permitiría adaptar los protocolos de refrigeración a diferentes condiciones de trabajo que se puedan presentar a nivel campo y que limitan el uso de los protocolos estándar (tiempo de equilibrado de 1 hora -Anel et al., 2010-). El tiempo de exposición de los espermatozoides al crioprotector antes de su congelación influye en la calidad postdescongelación (Herold et al., 2006). La velocidad y el grado de penetración del glicerol en los espermatozoides varían entre especies; puede penetrar en la membrana rápidamente en algunas especies y lentamente en otras (Toro: Berndtson y Foote, 1972, 3-4 min). Sin embargo, en un estudio con semen de oso negro japonés (Okano et al., 2006a) en el que se ha investigado el periodo de equilibrado, no se han observado diferencias en la calidad postdescongelación entre los tratamientos testados (1 h de refrigeración + 1 h de equilibrado frente a 3 h de refrigeración sin equilibrado). Okano et al. (2004b), usando semen de perro como especie de referencia para estudios de semen de oso, observaron que tras 16 horas de equilibrado, los espermatozoides presentaban una menor movilidad progresiva que con 0 ó 2 h. En el caso de eyaculados de oso panda gigante, Olson et al., (2003) evaluaron tres tiempos de equilibrado (0, 0,5 y 2,5 h) y no encontraron ninguna diferencia (movilidad y viabilidad precongelación). La mayoría de lo publicado en este sentido se ha centrado en las especies domésticas aportando, en todo caso, resultados contradictorios. Algunos autores (Toro: Wiggin & Almquist, 1975; verraco: Corcuera et al., 2007) lograron mejores resultados con tiempos de equilibrado cortos (0,5 h), mientras otros autores (Toro: Leite et al., 2010; macho cabrío: Choe et al., 2006; verraco: Yi et al., 2002; perro: Okano et al., 2004b) observaron que los espermatozoides necesitaban tiempos de equilibrado más prolongados (2-4 h). Sin embargo, Fabrocini et al. (2000) recomendaron tiempos de equilibrado mayores de 5 horas (búfalo mediterráneo) y Crockett et al. (2001) propugnan un equilibrado durante 12 horas para conseguir una mejor movilidad espermática postdescongelación (caballo). Kundu et al. (2000) congelaron directamente en nitrógeno líquido la papilla epididimaria de macho cabrío con diferentes modelos de refrigeración (refrigeración lenta vs no refrigeración) sin periodo de equilibrado y observaron una movilidad muy baja en todos los grupos experimentales ensayados.

Por otro lado, la aplicación de biotecnologías espermáticas para un mayor y más eficiente uso de los eyaculados, dada la disponibilidad restringida de centros tecnológicos de referencia, (como la selección de espermatozoides por sexaje) en

centros biotecnológicos de referencia requiere en la mayoría de los casos de un tiempo extra para el transporte y tratamiento de las muestras de las muestras refrigeradas (5 °C; "estrategia a largo plazo", tiempos de equilibrado hasta 48-72 h). Dicho envío podría realizarse con las muestras refrigeradas para realizar una congelación "diferida" tras el tratamiento o congeladas debiendo en este caso aplicar el tratamiento de mejora tras las descongelación y previamente a un segundo ciclo de congelación (recongelación). En varias especies se ha observado que las muestras espermáticas almacenadas durante tres o más días en refrigeración son capaces de mantener una movilidad por encima del 50 % (Perro: Iguer-Ouada et al., 2001, Ponglowphapan et al., 2004, Sahashi et al, 2011, Shahiduzzaman et al., 2007; ovino: Lopez-Saez et al., 2000; Tamayo-Canul et al., 2011; búfalo: Akhter et al., 2011). En relación a las muestras congeladas, varios autores realizaron un segundo ciclo de congelación (Toro: Saragusty et al., 2009, Underwood et al., 2009; oso pardo: Alvarez-Rodríguez et al., 2013b) y aunque observaron una caída importante en la calidad postdescongelación (movilidad y viabilidad) entre ambos ciclos, este modelo podría ser aceptable en ciertas condiciones. En una valoración global de estrategias, sería conveniente una evaluación comparativa de las dos técnicas: almacenamiento refrigerado a largo plazo y posterior congelación frente a una congelación inicial, envío y recongelación de las muestras seminales, que permitirían la aplicación de técnicas de mejora espermática en los especímenes a almacenar en los BRG.

Para poder aplicar protocolos eficaces de congelación diferida, son necesarias estrategias que permitan alargar el tiempo en el que los espermatozoides mantienen su capacidad funcional durante el almacenamiento en condiciones de refrigeración (Conejo: Rosato & Iaffaldano, 2011; elefante: Kiso et al., 2011); y en este punto es importante considerar las condiciones de conservación como temperatura, porcentaje de glicerol, o la tasa de dilución, así como sopesar la posibilidad del uso de medios sólidos, condiciones anaeróbicas o adición de antioxidantes, entre otras estrategias encaminadas a mejorar el rendimiento de los protocolos de criopreservación espermática que incluyen un almacenamiento precongelación de hasta 48-72 horas.

La temperatura es un factor clave para mantener la calidad espermática durante el almacenamiento líquido. Los espermatozoides son principalmente refrigerados a 4-5 °C, porque son metabólicamente menos activos, tienen una mayor vida útil y/o calidad post-descongelación aceptable (Toro: Vishwanath & Shannon, 2000; conejo: Rosato & Iaffaldano, 2011; carnero: O'Hara et al., 2010, Paulenz et al., 2002; caballo: Love et al., 2002; gato: Hermansson & Axner, 2007; perro: Bouchard et al., 1990). En concordancia, otros autores describen un descenso en la calidad con el almacenamiento precongelación a temperatura ambiente (Carnero: Paulenz et al., 2002; caballo: Love et al., 2002; gato:

Hermansson & Axner, 2007; perro: Bouchard et al., 1990) respecto a temperaturas más bajas. Sin embargo, varios autores encontraron la temperatura de 21 °C más adecuada para el envío de las muestras seminales previo al proceso de sex-sorting (Carnero: Hollinshead et al., 2004; ciervo rojo: Parrilla et al., 2010).

Otro factor importante en los protocolos de almacenamiento a largo plazo, es el glicerol. En este sentido, Alvarez-Rodríguez et al. (2011), en un estudio sobre el momento de adición del glicerol en el semen de oso, sugirieron que los espermatozoides de esta especie parecen poseer una buena tolerancia a este crioprotector, cuyo porcentaje de uso ronda el 4-8 % (mayor calidad con el 6 % de glicerol) (Okano et al., 2006; Anel et al., 2011; de Paz et al., 2012). Sin embargo, el efecto del glicerol en el almacenamiento a largo plazo, previo a la congelación espermática del oso pardo, no ha sido aún testado. En otras especies sí se ha estudiado el efecto de distintas concentraciones de glicerol en semen refrigerado con resultados variables (Perro: Hermansson & Forsberg, 2006; carnero: Purdy, 2006, Tamayo-Canul et al., 2011 a,b; mono: Dong et al., 2008; búfalo: Kumar et al., 1992).

Otro punto clave en el manejo pre-congelación del semen es la tasa de dilución. La dilución 1:1 podría ser útil para el envío de semen a los centros de referencia, en el caso de que se requieran altas concentraciones para posteriores tratamientos, evitando así la reconcentración que puede perjudicar la calidad seminal y disminuir el número de espermatozoides (Mono: Miro et al., 2009; oso: Alvarez et al., 2012). La dilución podría desestabilizar las membranas celulares, dando lugar a una pérdida de movilidad (Maxwell & Johnson, 1999) o a un aumento de la vulnerabilidad al estrés osmótico (Tamayo-Canul et al., 2011a). De Jong et al. (2005) estudiaron los efectos de la dilución en semen de zorro volador, y describen un descenso gradual en la movilidad, integridad de la membrana plasmática y el estado del acrosoma, cuando se usaba una mayor tasa de dilución. Sin embargo, Prathalingam et al., (2006) demostraron que la supervivencia del semen de toro se incrementaba a mayor dilución debido a una reducción de los productos metabólicos, aunque se ve afectada la integridad acrosomal. Miro et al. (2009) observaron en semen de burro que la viabilidad tras el almacenamiento hasta 72 horas a 5 °C se incrementó con la tasa de dilución (dilución 1;1, 1;5 o 1:10).

Teniendo en cuenta la problemática expuesta, existen diferentes alternativas que podrían mitigar el efecto deletéreo del almacenamiento a largo plazo (5 °C), tales como al almacenamiento sólido, los medios anaeróbicos o la adición de antioxidantes a los extender de criopreservación.

Durante el almacenamiento en medio líquido a largo plazo, se produce una sedimentación de células espermáticas en el fondo del tubo; por tanto, este tipo de

almacenamiento se producirían cambios deletéreos en el extender (ej. fluctuaciones del pH) así como un incremento de los metabolitos tóxicos (Conejo: Nagy et al., 2002; carnero: Paulenz et al., 2010). Los medios sólidos, como la gelatina, pueden ser una alternativa para prevenir esta problemática. Esta sustancia permitiría una distribución celular más uniforme (Paulenz et al., 2010; Salvador et al., 2006), e incluso minimizaría las demandas metabólicas a consecuencia de la movilidad disminuida (Conejo: López-Gatius et al., 2005; carnero: Yaniz et al., 2005).

El protocolo específico para oso desarrollado por nuestro grupo de investigación (Anel et al., 2008, 2010; de Paz et al., 2012) implica la adición del glicerol en dos pasos (una dilución 1:1 inicial a temperatura ambiente y hasta la concentración final a 5 ° C). Debido a que la gelatina solidifica por debajo de los 20 °C, para su manejo y congelación, la dilución final (6 % glicerol, 100×10^6 spz/mL) y el envasado en pajuelas se debe realizar a temperatura ambiente. Esta adición del glicerol a temperatura ambiente respecto a 4-5 °C produce un descenso/caída de la calidad post-descongelación ya descrito en otras especies (Carnero: Colas, 1974; cerdo: Almlid & Johnson, 1988), siendo considerado este manejo, clásicamente, una mala praxis (Hermansson & Axner, 2007; Gil et al., 2011). Sin embargo, bajo condiciones de campo, Crosier et al. (2006) observaron que los cambios en la temperatura (fluctuaciones durante el procesado precongelación) de la muestra se prevenían si la adición del glicerol se llevaba a cabo antes de la refrigeración (a temperatura ambiente) en lugar de a 5 °C. De este modo, algunos autores, también realizan la dilución final (glicerol final) a temperatura ambiente (Mono Cynomolgus: Li et al., 2005; búfalo africano: Herold et al., 2006; ocelote y tigrina: Baudi et al., 2008; oso pardo; Alvarez-Rodríguez et al., 2011), e incluso realizan el envasado en pajuela a temperatura ambiente (Guepardo de Namibia: Crosier et al., 2006; carnero: Camara et al., 2011) con una aceptable calidad post-descongelación.

En resumen, para establecer un banco de recursos genéticos "mejorado" en especies como el oso pardo mediante la aplicación de biotecnologías espermáticas complejas como el sexaje, hay que considerar que la recogida seminal se lleva a cabo bajo condiciones de campo que poco tienen que ver con las condiciones de laboratorio. Para dicha mejora, se debe ajustar la velocidad de refrigeración de las muestras hasta 5 °C y es preciso estudiar la permisividad de los espermatozoides tanto para la simplificación del proceso (omitir algún paso) como para alargarlo en el tiempo (hasta 48-72 horas a 5 °C) para permitir de esta manera su envío a centros biotecnológicos de referencia. En este sentido, es importante evaluar y adecuar los factores del almacenamiento a largo plazo, tales como la temperatura, el porcentaje de glicerol o la tasa de dilución. También sería interesante considerar medidas correctoras que permitan mitigar los efectos deletéreos de dicho almacenamiento precongelación como pueden ser el uso de

medios sólidos, con los que se podría evitar la sedimentación celular o reducir las demandas metabólicas por moción. Con todo ello, se podrían mejorar las características de las muestras que entran a formar parte de los bancos de recursos genéticos.

OBJETIVOS

El objetivo general de este trabajo de tesis doctoral es el estudio de diferentes modelos de manejo durante la precongelación de eyaculados de oso pardo con la finalidad de crear un Banco de Recursos Genéticos eficaz, aplicable a la población de osos de las montañas cantábricas.

Para ello, se plantean los siguientes objetivos específicos que han sido desarrollados en las publicaciones que se indican:

1.- Evaluar el efecto de la velocidad de refrigeración y el tiempo de equilibrado, antes de la congelación, en la calidad espermática postdescongelación en el oso pardo.

"Tolerance of brown bear spermatozoa to conditions of pre-freezing cooling rate and equilibration time" Theriogenology 81 (2014): 1229-38.

2.- Estudiar la repercusión de diferentes tiempos de almacenamiento precongelación (a corto -hasta 12 horas- o a largo plazo -hasta 48 horas-) a 5 °C sobre la calidad postdescongelación de los eyaculados de oso pardo.

"Alternative procedures for the cryopreservation of brown bear ejaculates depending on the flexibility of the "in cooling" period (5°C)" Cryobiology 69 (2014): 434-41.

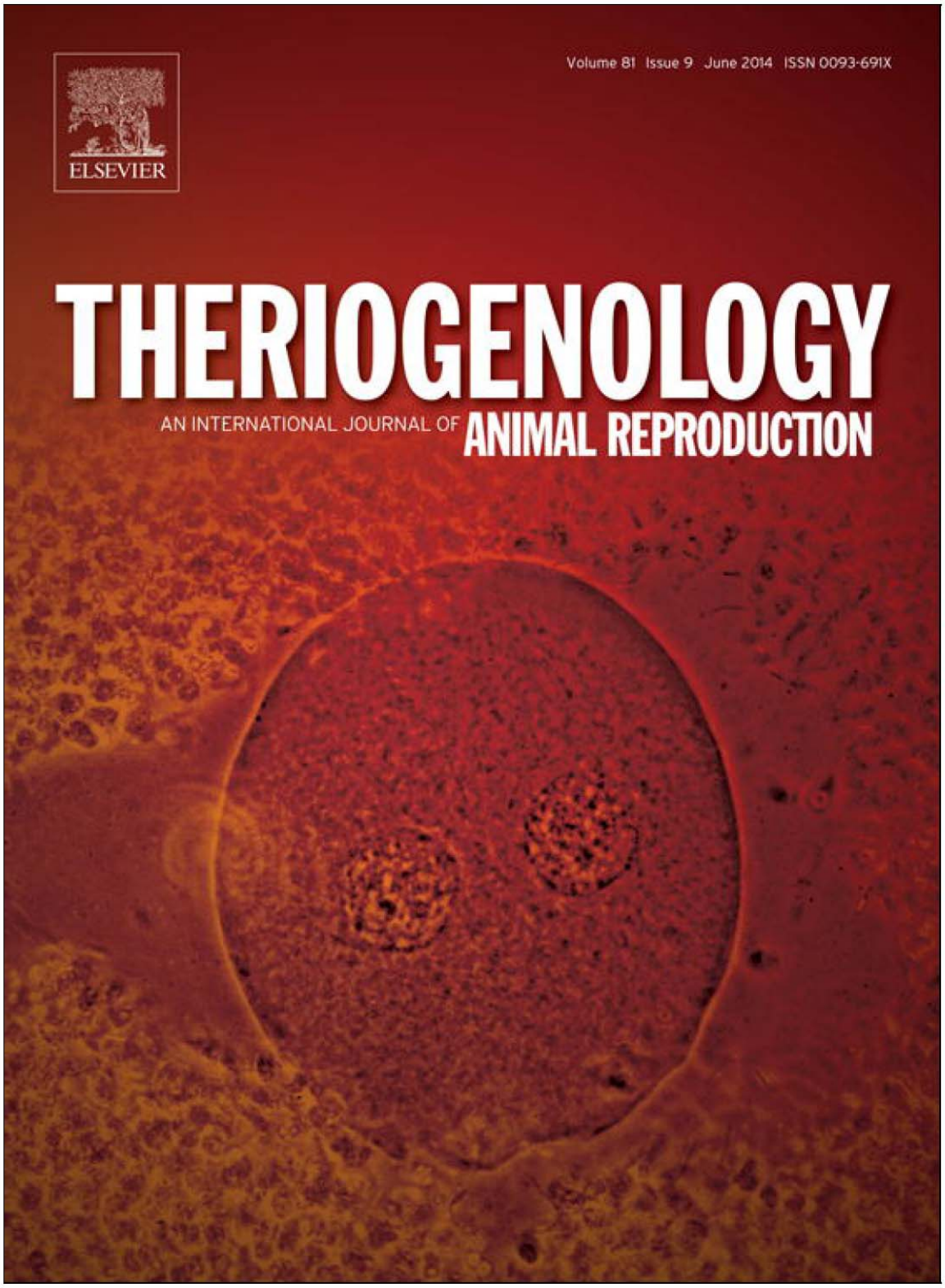
3.- Establecer las condiciones óptimas de temperatura, porcentaje de glicerol y tasa de dilución para el almacenamiento precongelación de muestras espermáticas de oso pardo.

"Optimization of conditions for long-term prefreezing storage of brown bear sperm before cryopreservation" Theriogenology 84 (2015): 1161-71.

4.- Estudiar el efecto de un medio sólido (gelatina) y sus métodos de manejo, sobre las condiciones del almacenamiento a largo plazo antes de la congelación de semen de oso pardo.

"Use of solid state extenders (gelatin) in long-term storage (up to 48 h) at 5 °C appears to improve prefreezing and post-thawing quality from brown bear electroejaculates" Enviado a publicación.

PUBLICACIONES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Tolerance of brown bear spermatozoa to conditions of pre-freezing cooling rate and equilibration time

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ABSTRACT

Specific protocols for the cryopreservation of endangered Cantabrian brown bear spermatozoa are critical to create a genetic resource bank. The aim of this study was to assess the effect of cooling rates and equilibration time before freezing on post-thawed brown bear spermatozoa quality. Electroejaculates from 11 mature bears were extended to 100×10^6 spermatozoa/mL in a TES–Tris–Fructose–based extender, cryopreserved following performance of the respective cooling/equilibration protocol each sample was assigned to, and stored at −196 °C for further assessment. Before freezing, after thawing, and after 1 hour's incubation post-thawing at 37 °C (thermal stress test), the quality of the samples was assessed for motility by computer-assisted semen analysis, and for viability (SYBR-14/propidium iodide), acrosomal status (peanut agglutinin–fluorescein isothiocyanate /propidium iodide), and sperm chromatin stability (SCSA) by flow cytometry. In experiment 1, three cooling rates (0.25 °C/min, 1 °C/min, and 4 °C/min) to 5 °C were assessed. After thawing, total motility (%TM) was higher and percentage of damaged acrosomes (%dACR) was lower ($P < 0.05$) for 0.25 °C/min than for 4 °C/min. The thermal stress test data indicated equally poor quality ($P < 0.05$) for the 4 °C/min cooled samples in viability (%VIAB), %dACR, %TM, and progressive motility (%PM). In experiment 2, the effect of a pre-freezing equilibration period at 5 °C for 1 hour (cooling at 0.25 °C/min) was evaluated. Samples kept at 5 °C for 1 hour showed higher ($P < 0.05$) values than the nonequilibrated ones for both thawing (%dACR) and thermal stress test (%VIAB, %TM, and %PM). In experiment 3, samples stored without cooling and equilibration (direct freezing) were compared with the samples cooled at 0.25 °C/min and equilibrated for 1 hour (control freezing). Using thermal stress test, we observed that direct freezing causes damage in viability, acrosomal status, and motility of spermatozoa compared with the control group ($P < 0.05$). In conclusion, our results suggest that slow cooling rates to 5 °C and at least 1 hour equilibration time are necessary for the effective cryopreservation of brown bear sperm.

1. Introduction

The development of a specific protocol for sperm cryopreservation adapted to endangered species (i.e., Cantabrian brown bear (*Ursus arctos*) in Spain [1]) is essential to

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create a genetic resource bank. Diverse factors influence the quality of sperm after freezing–thawing. Studies of bear sperm cryopreservation have mainly been focused on the collection method and extenders (black Japanese bear [2–5]; Hokkaido's brown bear [6]) and handling pre-freezing (black Japanese bear [4,7]; giant panda [8]). In brown bear, our research group evaluated, among other aspects of sperm cryopreservation, glycerol addition timing [9], glycerol concentration and freezing rates [10], and a post-thawing incubation (thermal stress test) [11,12].

The specific adaptation of sperm cryopreservation protocols in endangered species aims for a simplification of procedures and a reduction in times, desirable in fieldwork conditions, to achieve the best seminal quality post-thawing. In this sense, a high cooling rate, the removal of equilibration times or omission of cooling, and equilibration periods should be evaluated.

Spermatozoa cooling can result in a damage known as cold-shock, which results in a loss in viability in a number of spermatozoa and decreased fertilizing capacity [13]. In domestic cat, rapid cooling of spermatozoa to 5 °C induced significant acrosomal damage, but slow cooling maintained a high proportion of spermatozoa with intact acrosomes and motility remained unaffected [14]. In a more recent study, Hermansson and Axnér [15] concluded that cat spermatozoa were tolerant to cold-shock at the rates usually applied. Not all species respond in the same way to cold-shock; spermatozoa from ungulate species seem to be particularly sensitive, whereas those of rabbit, dog, bird, and human show a higher resistance [16].

Previous studies of mammal spermatozoa have analyzed cooling rate effects in sperm quality and very different results can be seen. Some authors yielded more scores with slow cooling rate (<0.5 °C/min): dog [17], Bactrian camel [18], bull [19], and stallion [20]. Other researchers showed better results with rapid cooling (≈4 °C/min): red deer [21,22]. However, in some studies no differences have been found between tested cooling rates: cat [15]. High variability in cold-shock resistance is observed among species. Baudi et al. [23] investigated the effect of cooling rates in sperm from two species of the genus *Leopardus* (ocelot: *Leopardus pardalis*, and tigrina: *Leopardus tigrinus*). They found that the quality of ocelot spermatozoa achieved higher scores with 0.7 °C/min cooling rate, whereas for tigrina sperm this rate was 0.16 °C/min.

Another basic aspect of cryopreservation protocols concerns the pre-freezing equilibration period; reduction or omission of this step would simplify the cryopreservation protocol enormously. The exposure time of spermatozoa to a cryoprotectant before freezing influences post-thawing quality [24]. Equilibration times that are too short prevent the permeation of sufficient amounts glycerol through the spermatozoal membrane to achieve protective concentrations. However, excess exposure could be toxic [25]. The effects of equilibration times on semen cryopreservation remain controversial, and the duration of equilibrium required for satisfactory results in post-thaw sperm quality varies among species: bull, 0.5 hours [26] and 4 hours [27]; Mediterranean buffalo, >5 hours [28]; boar, 2–3 hours [29]; dog, >3 hours [30]. In brown bear spermatozoa, only one study of the equilibration period has been reported [4]. The authors found no difference in post-thawing sperm quality between a treatment with 1 hour cooling plus 1 hour equilibration, and another with 3 hours cooling without equilibration.

In wild species such as buffalo, sperm collection could be carried out in the field where this has little to do with the ideal conditions of a laboratory [24]. Due to this premise, it is necessary to simplify the cryopreservation protocol and even omit the cooling and equilibration steps (direct freezing). Kundu et al. [31] directly froze goat epididymal spermatozoa at different cooling rates by a four-step cooling technique without an equilibration period and observed a marked decrease in motility.

The aim of this work was to shorten and/or simplify the semen handling in the field, and for this we evaluated the following: (1) different cooling rates to 5 °C, (2) the influence of equilibration time, and (3) the effect of direct freezing in the cryopreservation of brown bear ejaculates.

2. Materials and methods

2.1. Materials

All chemicals were of at least reagent grade and were acquired from Sigma (Madrid, Spain), unless otherwise stated.

2.2. Animals and sample collection

Animal manipulations were performed in accordance with the Spanish Animal Protection Regulation RD1201/2005, which conforms to the European Union Regulation 2003/65. All experiments were performed after obtaining ethical committee approval from the Ethical Committee for Experimentation with Animals of León University, Spain (03–02/2010).

Sperm samples from 11 sexually mature male (≥6 years old) of brown bear (*Ursus arctos*) were obtained by electroejaculation in two or three sessions, with an interval of at least 3 weeks, during the breeding season (end of April to early July) over two consecutive years. Animals were housed in a half-freedom regime in Cabarceno Park (Cantabria, Spain; 43° 21′ N, 3° 50′ W; altitude: 143 m) and fed with a diet based on chicken meat, bread, and fruits.

The males were immobilized by teleanesthesia, using 750 mg zolazepam HCl + tiletamine HCl (Zoletil100; Virbac, Carros, France) and 6 mg medetomidine (Zalopin, Orion Pharma Animal Health, Finland, 10 mg/mL). After immobilization, the bears were weighed and monitored during anesthesia (pulse, saturation of peripheral oxygen, and breathing). Before electroejaculation, the pubic region was cleaned, the penis washed with sterile physiological saline, and the rectum emptied of feces. The bladder was catheterized during semen collection. Electroejaculation was carried out with a PT Electronics1 electroejaculator (PT Electronics, Boring, OR, USA). The transrectal probe was 320 mm long, with a diameter of 26 mm. Electric stimuli were applied until ejaculation (6–10 V and 250–300 mA, on average). The ejaculates were collected in a graduated glass tube.

2.3. Experimental design

Immediately after collection, the volume and concentration of each ejaculate were recorded. Sperm concentration was assessed using a Bürker hemocytometer (Marienfeld GmbH, Marienfeld, Germany) by computer-assisted semen analysis (CASA, ISAS v. 1.2, Integrated Semen Analyser System; Proiser, Valencia, Spain). The motility and kinematic parameters were assessed using CASA and a phase-contrast microscope (Section 2.6.1); urospermia was evaluated by means of a rapid urea test (urea test strips, DiaSys Ecoline GmbH, Holzheim, Germany). Samples of low total motility (<50%) or urine-contaminated samples (>80 mg urea/dL) were rejected [32]. The selected samples were centrifuged at 600× *g* for 6 minutes. The supernatant was discarded and each pellet was resuspended 1:1 with a TES–Tris–Fructose (TTF) extender as described in a previous study [10]. Further treatment was preformed according to the experiment each sample was assigned to.

2.3.1. Experiment 1: Effect of cooling rates to 5 °C

Fifteen electroejaculates from six brown bears were processed. Extended samples were divided into three aliquots and cooled down to 5 °C at different cooling rates in programmable refrigerated baths (refrigerating/heating circulators, Model 9512, Niles, IL, USA): 0.25 °C/min (slow, ~70 min), 1 °C/min (medium, ~15 min), and 4 °C/min (rapid, ~5 min). The sperm samples were diluted again to the final concentration (100 × 10^6 spermatozoa/mL) and equilibrated for 1 hour at 5 °C before freezing (Section 2.4).

2.3.2. Experiment 2: Effect of equilibration time

The samples extended were cooled to 5 °C at a rate of 0.25 °C/min. Then, the samples were diluted to the final concentration (100 × 10^6 spermatozoa/mL) and divided into two aliquots. The first aliquot was filled in 0.25-ml straws and frozen immediately. The second aliquot was equilibrated at 5 °C for 1 hour and frozen thereafter (see Section 2.4). For this experiment, eight electroejaculates from four brown bears were processed.

2.3.3. Experiment 3: Direct freezing

Twenty electroejaculates from nine brown bears were used. Extended semen samples were divided into two aliquots, of which one was frozen immediately without cooling and equilibration period, whereas the second was cooled to 5 °C at a rate of 0.25 °C/min, extended to final dilution for 1 hour before freezing (control sample).

2.4. Spermatozoa cryopreservation

Once at 5 °C, a second 1:1 dilution was performed, using the TTF extender at 9% glycerol, in order to reach a glycerol concentration of 6%. More TTF at 6% glycerol was added to obtain a final concentration of 100 × 10^6 spermatozoa/mL. After equilibration for 1 hour at 5 °C or no equilibration (depending on the experiment), the semen was packaged into 0.25-mL plastic straws. The samples were frozen in a programmable biofreezer (Kryo 560–16 Planer, Planer plc., Sunbury-On-Thames, UK) at −20 °C/min down to −100 °C, and then transferred to liquid nitrogen containers for a minimum of 1 week. Thawing was performed by dropping the straws in water at 65 °C for 6 seconds.

2.5. Thermal stress test

The spermatozoa were analyzed for motility, viability, and acrosomal status at three points of processing: before freezing, after thawing, and after incubation of thawed spermatozoa for 1 hour at 37 °C, assay named "thermal stress test" (ThS test). The purpose of the thermal stress test is to amplify the sublethal damage induced in cells by freezing. Chromatin structure was only evaluated after thawing.

2.6. Semen evaluation

2.6.1. Motility assessment

Motility and kinetic parameters were assessed using CASA. A 5-μL sperm sample was placed in a Makler cell counting chamber (10 μm depth; Sefi Medical Instruments, Haifa, Israel) and examined using a negative phase-contrast microscope (×10) with a warmed stage (38 °C). The standard parameter settings were as follows: 25 frames/sec; 5 to 50 μm^2 for head area; curvilinear velocity >10 μm/sec to classify a spermatozoon as motile. At least five fields or 200 spermatozoa were saved and analyzed afterward. The reported parameters were total motility (TM, %), progressive motility (PM, %; spermatozoa were considered progressive if VCL > 25, STR > 80), average path velocity (VAP, μm/sec), and linearity (LIN, %).

2.6.2. Viability assessment

Analysis of semen viability was performed as described previously [32]. Briefly, a double staining of SYBR-14 (Molecular Probes, L-7011 LIVE/DEAD Sperm Viability Kit; Invitrogen, Barcelona, Spain) and propidium iodide (PI). The semen samples were diluted to 5 × 10^6 sperms/mL with PBS, and 300 μL were transferred to a polypropylene tube, where 3 μL PI (24 mM) and 1.5 μL SYBR 14 (100 nM) were added. The tubes were maintained at 37 °C for 20 minutes in the dark. The percentage of viable sperm (VIAB) was determined by means of a flow cytometer (see Section 2.6.5).

2.6.3. Acrosomal status assessment

For assessment of acrosomal status, we used the double stain of peanut agglutinin–fluorescein isothiocyanate and PI. Sperm samples were diluted with PBS (5 × 10^6 sperms/mL), and 300 μL was transferred to a polypropylene tube to which 2.5 μL PI (1 mg/mL in water) and 2.5 μL peanut agglutinin–fluorescein isothiocyanate (0.2 mg/mL in water) was added. Flow cytometer rendered the percentage spermatozoa with damaged acrosomes (PNA+, named dACR).

2.6.4. Chromatin structure assessment

For SCSA, we used acridine orange to detect damage in sperm chromatin [33]. Sperm samples were submitted to a DNA denaturation step. All samples were diluted in a TNE buffer (1 × 10^6 sperms/mL) and stored at −80 °C. For

analysis, samples (200 μL) were diluted with acid–detergent solution (0.4 mL), and just 30 seconds after this dilution, they were stained with acridine orange solution (1.2 mL). Flow cytometry was performed, and the results were expressed as %DFIt (total DNA fragmentation index; red/[total fluorescence] ratio).

2.6.5. Flow cytometry evaluation

Viability, acrosomal status, and chromatin structure were analyzed using a FACScalibur flow cytometer (Becton Dickinson Immunochemistry Systems, San Jose, CA, USA), equipped with standard optics and an argon ion laser, tuned at 488 nm, and running at 200 mW. Calibration was carried out periodically using standard beads (Calibrites; Becton Dickinson). Data corresponding to the red (FL3 photodetector) and green fluorescence (FL1 photodetector) of acquired particles were recorded. In all cases, 10,000 events per sample with a flow rate of 200 cells/sec were assessed.

2.7. Statistical analysis

Data were analyzed using the SAS v. 9.1 package. Our hypotheses for cooling rates, equilibration, and direct freezing were tested by using mixed-effects linear models after transforming the data (arc sine for percentages and natural logarithm for the rest of the analyzed parameters). Male was considered a random effect. Least-squares means were computed for each effect listed, and P-values for differences in the multiple comparisons were calculated by Tukey's test. Values were considered to be statistically significant at $P < 0.05$. All results are presented as mean ± SEM.

3. Results

Fresh brown bear semen samples showed an average volume of 3.5 ± 0.5 mL and an average concentration of 183.0 ± 30.5 × 10^6/mL.

3.1. Experiment 1: Effect of cooling rates

After the cooling and equilibration stages, differences among cooling rates were found only for TM (68.6% vs. 73.0%; $P < 0.05$) and PM (42.1% vs. 45.1%; $P < 0.001$) between slow and rapid rates (Fig. 1). In the post-thawing sample, the %dACR for the slow rate was lower ($P < 0.01$) than for the rapid one (7.5% vs. 10.9%, respectively). In motility, slow and medium cooling rates were better options to preserve the TM (52.2% vs. 46.4%; $P < 0.05$) and VAP (64.7% vs. 61.2%; $P < 0.05$) of cryopreserved sperm.

After the thermal stress test, the spermatozoa chilled at a slow rate showed higher values for VIAB (31.0% vs. 26.5% [$P < 0.05$] and 25.0% [$P < 0.05$] for medium and rapid rates, respectively). The percentage of dACR was higher in the samples cooled at a rapid rate (25.1%; $P < 0.001$) compared with the samples chilled at slow and medium rates. Similarly, samples chilled at a rapid rate showed lower values for TM and PM (16.7%, and 8.7%, respectively; $P < 0.05$). Regarding the analyzed kinetic parameters, the thermal stress test only marked differences for LIN (29.7% vs. 31.3%, medium and rapid rate, respectively; $P < 0.05$).

SCSA did not reveal any differences between cooling rates (Fig. 2).

3.2. Experiment 2: Effect of equilibration time

After the cooling, both VAP and LIN yielded higher scores ($P < 0.05$) when the samples were not equilibrated (VAP: 86.8 μm/sec; LIN: 46.7%, Fig. 3). After thawing, the samples equilibrated for 1 hour had lower percentage of dACR than those without the equilibration period (8.6% vs. 12.7%, respectively; $P < 0.05$). TM (17.4%) and PM (7.5%) and VIAB (33.1%), evaluated after the thermal stress test, were higher when the seminal sample was frozen after 1 hour equilibration ($P < 0.01$). After thawing, DFI did not show any differences in relation to equilibration time (18.1 vs. 19.3%, Fig. 2).

3.3. Experiment 3: Direct freezing

Before freezing, the TM, PM, VAP, and LIN parameters showed higher values for directly frozen samples than control samples (Fig. 4; $P < 0.01$), whereas after thawing, the percentage of damaged acrosomes was higher and the values for PM and LIN were lower than in the control group ($P < 0.05$). Nevertheless, after the thermal stress test, these samples showed great deterioration for all evaluated parameters, whose values were lower than those observed in standard freezing samples (Fig. 4; $P < 0.001$).

Direct freezing had no effect on chromatin status evaluated by DFI (Fig. 2).

4. Discussion

Sperm cryopreservation protocols in endangered species require a simplification of procedures that is recommended in fieldwork conditions. In the present study, we evaluated various cooling rates, the removal of equilibration time, or omission of cooling and equilibration periods in brown bear semen cryopreservation protocol.

Cold-shock induced by semen cooling to a temperature range above 0 °C affects a great number of species [13,34], and the species-dependent susceptibility of spermatozoa to cold-shock has been described [35]. Our results showed that viability, acrosomal status, and motility values of frozen-thawed brown bear semen dropped upon the increase of the cooling rate. In contrast to this, brown bear spermatozoa do not respond to cold-shock in the same way as described in other species. Fernandez-Santos et al. [22] observed that after cooling red deer epididymal semen, motility preservation was better for rapid rates than slow ones (4.2 °C/min vs. 0.23 °C/min), although there were no differences with regard to plasma membrane and acrosome integrity. In bovine semen preservation, some researchers did not find a significant impact of two cooling rates (slow and rapid) on sperm quality and fertility after AI of bull semen [19], whereas others found a slow cooling rate to provide superior motility parameters for cattle spermatozoa [37]. In equine reproduction technology, a slow cooling

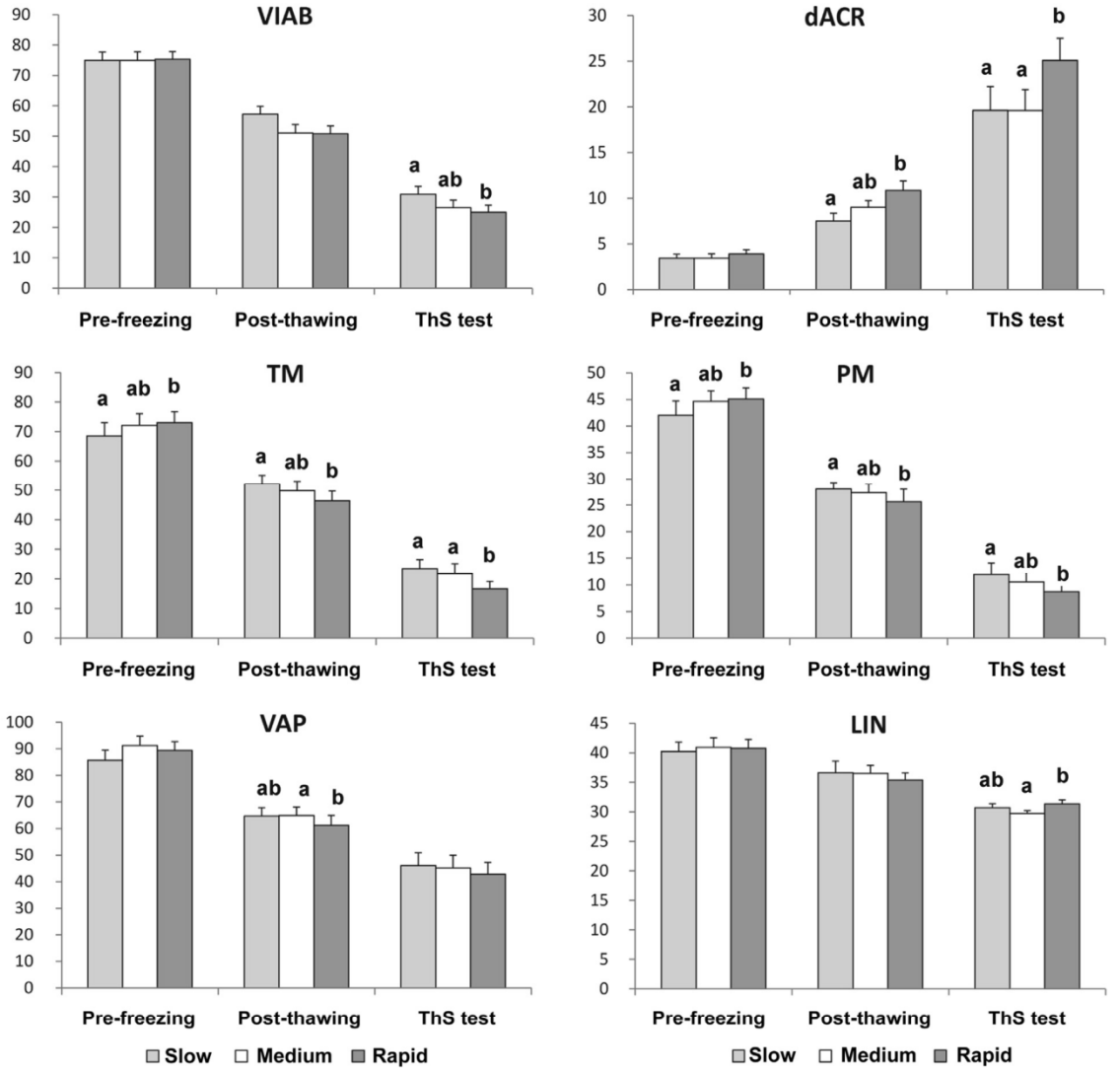

Fig. 1. Experiment 1: Effect of cooling rates (slow, −0.25 °C/min; medium, −1 °C/min; and rapid, −4 °C/min) on the quality of pre-freezing, post-thawing, and after the thermal stress test (ThS test) on viability, acrosomes, and motility parameters of brown bear ejaculates ($n = 15$). dACR, % damaged acrosomes; LIN, % linearity; PM, % progressive motility; TM, % total motility; VAP, velocity average path –(μm/sec); VIAB, % viability. [a,b] Different letters indicate that means of each cooling rate differ for each analysis time ($P < 0.05$).

rate was recommended to preserve *in vitro* motility of liquid equine semen [20].

Carnivore spermatozoa seem less susceptible to cooling than those of other mammalian species [36]. Bouchard et al. [17] assayed cooling rates of 0.3 °C/min and 1 °C/min for canine sperm and did not observe differences in spermatozoa motility in chilled samples at 6 hours after collection. Hermansson and Axnér [15] studied resistance to cold-shock in cat semen by applying 0.5 °C/min and 3 °C/min cooling rates and concluded that feline spermatozoa are not susceptible to cold-shock in the temperature range usually used for liquid sperm preservation. Pukazhenthi et al. [14] observed damage in the acrosome of cat spermatozoa chilled at a rapid (4 °C/min) or ultra-rapid (14 °C/min) rate in comparison with a slow (0.5 °C/min) rate.

During the cooling process, the differences observed between the aforementioned studies could be due to differences in the source of the spermatozoa or in the composition of the extenders used. Gilmore et al. [38] concluded that epididymal spermatozoa of selected African mammalian are clearly more resistant to cold-shock than ejaculated spermatozoa. However, in cat, epididymal and ejaculated spermatozoa were not seen to differ in their susceptibility to cold-shock at the cooling rates usually

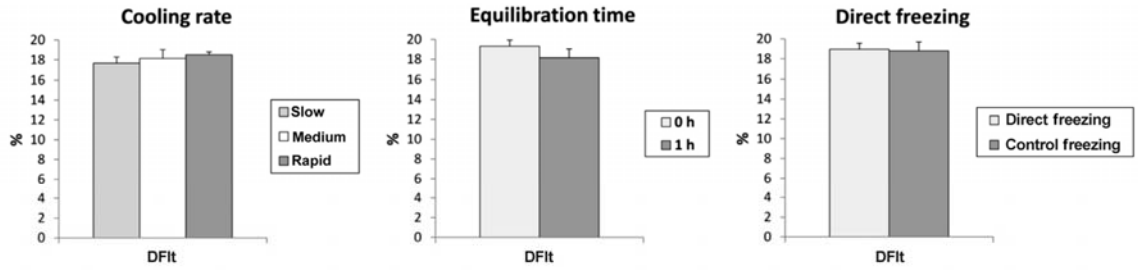

Fig. 2. Experiments 1, 2, and 3: Effect of different cooling rates ($n = 15$), 0 or 1 hour of equilibration pre-freezing ($n = 8$) and direct freezing ($n = 20$) on DNA fragmentation index (DFI) in post-thawed brown bear spermatozoa.

applied [15]. The protective effects of egg yolk in extender during semen cooling have been described in diverse studies [22,39]. In the present study, we have used an extender with 20% egg yolk, but this did not prevent the deleterious effect of the rapid cooling rate on brown bear spermatozoa. In contrast, an extender containing 20% egg yolk showed beneficial effects in red deer semen during rapid cooling [22].

Different studies have suggested that the response of spermatozoa to cold-shock may be related to the lipid composition of their plasma membrane. Temperature variations during cooling cause changes in the fluidity of cell membrane and render the sperm membrane more permeable [40]. In the present study, the chilled sample did not show any changes in viability and acrosome status of brown bear spermatozoa regardless of cooling rates, but these changes were observed in the post-thawing semen. Possibly, this sublethal change in the chilled sample might be a result of membrane reorganization depending on temperature induced during cooling to 5 °C, which sensitizes the membrane to a post-thawing change induced by freezing. This change could be explained by membrane permeabilization taking place once the samples reach the threshold temperatures during the subzero phase of the protocol [41]. As the specific membrane composition of brown bear spermatozoa is not known till date, this has to remain subject of speculation until scientific evidence has been made.

The equilibration time of spermatozoa in defined extender might affect the survival of post-thawing spermatozoa [42,43]. The results of our study indicated that TM, PM, VIAB, and acrosomal status of bear spermatozoa observed after the thermal stress test were significantly better for samples with 1 hour equilibration time. In post-thawing analysis, this difference was also observed as acrosomal status and viability exhibited a clear trend in the same direction. In contrast, Okano et al. [4] evaluated the effect of two different incubation times (1–1 and 3–0 hours of cooling–glycerol equilibration times) on motility, viability, and morphology of frozen-thawed spermatozoa of Japanese black bear and reported that no difference was observed between the two incubation times. One notable difference between these two studies is the procedure of glycerol addition: we used a two-step protocol, whereas Okano et al. [4] employed a simple procedure adding glycerol in equilibration period. In this respect, in a previous study, our group tested the time (before or after cooling to 5 °C) when glycerol was added [9]. Our results suggested that brown bear semen shows good tolerance if a low proportion of glycerol is added at ambient temperature.

Generally, sperm cryopreservation procedures utilize an extended period of time at temperatures above 5 °C, during which the sperm are exposed to a cryoprotectant, for example, glycerol, to allow a protective effect on membranes during cold-shock. However, exposure to glycerol might induce damage in spermatozoa, and therefore, its use has been analyzed extensively. Post-thawing motility in bull spermatozoa was significantly higher after 10 seconds of glycerol exposure compared with an exposure of 2 minutes or longer [42]. Corcuera et al. [44] studied the effect of glycerol (4%, 6%, 8%, and 10%) on boar sperm depending on equilibration time (0 and 30 minutes). They observed that longer equilibration time was favorable for motility when freezed in the 4% glycerol extender, but was detrimental when using the 8% glycerol extender. In canine semen, Okano et al. [30] evaluated the optimal times required for cooling and the time for equilibration with glycerol to 4 °C and concluded that various times for equilibration (0–4 hours) with 3 hours of cooling had no effect on the post-thaw characteristics of canine spermatozoa.

The differences in these studies should be interpreted based on the species-dependent differences in sensitivity of spermatozoa to glycerol [35]. Our data suggest that, unlike other species, an equilibration period with glycerol of 1 hour at 5 °C is superior to total omission of equilibration period and provides the conditions for a better cryosurvival of brown bear spermatozoa. Some authors obtained better results with longer equilibration time, for example, 5 hours in goat [45] and buffalo semen [28] and 4 hours in bulls [27]. This period may be important for optimizing the handling conditions during fieldwork.

Kundu et al. [31] tried to develop a simple model of goat semen cryopreservation by direct freezing of extended sample with a programmable biofreezer, and they observed very low recovery of sperm motility, which was dose-dependent on glycerol. To our knowledge, the omission of cooling and equilibration periods for a cryopreservation protocol has been studied little. Our results have shown that direct freezing drastically reduces sperm viability, acrosomal status, and motility in comparison with conventional freezing. This damage is only partially visible in the post-thawing sample, but the sample subjected to thermal stress test reveals the serious injury suffered. These observations suggest that brown bear spermatozoa

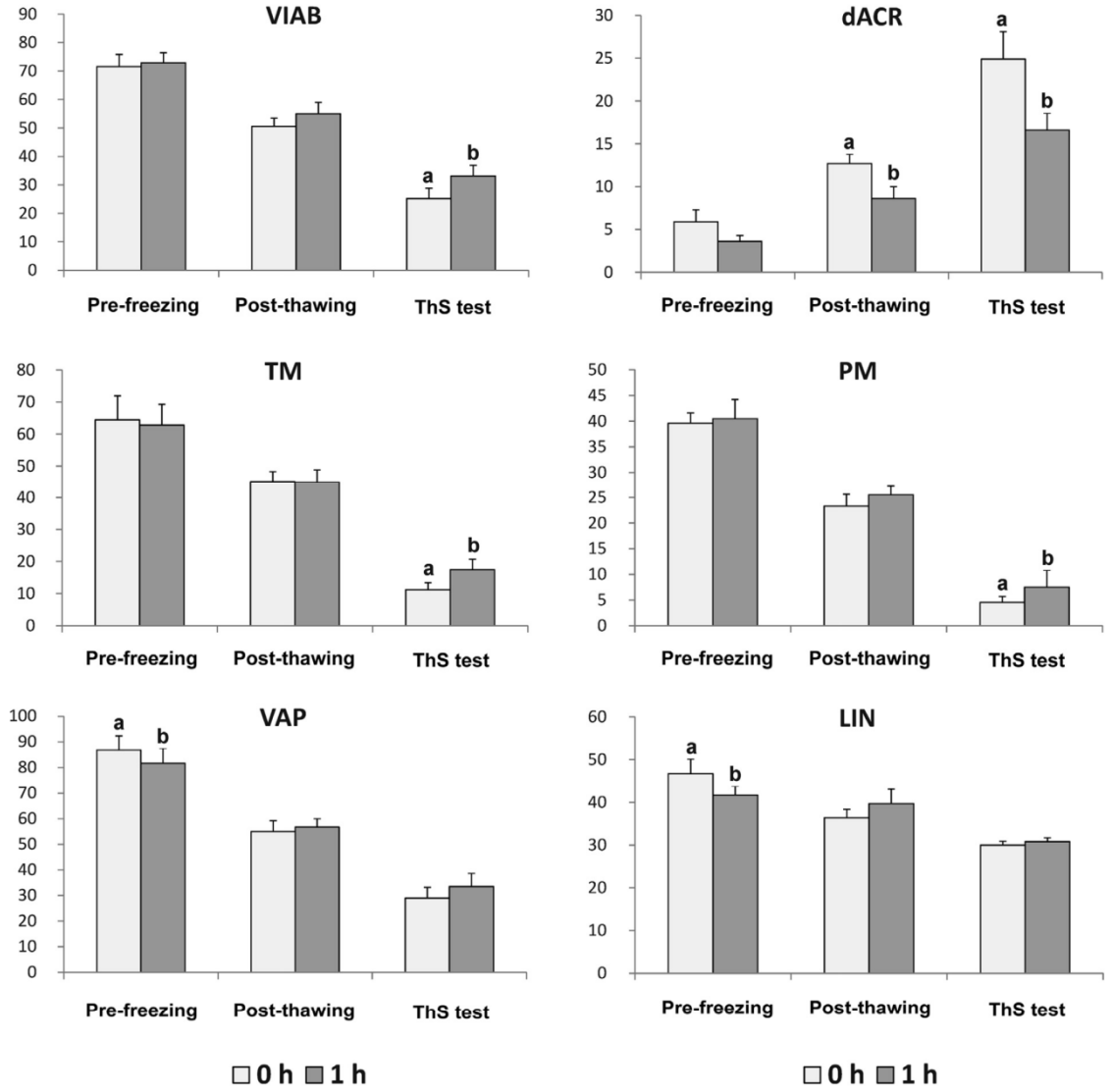

Fig. 3. Experiment 2: Viability, acrosomes, and motility (mean ± standard error) parameters at pre-freezing, post-thawing, and after the thermal stress test (ThS test) on brown bear ejaculates ($n = 8$), where samples were equilibrated during 0 or 1 hour. dACR, % damaged acrosomes; LIN, % linearity; PM, % progressive motility; TM, % total motility; VAP, velocity average path –(μm/sec); VIAB, % viability. [a,b] Different letters indicate differences for each sampling time ($P < 0.05$).

do not resist a drastic cryopreservation protocol under the conditions of our experiment. Other formulations should be assayed by improving the performance of direct freezing.

The chromatin status (%DFIt) evaluated by SCSA was not affected by any of the conditions analyzed in the present study. Similar results have been observed previously in cold storage of canine sperm by Kmenta et al. [46], who observed that chromatin status of cells was not changed by cooling within 8 days. Chromatin status of brown bear spermatozoa varies markedly between seasons [32], and, in the breeding season, brown bear rendered spermatozoa with loose chromatin. Our data confirm this statement and also showed that there are no significant differences in chromatin status between the different methods of sample handling. It is accepted that DNA can be damaged by oxidative stress during cryopreservation, and therefore several studies have been conducted to evaluate the methods of protecting the integrity of sperm DNA during cooling [47].

We observed that the freezing of brown bear semen requires a slow cooling to 5 °C and a pre-freezing equilibration time of 1 hour to prevent significant deterioration. In conclusion, the semen handling time cannot be

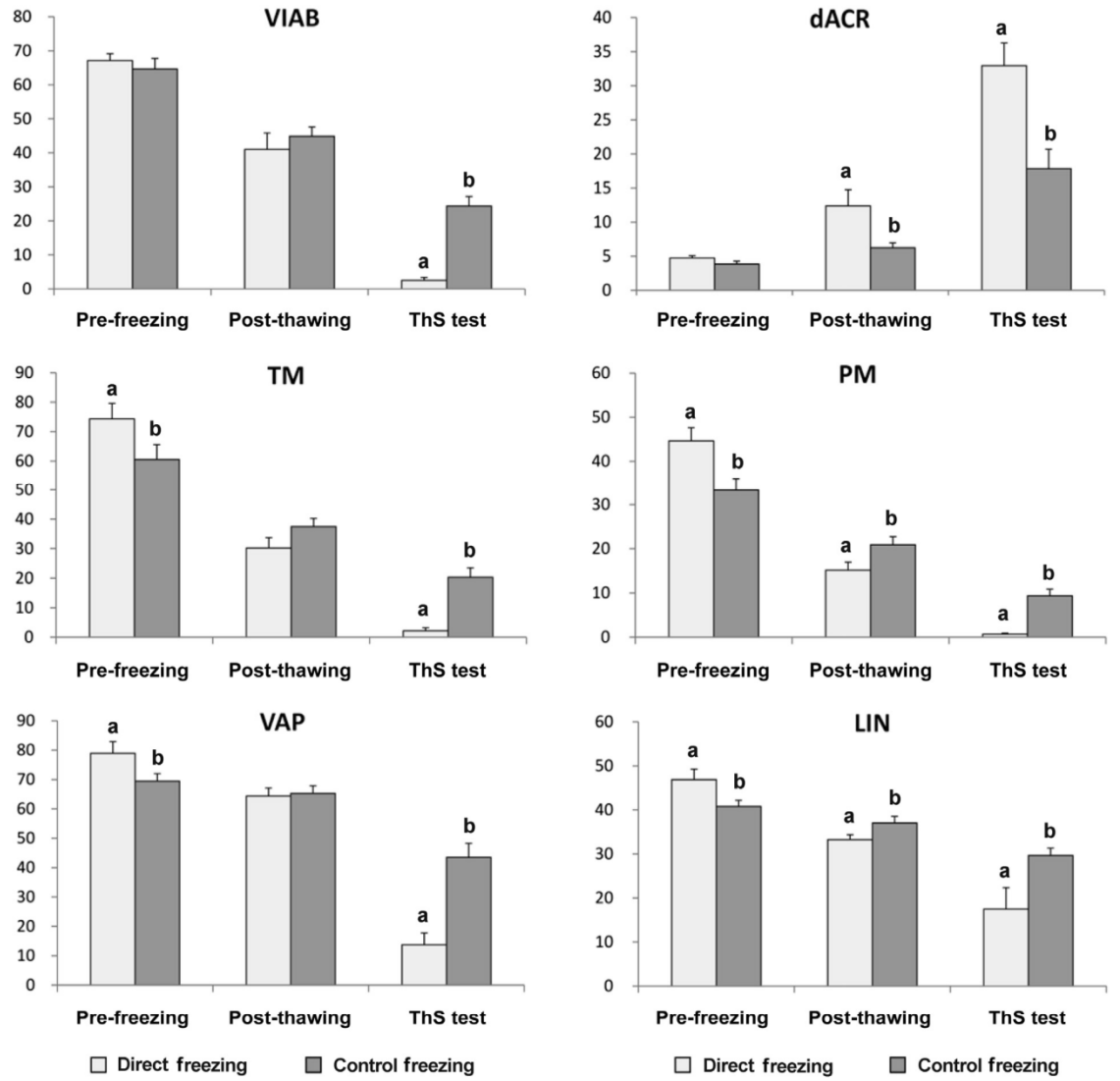

Fig. 4. Experiment 3: Direct freezing in brown bear ejaculates ($n = 20$). Pre-freezing, post-thawing, and post-incubation data for viability, acrosomes, and motility parameters of brown bear ejaculates frozen by control or direct freezing. dACR, % damaged acrosomes; LIN, % linearity; PM, % progressive motility; TM, % total motility; VAP, velocity average path (μm/sec); VIAB, % viability. [a,b] Different letters indicate differences for each sampling time ($P < 0.05$).

shortened with faster cooling rates. We recommended to include slow cooling rates to 5 °C and equilibration period of 1 hour into bear semen preservation. In future research, the effect of longer equilibration time should be explored in this species given the opportunity of sample handling that this would allow.

Acknowledgments

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Alternative procedures for the cryopreservation of brown bear ejaculates depending on the flexibility of the "in cooling" period (5 °C) ☆

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ABSTRACT

The adaptability of cryopreservation protocols for brown bear spermatozoa collected under field conditions and frozen in a nearby laboratory (transported for a few hours) or shipped to a reference laboratory for sex sorting (transported for a few days) was evaluated. Forty-nine electroejaculates from 15 mature brown bears were extended to 100×10^6 sperm/mL in a TES-Tris-Fructose based extender and cryopreserved (−20 °C/min to −100 °C and stored at −196 °C). After thawing, the quality of the seminal samples was assessed for total (TM), progressive (PM) motility and kinetic parameters – by CASA –, and viability (VIAB), viable and non-apoptotic status (YOPRO−), high membrane mitochondrial potential (MIT) and intact acrosomes (iACR) – by flow cytometry –. In Experiment 1, we assessed different storage times (0, 0.5, 1 – control –, 4–5, 7–8 and 11–12 h) at 5 °C from final dilution to freezing. After thawing, non-equilibrated samples (0 h) showed lower values of iACR, TM and PM. No significant differences were found for the different periods of equilibration tested. In Experiment 2, we evaluated three long-term storage times (24, 48 and 72 h) at 5 °C before freezing using storage for 1 h as control. The post-thawing quality of brown bear spermatozoa declined markedly after 48–72 h of pre-freezing. In conclusion, our findings suggest the possibility of extending the pre-freezing cooling period up to 24 h post-collection without freezing. This knowledge should enable the adaptation of the freezing protocols for when a special handling conditions are required such as the shipment of seminal samples to technological centers for the pre-freezing application of enhancer spermatic biotechnologies.

Introduction

Genetic resources banks (GRB) are an important tool for the conservation of threatened species, such as the Cantabric brown bear population [4], since they adequately safeguard genetic variability. The first step to establish an effective GRB is the development of an adapted sperm cryopreservation protocol for this species, which will allow long-term conservation of good quality spermatozoa.

In wild species, sperm collection can be carried out in the field which has little to do with the ideal conditions of a laboratory [12,19]. Standard cryopreservation protocols might be adapted to variable working conditions; for this reason, it is necessary to know the permissiveness of spermatozoa in order to cryopreserve them by non-standard protocols reaching an acceptable level of post-thawing quality [22]. In the present work, we have studied the flexibility of the cooling period (5 °C) in order to improve the efficiency of the cryopreservation procedure for brown bear ejaculates under field conditions. Sometimes, it is necessary to provide extra time after semen collection to adapt the cryopreservation protocol to available infrastructure (short-term strategy, equilibration times from 0 to 12 h). On the other hand, the application of biotechnologies for a better and more efficient use of ejaculates (such as sex-sorted spermatozoa) in reference technological

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centers requires an extra time to ship the cooled samples (5 °C; long-term strategy; equilibration times up to 72 h).

Short-term strategy would allow cooling protocols to adapt to different conditions that limit the use of control procedures (1 h of equilibration time [5]). Berndtson and Foote [7] demonstrated that glycerol could penetrate bull sperm in 3–4 min (both 25 and 5 °C). These results suggested that equilibration time seems to be more important for adapting sperm membrane to low temperatures than for penetration of glycerol into spermatozoa [27]. However, Okano et al. [30] assessed 1 h cooling and 1 h equilibration (protocol 1) or 3 h cooling without equilibration (protocol 2) for Japanese black bear sperm, and the results did not show any differences between the protocols. Okano et al. [29], using dog semen as reference in studies on bear sperm, observed that after 16 h equilibration time, dog sperm had less progressive motility than with 0 or 2 h. For giant panda semen, Olson et al. [31] tested 3 equilibration times (0, 0.5 and 2.5 h) and they did not find any significant differences for motility and viability, although these results refer only to pre-freezing data. Most published research on this topic has been conducted with domestic species, and conflicting results have been observed. Some authors (Bull [45]; boar [10]) found best results with short equilibration time (0.5 h), while other authors (Bull [24]; buck [8]; boar [46]) observed spermatozoa needed more time of equilibration (2–4 h). However, Crockett et al. [11] concluded that stallion semen required overnight equilibration for a higher motility (progressive motility at 12 h higher than for 2 or 6 h).

A long-term storage strategy to facilitate sample shipment to biotechnological centers to improve the efficiency of semen doses in GRBs should be considered. Transport could be from cooled or frozen seminal samples. Sperm cooled for 3 or more days has been evaluated in several species and has been found to retain over 50% motility (Dog [23,34,37,39]; Ram [26,40]; Buffalo [1]). In relation to frozen samples, several authors evaluated the effect of the semen refreezing (bull [38,41]; brown bear [3]) and they observed a drop in quality (motility and viability) between two freezing cycles, which could be acceptable in some situations. A comparative evaluation enabling one of the two techniques to be selected is needed: (1) long-term cooled storage and posterior freezing or (2) initial freezing, shipping and refreezing of semen samples.

In this work, we evaluate different storage time at 5 °C for brown bear semen in comparison with the control cryopreservation protocol (storage 1 h), considering the different situations that we can find during the semen collection under field conditions. Thus, we analyze the effect of (1) short storage times (0, 0.5, 1 – control –, 4–5, 7–8 and 11–12 h at 5 °C) and (2) long-term storage (1 – control –, 24, 48 and 72 h at 5 °C) in the freezing protocol for brown bear ejaculates.

Materials and methods

Materials

All chemicals were of at least reagent grade and were acquired from Sigma–Aldrich (Madrid, Spain), unless otherwise stated.

Animals and ejaculate collection

Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. All experiments were performed after obtaining ethical committee approval from the Ethical Committee for Experimentation with Animals of León University, Spain (03-02/2010).

Ejaculates from 15 sexually mature male brown bears (*Ursus arctos*) were obtained by electroejaculation during the breeding season (end of April to early July). Animals were housed in a half-freedom regime in Cabarceno Park (Cantabria, Spain; 43° 21′ N, 3° 50′ W; altitude: 143 m), and fed with a diet based on chicken meat, bread and fruits.

The animals were immobilized by teleanaesthesia with 750 mg zolazepam HCl, tiletamine HCl (Zoletil 100®; Virbac, Carros, France) and 6 mg medetomidine (10 mg/mL Zalopine®, Orion Pharma Animal Health, Finland). After immobilization, the bears were weighed and monitored during anesthesia (pulse, saturation of peripheral oxygen and respiration). Prior to electroejaculation, the pubic region was cleaned, the penis was washed with sterile physiological saline and the rectum was emptied of feces. The bladder was catheterized during semen collection. Electroejaculation was carried out with a PT Electronics1® electroejaculator (PT Electronics, Boring, OR, USA). The transrectal probe was 320 mm long with a diameter of 26 mm. Electric stimuli were applied until ejaculation (6–10 V and 250–300 mA, on average). Ejaculates were collected in a graduated glass tube.

Ejaculates management

Immediately after collection, the volume and concentration of each ejaculate were recorded. Sperm concentration, the motility and kinematics parameters was assessed by Computer-Aided Sperm Analysis (CASA) (ISAS v.1.2, Integrated Semen Analyser System; Proiser, Valencia, Spain) using a Bürker hemocytometer (Marienfeld GmbH, Marienfeld, Germany) and a phase contrast microscope. Urospermia was evaluated by means of a rapid urea test (Urea test strips, DiaSys Ecoline® GmbH, Holzheim, Germany). Nine samples of low motility (<50%) or contaminated urine (>80 mg urea/dL) were rejected [18]. Sperm concentration of the ejaculates used in this study was $341.5 \pm 50.8 \times 10^6$ mL^{-1}. The selected samples were centrifuged at 600×*g* for 6 min. The supernatant was discarded and each pellet was resuspended with the same volume of TES-Tris-Fructose based extender at room temperature (dilution 1:1), and cooled at 0.25 °C/min to 5 °C (~70 min). The extender was prepared using TES-Tris-Fructose, 300 mOsm/kg, pH 7.1 (TTF extender); 6% glycerol, 20% egg yolk, supplemented with 2% EDTA, 1% Equex (Equex STM Paste; Minitüb, Tiefenbach, Germany), 0.302 mg penicillin G sodium salt/ml and 0.625 mg dihydrostreptomycin sesquisulfate/ml), as in de Paz et al. [13].

Sperm cryopreservation

Once at 5 °C, a second 1:1 dilution was performed, using the TTF extender at 9% glycerol, in order to reach a final glycerol concentration of 6%. More TTF at 6% glycerol was added to obtain a final concentration of 100×10^6 sperm/mL. After equilibration (see Experimental design), the diluted sperm sample was packaged into 0.25-mL plastic straws. The samples were frozen in a programmable biofreezer (Kryo 560-16 Planer™, Planer plc., Sunbury-On-Thames, UK) at −20 °C/min down to −100 °C, and then transferred to liquid nitrogen containers. The frozen sperm samples were stored in liquid nitrogen for a minimum of one month. Thawing was performed by dropping straws in water at 65 °C for 6 s.

Experimental design

Experiment 1: short storage of pre-freezing sample

Thirty-four electroejaculates from 15 brown bears were processed for this experiment. After the final dilution (at 5 °C) of the sperm sample, a 0.25 ml-straw was filled and immediately frozen (sample without equilibration time – 0 h –); the remaining volume

was kept at 5 °C during different equilibration times (0.5, 1, 4–5, 7–8 and 11–12 h) before freezing.

Experiment 2: long-term storage of pre-freezing sample

For this experiment, 15 electroejaculates from 7 brown bears were processed. Sperm samples were extended to the final concentration and kept at 5 °C for 1 h, then a 0.25 ml-straw was filled and frozen (1 h, control freezing sample); the remaining volume was stored in a glass tube at 5 °C up to 72 h during the equilibration period, and a 0.25 ml-straw was filled and frozen every 24 h. To describe the changes in sperm quality induced by handling the sample, we calculated normalized data dividing each value of pre-freezing or post-thawing sample by the corresponding value of control freezing sample and multiplying by 100 (Recovery rate).

Finally, pre-freezing data of samples cooled for 24, 48 and 72 h were compared with post-thawing data of a control freezing sample (1 h) considered as a reference protocol to biotechnological application (sex-sorting) prior to storage in GRB's.

Semen evaluation

Before freezing and post-thawing (at 10 min), sperm samples were analyzed for motility, viability, apoptosis, and acrosomal and mitochondrial status.

Motility assessment

The motility and kinematic parameters were assessed using CASA. A 5-μl sperm sample was placed into a Makler counting cell chamber (10 μm depth; Sefi Medical Instruments, Haifa, Israel) and examined using a negative phase contrast microscope (×10) with a warmed (38 °C) stage. The standard parameter settings were as follows: 25 frames/s; 5–50 μm^2 for head area; curvilinear velocity > 10 μm/s to classify a spermatozoon as motile. At least 5 fields or 200 spermatozoa were saved and analyzed afterwards. Reported parameters were total motility (TM, %), progressive motility (PM, %; spermatozoa were considered progressive if VCL > 10, STR > 65), average path velocity (VAP, μm/s), and linearity (LIN, %).

Viability assessment

We applied a double staining of SYBR-14 (Molecular Probes, L-7011 LIVE/DEAD® Sperm Viability Kit; Invitrogen, Barcelona, Spain) and propidium iodide (PI) according García-Macías et al. [18]. The semen samples were diluted to 5×10^6 sperm/mL with PBS, and 300 μL were transferred to a polypropylene tube, and 3 μL IP (24 mM) and 1.5 μL SYBR 14 (100 nM) were added. The tubes were maintained at 37 °C for 20 min in the dark. We evaluated the percentage of viable sperm (SYBR-14+/IP-, named VIAB).

Acrosomal status assessment

We used the double stain of peanut agglutinin-fluorescein isothiocyanate (PNA-FITC) and PI according García-Macías et al. [18]. Seminal samples were diluted with PBS (5×10^6 sperms/mL), and 300 μL were transferred to a polypropylene tube and 0.3 μL of stock solution (PNA-FITC at 1 mg/mL in PBS and 1.5 mM IP in a PBS) added. The tubes were incubated for 10 min at room temperature and analyzed. We used the percentage of spermatozoa with intact acrosomes (PNA-, named iACR) observed in the flow cytometer.

Mitochondrial status assessment

JC-1 (Invitrogen, Barcelona, Spain) was used to assess mitochondrial status [18], identifying mitochondria with high membrane potential. Samples were diluted in 300 μL of PBS (5×10^6 sperms/mL), adding 0.3 μL of JC-1 stock solution (3 mM JC-1 in DMSO), for 30 min at 37 °C and analyzed. Only the percentage of red stained sperm (high membrane mitochondrial potential, named MIT) was used.

Apoptosis assessment

Changes in sperm membrane permeability were analyzed with double staining YO-PRO-1 (Invitrogen, Barcelona, Spain) and IP [28]. The YO-PRO-1 can be used to track the destabilization of sperm membrane during cryodamage [32]. Sperm samples were diluted to 5×10^6 sperms/mL in polypropylene tubes (300 μL/tube), adding 2 μL of YO-PRO-1 at 25 μM in DMSO and 0.3 μL of 1.5 mM IP in a PBS. The tubes were incubated at 37 °C for 10 min until analysis by flow cytometry. We used the percentage of viable and non-apoptotic spermatozoa (YO-PRO-1-/IP-, named YOPRO−).

Flow cytometry evaluation

A FACScalibur flow cytometer (Becton Dickinson Immunochemistry Systems, San Jose, CA, USA), equipped with standard optics and an argon ion laser, tuned at 488 nm, and running at 200 mW was used. Calibration was carried out periodically using standard beads (Calibrites; Becton Dickinson). The fluorescence emitted by SYBR-14, YO-PRO-1 and PNA-FITC was quantified using the FL1 photodetector (530/28BP filter), JC-1 (red) with the FL2 photodetector (585/42BP filter) and PI fluorescence using the FL3 photodetector (670LP filter). The FSC/SSC (Forward Scatter/Side Scatter) signals were used to discriminate the sperm population from other events. For each sample, we have acquired 10,000 spermatozoa per sample with a flow rate of 200 cells/s, using Cell Quest Pro v. 3.1 (BD Biosciences) software. The analysis of the flow cytometric data was performed using the Weasel v.2.6 program (the Walter and Eliza Hall Institute of Medical Research, Victoria, Australia).

Statistical analysis

Data were analyzed using the SAS™ V.9.1 package. Data were tested for normality (Shapiro–Wilk test) and arcsin square-root transformation of the percentage data was used to normalize the data before analysis when was necessary. Our hypotheses for equilibration times and long-term storage before freezing were tested by using mixed-effects linear models. Each male was considered as a random effect. Least-squares means were computed for each effect listed and *P*-values for differences of the multiple comparisons were calculated by Tukey's test. Values were considered to be statistically significant at $P < 0.05$. All the results of the present study are presented as mean ± SEM.

Results

Experiment 1: short storage of pre-freezing sample

In pre-freezing, differences were only found for YOPRO− spermatozoa (81.9% vs. 65.0%; $P < 0.05$) between the control sample (1 h) and the sample equilibrated 7–8 h (Fig. 1). After thawing, the control sample showed higher motility than the non-equilibrated ones – sample 0 h – (TM: 72.0% vs. 40.2% and PM: 32.3% vs. 16.9%; respectively, $P < 0.05$). Equilibrated samples showed higher iACRO parameters (ranging between 87.2% and 90.2%, $P < 0.05$) than the non-equilibrated ones (75.6%).

Experiment 2: long-term storage of pre-freezing sample

Samples stored for 1 and 24 h showed similar post-thawing results in motility, kinetic parameters and mitochondrial status (Fig. 2). Samples stored during 48 h yielded lower scores than the control group ($P < 0.05$) for VIAB (36.0%), YOPRO− (40.4%), TM (39.2%) and PM (14.2%) parameters. Samples stored for 72 h

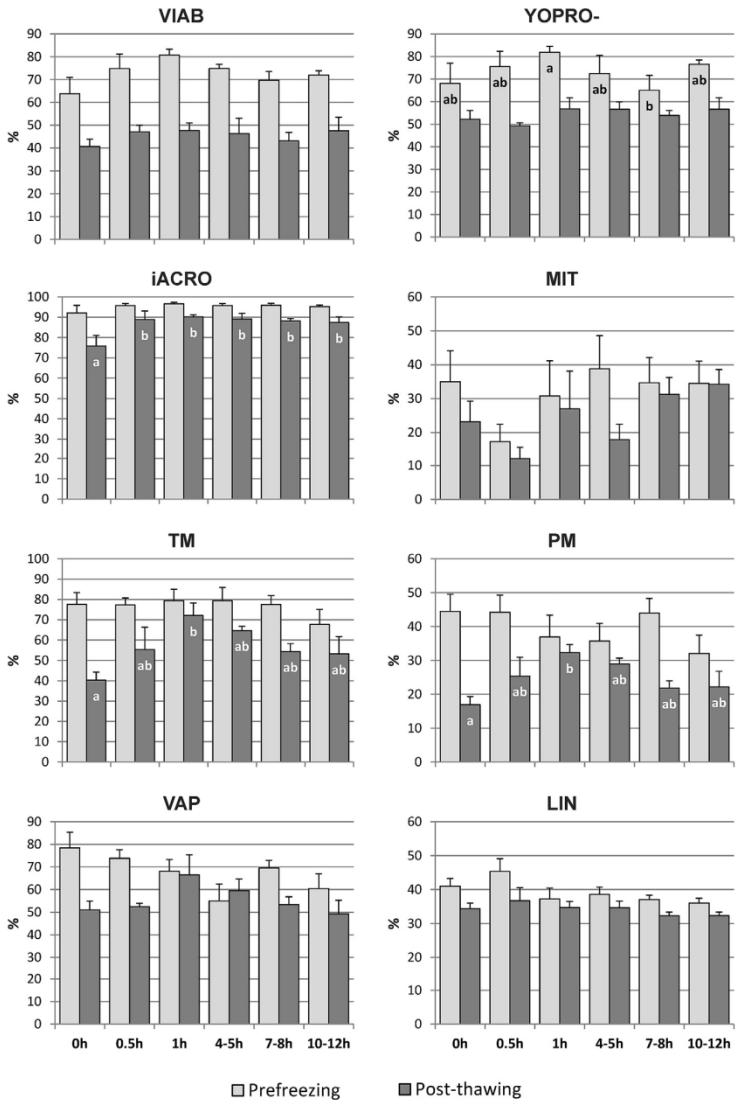

Fig. 1. Effect of equilibration times (0, 0.5, 1 – control –, 4–5, 7–8 and 10–12 h) at 5 °C in the quality of pre-freezing and post-thawing brown bear spermatozoa (VIAB: % viability; YOPRO−: % viable and non-apoptotic spermatozoa; iACR: % intact acrosomes; MIT: % high mitochondrial membrane potential; TM: % total motility; MP: % progressive motility; VAP: velocity average path – μm/s –; LIN: % linearity). [a,b] Different letters indicate that means of each equilibration time differ for each analysis time ($P < 0.05$).

showed the lowest results for all evaluated parameters except LIN (Fig. 2).

Comparing data for storage at 5 °C at different long-term times versus the post-thawing values of a control freezing (Table 1), we found that 24 h stored sperm samples showed better pre-freezing values for VIAB (62.9%), YOPRO− (65.9%) and iACRO (94.1) ($P < 0.05$). Control freezing showed higher scores for all motility parameters than spermatozoa stored up to 72 h ($P < 0.05$).

When TM and PM recovery rates were analyzed (see Fig. 3), the post-thawing values decreased significantly with increasing pre-freezing storage time (TM: 93.2%, 66.9% and 36.6%; PM: 88.9%, 54.3% and 33.8%; 24, 48 and 72 h, respectively). Moreover, we observed that samples stored for 24 h showed higher post-thawing recovery rates ($P < 0.05$) for VIAB (92.8 vs. 65.1), YOPRO− (84.1 vs. 66.0) and iACRO (101.6 vs. 92.5), than those 72 h.

Discussion

The effect of equilibration time and pre-freezing long-term storage of semen samples at 5 °C were studied, in order to design an adequate cryopreservation protocol for brown bear ejaculates

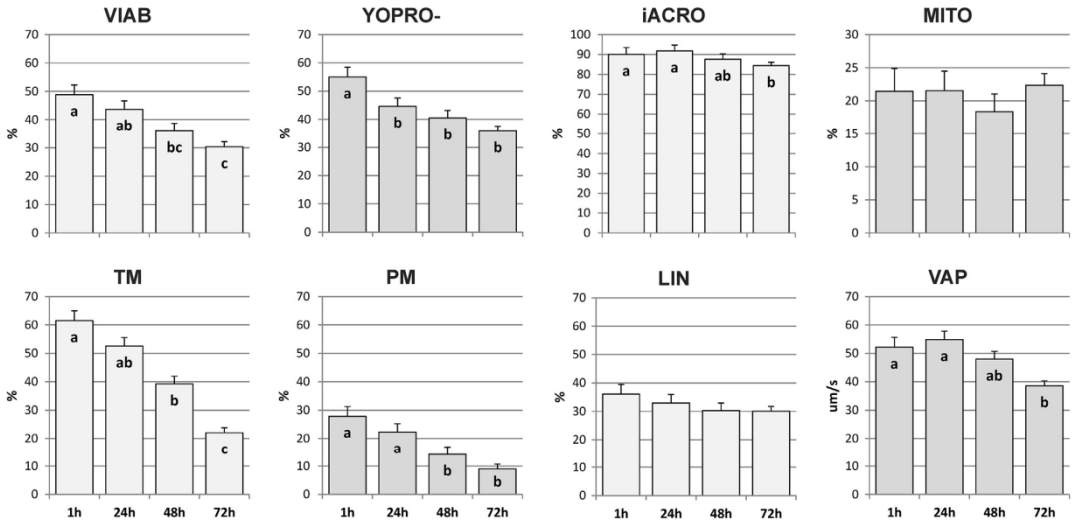

Fig. 2. Post-thawing quality of brown bear spermatozoa stored at 5 °C up to 72 h (1 h – control –, 24, 48 and 72 h) (VIAB: % viability; YOPRO–: % viable and non-apoptotic spermatozoa; iACR: % intact acrosomes; MIT: % high mitochondrial membrane potential; TM: % total motility; MP: % progressive motility; VAP: velocity average path – μm/s –; LIN: % linearity). [a,b]Different letters indicate that means of each time of long-term storage differ for each parameter ($P < 0.05$).

Table 1
Pre-freezing data of sample stored for 24, 48 and 72 h at 5 °C versus post-thawing data of control freezing. [VIAB: % viability; YOPRO–: % viable and non-apoptotic spermatozoa; iACR: % intact acrosomes; MIT: % high mitochondrial membrane potential; TM: % total motility; MP: % progressive motility; VAP: velocity average path – μm/s –; LIN: % linearity.]

	Pre-freezing (5 °C)			Post-thawing
	24 h	48 h	72 h	Control freezing
VIAB	62.9 ± 3.7[a,*]	56.3 ± 4.7[a,b]	42.2 ± 5.9[b]	48.8 ± 3.4
YOPRO–	65.9 ± 3.8[*]	58.6 ± 4.9	49.7 ± 5.6	55 ± 3.3
iACR	94.1 ± 0.7[*]	93.8 ± 0.9[*]	90.6 ± 1.1	89.9 ± 0.7
MIT	20.6 ± 5.4	32.6 ± 7.1	25.2 ± 6.3	21.5 ± 6.6
TM	57.6 ± 7.8	53.5 ± 8.1	42.5 ± 6.2[*]	61.5 ± 3.1
PM	24.3 ± 4.6	21.5 ± 3.2	16.1 ± 2.5[*]	27.8 ± 1.7
VAP	46.4 ± 3.5[a,b]	55.6 ± 6.4[a]	38.3 ± 3.9[b,*]	52.2 ± 2.2
LIN	31.9 ± 2.2	32.1 ± 1.3[*]	27.9 ± 1[*]	36.1 ± 1

[*] An asterisk indicates that means of each time of cooling storage (pre-freezing data) differ from post-thawing data of control freezing (1 h).
[a,b] Different letters indicate that means of each time of long-term storage differ within pre-freezing analysis ($P < 0.05$).

handled under different conditions. We carried out two experiments in which sperm samples were equilibrated to 10–12 h at 5 °C (Experiment 1) or stored at 5 °C up to 72 h before freezing (Experiment 2).

In the first experiment, we observed a significant reduction in sperm motility and greater acrosome damage after the freezing-thawing process in sperm samples cooled at 5 °C without a posterior equilibration period (0 h). In general, samples at 5 °C equilibrated for 1 h (control) yielded higher sperm quality and our results showed a gradual decrease in quality as the equilibration time increased, although no differences were found up to 10–12 h. Okano et al. [29] after cooling dog semen for 3 h at 4 °C followed by equilibration for different times (0–4 h), found that an equilibration time was not necessary. In Japanese black bear semen studies, Okano et al. [30] testing 1 h cooling followed by 1 h equilibration (protocol 1) versus 3 h cooling without equilibration (protocol 2); observed that post-thawing data showed no significant differences (motility, viability, abnormal morphology and intact acrosomes) and they suggested that the glycerol equilibration time can be omitted. Their results do not coincide with those observed in the present study, which may partly be explained by the fact that we applied a single cooling time for 1 h and they used two times (3 or 1 h) in combination with 0 or 1 h equilibration time respectively. Olson et al. [31] studied 4 cooling methods using electroejaculates from one giant panda (0.5 or 2.5 h cooling combined with 0, 0.5 or 2.5 h equilibration) and no differences were found for motility or viability in pre-freezing data. These data only have a descriptive value and lack statistical validity, so a comparison with our results is not possible.

Dhami and Sahni [15] studied the effects of four cooling rates and two equilibration periods (0 and 2 h) on the motility and fertility of bull spermatozoa and concluded that slow cooling over a 2 h period from 30 °C followed by at least 2 h of equilibration at 5 °C was essential for optimal results. In goat semen, Deka and Rao [14] observed that cooling diluted semen from 35 °C to 5 °C for 1.5 h and after 1, 3 or 5 h of equilibration, the best post-thawing results were obtained with a short equilibration time for acrosome status (1 h) or a long equilibration time for motility (5 h) depending on the glycerol rate used. In these two papers, sperm were first diluted at room temperature with non-glycerolated extender and then at 5 °C adding extender with glycerol; in contrast, in our study the first dilution (1:1) was performed with 6% glycerol and refrigeration was performed at 3% glycerol.

Membrane permeability to glycerol appears to be specific for each species, so glycerol can rapidly penetrate through spermatic membrane in some species but slowly in others [25]; however, no studies on the glycerol penetration into bear spermatozoa have been carried. Our results showed that semen without equilibration (0 h group) presented a significant reduction in post-thawing motility and intact acrosomes, while a short equilibration time (0.5 h) in a diluent with glycerol seems to be sufficient for a better cryopreservation of motility and acrosome of bear spermatozoa. This fact leads us to think that glycerol may need a minimum period to stabilize sperm membranes as described by others authors [16], although other factors may affect to spermatozoa

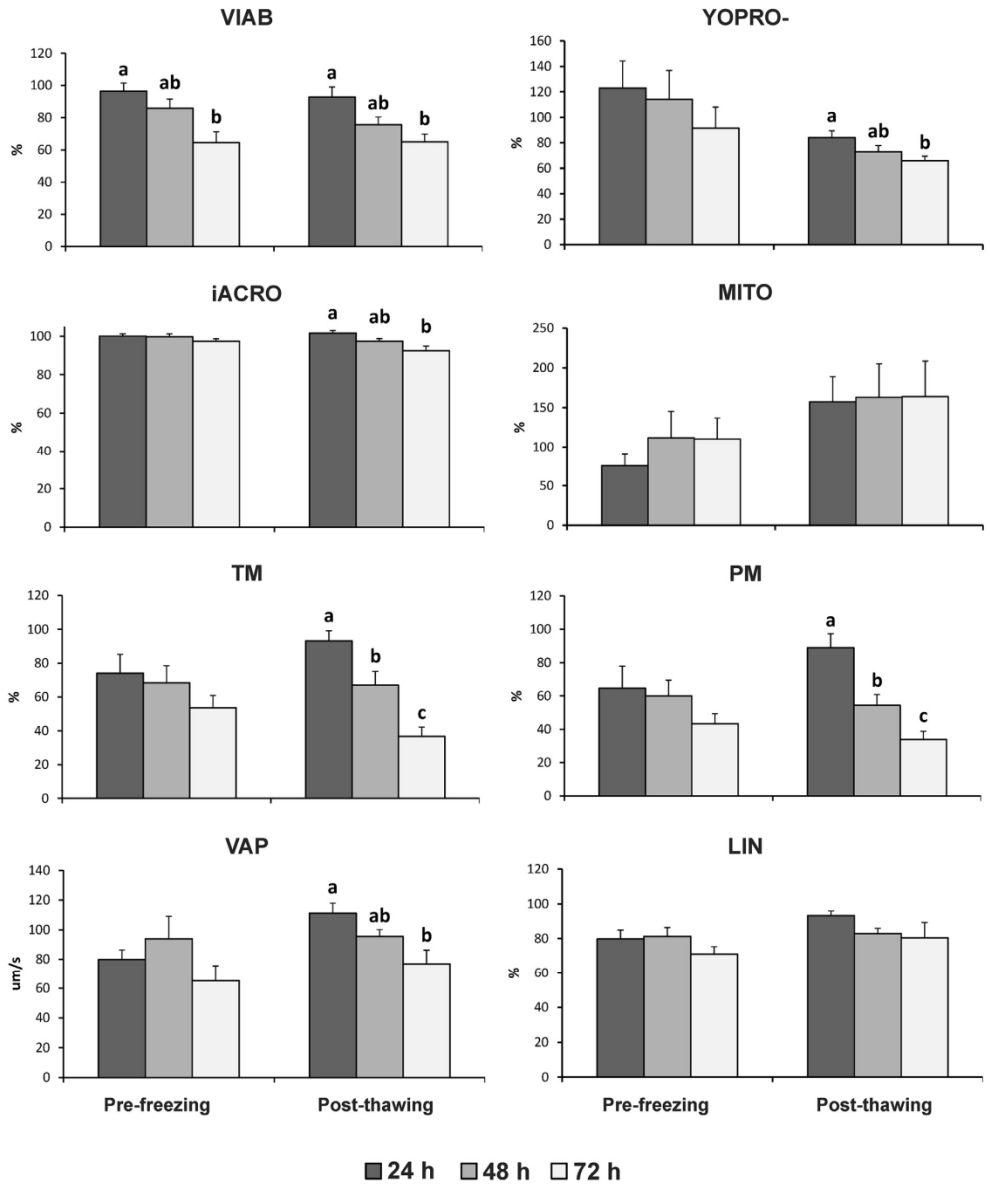

Fig. 3. Recovery rates in comparison with control freezing group (1 h) of semen stored during 24, 48, 72 h at pre-freezing and post-thawing (long-term group value/control freezing value × 100). (VIAB: % viability; YOPRO−: % viable and non-apoptotic spermatozoa; iACR: % intact acrosomes; MIT: % high mitochondrial membrane potential; TM: % total motility; MP: % progressive motility; VAP: velocity average path – μm/s –; LIN: % linearity). [a,b] Different letters indicate that means of each time of long-term storage differ for each analysis time ($P < 0.05$).

cryosurvival [17]. Concerning to glycerol toxicity, the samples with long equilibration times (10–12 h) showed no significant differences in any of the evaluated parameters in comparison with short times, although there is a gradual decrease from 1 to 10–12 h. In this sense, Herold et al. [22] tested equilibration times between 2 and 9 h when freezing buffalo epididymal sperm using two glycerolated extenders and they did not observed any differences in motility and acrosomal status. Alvarez-Rodríguez et al. [2] suggested that brown bear semen seems to possess a good tolerance to glycerol since no differences in semen quality were observed among three protocols for glycerol addition: (1) all at room temperature, (2) half of the final concentration at room temperature and the rest at 5 °C, and (3) all at 5 °C.

In Experiment 2, our post-thawing results (VIAB, dACR, motility) showed a decline in sperm quality after 24 h of storage at 5 °C although recovery rates were close to 100% for most of the

parameters. Storage for 72 h resulted in a dramatic decline in sperm quality at both pre-freezing and post-thawing. In discrepancy with our results, some authors found that semen samples remained at ⩾50% of motility for 3 or more days of cooling storage (Dog [23,34,37,39]; Ram [26,40]; Buffalo [1]). These differences may be based on specific differences, in collection method or in the use of glycerolated extender. In our study, semen samples were collected by electroejaculation while in the studies cited above they were obtained by artificial vagina, post-mortem recovery (epididymal) or digital manipulation, and obtained from other mammal species than brown bear.

On the other hand, some authors have studied cryopreservation of spermatozoa after long-term storage and practically found no differences after the freezing-thawing procedure between assessed times (Purdy [36]: 0, 24 and 48 h, in ram. Guthrie and Welch [20]: 3 and 24 h, in boar. Hermansson and Forsberg [21]: 24 and 48 h, in dog. Ponglowhapan et al. [35]: 0 and 48 h, in dog). Even, Anzar et al. [6] achieved even higher post-thawing quality (motility, membrane and acrosome integrity) for bull semen stored for 24 h than those for 0 h. However, Crockett et al. [11] observed stallion sperm had higher motility when seminal samples were frozen after 2.5 h than those stored for 24 h. In our study, post-thawing quality at 24 h remained similar to post-thawing control freezing-(1 h), so brown bear spermatozoa could be frozen after 24 h collection if these samples were obtained at a considerable distance from the laboratory. Verstegen et al. [42] evaluating changes in glucose concentration in dog ejaculates stored at 4 °C for several days, observed that during the first 10 days semen motility percentages did not change significantly but glucose concentration decreased abruptly on the second day of storage in comparison with day 1 although acceptable fertility was achieved after storage for 6 days. They suggested that glucose consumption during day 1 was related with a high spermatozoa activity but glucose was probably not the only parameter involved in semen motility.

When bear semen needs to be transported for the application of spermatic enhancer biotechnology (eg sex-sorting), it is important to know which methodology renders the best results: (1) cooling-freezing/thawing, sex-sorting and refreezing, or (2) cooling, long-term storage at 5 °C, sex-sorting and freezing. We compared post-thawing data from control freezing versus several long-term storage times at 5 °C and our results indicated that quality (viability, viable and non-apoptotic spermatozoa and acrosome status) was diminished after the freezing/thawing process (control freezing) in comparison with 24 h storage time and was similar to storage for 48 h at 5 °C. Thus, when enhancer biotechnologies are to be applied within 24 h post-collection, we recommended shipment at 5 °C. Storage at 5 °C up to 48 h or the control freezing-thawing presented similar scores, so the choice would depend on the possibility of shipment to the reference laboratory and the freezability of the semen. However, when the reference laboratory is further away (semen shipment around or more than 72 h) the seminal sample should be frozen with a control protocol instead of pre-freezing storage at 5 °C. After control freezing of brown bear spermatozoa, 75% of initial viability and 79% of initial motility were recovered, but we did not know what the level of freezability would be after a second freezing cycle.

In a previous study [3], we refroze brown bear semen and found a significant detrimental effect in the evaluated parameters; motility and acrosome status were more negatively affected (recovery rates between the two thawing afforded around 50%). Several authors observed a clear drop in quality between first and second freezing-thawing processes in human (Polcz et al. [33], TM: 24.4 vs. 8.0, VIAB: 39.1 vs. 25.3; Verza and Esteves [43], TM: 42 vs. 22.5, PM: 34 vs. 9.5, VIAB: 45 vs. 20; Verza et al. [44], TM: 50.6 vs. 26.9), stallion (Choi et al. [9], TM: 36 vs. 16) and bull spermatozoa (Underwood et al. [41], TM: 70 vs. 55, PM: 50 vs. 30, VIAB: 50 vs. 40). In our experiment, the comparison is not complete because we did not carry out refreezing with semen frozen using a control protocol, but it is an initial attempt to establish handling guidelines for spermatic samples of this species.

In conclusion, our results suggested that brown bear spermatozoa tolerate an equilibration time of up to 10–12 h; period that will allow us to move the semen obtained from the site of capture of the bear in the field to the position of the mobile laboratory. On the other hand, pre-freezing seminal samples can be stored during 24 h at 5 °C (even 48 h could be an acceptable option), which will facilitate their submission to a distant Technology Center for handling.

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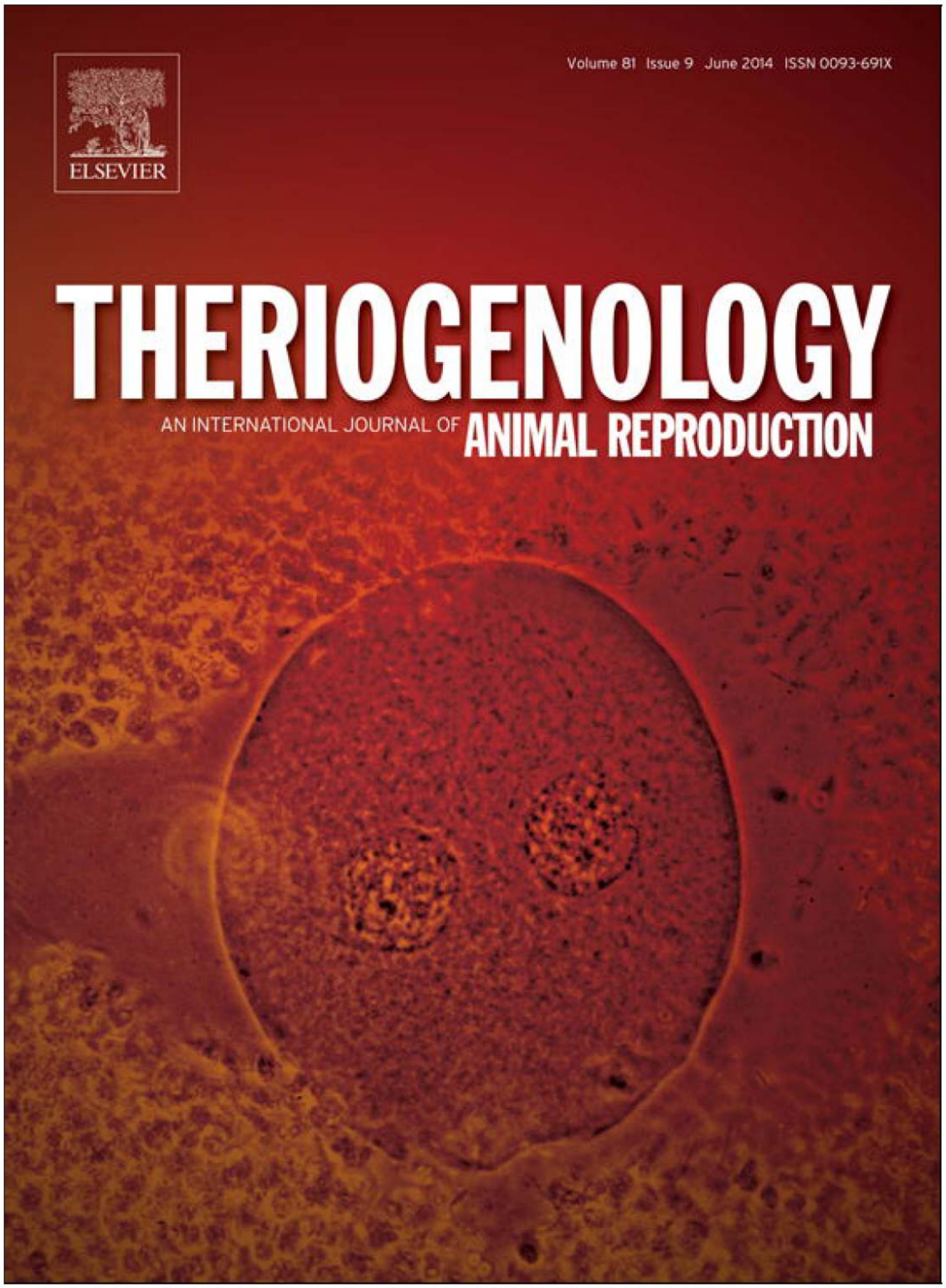
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Optimization of conditions for long-term prefreezing storage of brown bear sperm before cryopreservation

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ABSTRACT

Brown bear ejaculates are usually collected in field conditions and may need to be shipped to a laboratory for the application of reproductive biotechnologies before cryopreservation. The aim of this study was to extend the prefreezing step to 48 hours (1 hour vs. long-term storage [LS] to 24 and 48 hours) to enable the sample to be transported. The effects of storage temperature (experiment 1), glycerol concentration (experiment 2), and dilution rate (experiment 3) on sperm were evaluated. Electroejaculates from brown bears were stored under different experimental conditions and cryopreserved. The sperm motility and viability, apoptotic status, and acrosomal status of sperm were assessed before freezing (prefreezing), after thawing, and after 2-hour incubation at 37 °C (thermal stress test). In all experiments, one control sample was frozen using a standard protocol (control). In experiment 1, three temperatures during LS with 6% glycerol were tested: 5 °C (T5), 15 °C (T15), and room temperature (RT). The LS-T5 sample yielded the highest postthawing results for viability (42.4%), progressive motility (15.6%), and intact acrosome (83.1%) after 24 hours in comparison with the other temperatures ($P < 0.05$); for 48 hours, the LS-T5 sample reached higher total and progressive motility (25.9% and 9%, respectively) and nonapoptotic values (36.5%). Recovery rates revealed susceptibility to freezing at LS-15 or LS-RT samples at 24 hours (viability) or 48 hours (viability and motility). In experiment 2, samples were stored at 5 °C up to 48 hours and three glycerol concentrations were evaluated: 0% (0Gly), 3% (3Gly), and 6% (6Gly). Postthawing viability and motility increased progressively with the percentage of glycerol for 24 hours at 5 °C; 6% glycerol during 48-hour storage had beneficial effects on sperm cryopreservation. Besides, 6% glycerol had a clearly superior freezability for viability (42.7% and 40.8% for 24 hours and 48 hours, respectively) and motility (24 hours: total, 44.1%; progressive, 17.1%; 48 hours: total, 38.4%; progressive, 16%). In experiment 3, samples were stored up to 48 hours at 5 °C with 6% of glycerol and two dilution methods were evaluated: dilution 1:1 (average: 1782×10^6 sperm/mL; low) or final dilution (100×10^6 sperm/mL; high). Both dilution rates showed similar postthawing and postincubation results within 24 hours of long-term storage. After 48 hours, high dilution supported better postthawing quality. Both dilutions showed similar resistance to cryopreservation, except after 48 hours, when the high dilution reached a higher percent recovery rate of viability (38.8% vs. 21.6%, $P < 0.05$). In conclusion,

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our results suggested that the best conditions for long-term prefreezing storage (up to 48 hours) of brown bear electroejaculates are at 5 °C, at a concentration of 100 × 10^6 sperm/mL, and with 6% glycerol.

1. Introduction

Brown bear, distributed in two subpopulations in the Cantabrian Mountains, is an endangered species in Spain. Sperm collection could be carried out in the field and we need to adapt standard protocols of sperm cryopreservation to achieve the conditions that allow specific biotechnologies (e.g., sex sorting) to be applied before freezing in distant centers. Before being used, semen may be extended, cooled, and stored at 5 °C or frozen at −196 °C for transportation [1,2].

It is necessary to review different aspects of the extended prefreezing period to improve the quality of seminal samples at the time of freezing. Temperature is a key factor for sperm liquid storage. Many reports have determined that sperm stored at 4 °C to 5 °C are metabolically less active and show longer life span and/or higher postthawing quality (bulls [3]; rabbits [4]; rams [5,6]; stallions [7]; cats [8]; dogs [9]). A decline in sperm quality has been observed with storage at room temperature (RT) [6–9]. This decline may be the result of more active metabolic reactions at higher temperatures [1,10]. In some species, the most adequate temperature for sperm storage is around 15 °C (boars [11–13]; rabbits [14]), whereas in others, similar quality was observed at 5 °C and 15 °C storage (stallions [15,16]; elephants [17]). Furthermore, some researchers reported 21 °C as a most adequate temperature to ship sperm before it is subjected to a sex-sorting process (rams [18]; red deer [19]).

Another important factor is the use of glycerol during prefreezing storage. Li et al. [20] reported that the highest percentages of sperm motility and membrane integrity in monkey spermatozoa were achieved using an extender containing 5% glycerol at RT and 30 minutes of equilibration but an excessive exposition might be toxic. Previous studies on the cryopreservation of brown bear sperm indicated that spermatozoa can tolerate 3% glycerol at RT [21] and exposure to 6% glycerol for 10 to 12 hours at 5 °C [22]. An optimal glycerol rate ranging between 4% and 8% was found for brown bear sperm cryopreservation [23–25]. However, the glycerol effect for long-term storage before brown bear sperm cryopreservation has not yet been tested. Different glycerol concentrations for long-term storage of sperm have been tested with variable results in sperm quality (dogs [26]; rams [27–29]; monkeys [30]; buffalo [31]).

The sample dilution rate is another key point to evaluate in prefreezing sperm handling. In the present study, the 1:1 dilution may be useful for shipment of sperm to biotechnology centers if high concentrations of sperm are required in posterior treatment (donkeys [32]; bear [33]). Dilution of semen could destabilize sperm membranes, resulting in loss of motility [34], or increase their vulnerability to osmotic stress [28]. de Jong et al. [35] carried out a study on the effects of dilution in flying fox sperm; they found a gradual decrease in motility, plasmatic membrane integrity, and acrosome status when a higher dilution rate was used. However, Prathalingam et al. [36] reported that bull sperm survival increased at higher dilutions because of a reduction in metabolic products, although acrosome integrity decreased. Miro et al. [32] found that viability after storage increased with the dilution rate in donkey sperm diluted at 1:1, 1:5, or 1:10 up to 72 hours at 5 °C.

Our aim was to extend the cooling period before brown bear sperm cryopreservation to allow the shipment of sperm samples to the laboratory. Therefore, we evaluated the effect of temperature (RT, 15 °C, and 5 °C), glycerol concentration (0%, 3%, and 6%), and dilution rate (1:1 and final dilution) on brown bear sperm during a long-term storage of up to 48 hours.

2. Materials and methods

2.1. Materials

All chemicals were of at least reagent grade and were acquired from Sigma–Aldrich (Madrid, Spain), unless otherwise stated.

2.2. Animals and sperm collection

Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. All experiments were performed after obtaining ethical committee approval from the Ethical Committee for Experimentation with Animals of León University, Spain (03-02/2010).

Ejaculates from 24 sexually mature males (aged ≥6 years) of brown bears (*Ursus arctos*) were obtained by electroejaculation in one or two sessions, at least 3 weeks apart, during the breeding season (end of April to early July) over two consecutive years. The animals were housed in a half-freedom regime in Cabárceno Park (Cantabria, Spain; 43° 21′ N, 3° 50′ W; altitude: 143 m) and fed with a diet based on chicken meat, bread, and fruits.

The animals were immobilized by teleanesthesia, using 750 mg of zolazepam HCl + tiletamine HCl (Zoletil 100, 50 mg/mL of both zolazepam and tiletamine; Virbac, Carros, France) and 6 mg of medetomidine (Zalopine 10 mg/mL; Orion Pharma Animal Health, Finland). After immobilization, the bears were weighed and monitored during anesthesia (pulse, saturation of peripheral oxygen, and respiration). Before electroejaculation, the pubic region was cleaned, the penis was washed with sterile physiological saline, and the rectum was emptied of faeces. The bladder was catheterized during sperm collection. Electroejaculation was carried out with a PT Electronics1 electroejaculator (PT Electronics, Boring, OR, USA).

The transrectal probe was 320 mm long, with a diameter of 26 mm. Electric stimuli were applied until ejaculation (6–10 V and 250–300 mA). The ejaculates were collected in a graduated glass tube.

2.3. Experimental design

Immediately after collection, the volume and concentration of each ejaculate were recorded. Sperm concentration was assessed using a Bürker hemocytometer (Marienfeld GmbH, Marienfeld, Germany) by computer-assisted semen analysis (CASA; ISAS v.1.2, Integrated Semen Analysis System; Proiser, Valencia, Spain). The motility and kinematics parameters were assessed using CASA and a phase-contrast microscope (Section 2.5.1); urospermia was evaluated by means of a rapid urea test (Urea test strips; DiaSys Ecoline GmbH, Holzheim, Germany). Low-motility (<50%) or urine-contaminated (>80 mg urea/dL) samples were rejected [37]. The selected ejaculates were centrifuged at 600 × g during 6 minutes. The supernatant was discarded, and each pellet was resuspended with the same volume of TTF (TES-Tris-fructose) extender at RT (dilution 1:1) and cooled according to each experimental design. The extender was prepared as described by de Paz et al. [25] (TES-Tris-fructose, 300 mOsm/kg, pH 7.1; 20% egg yolk, supplemented with 2% EDTA, 1% Equex [Equex STM Paste; Minitüb, Tiefenbach, Germany], 0.302-mg penicillin G sodium salt/mL, and 0.625-mg dihydrostreptomycin sesquisulfate/mL; the glycerol concentration is specified within each experimental design).

2.3.1. Experiment 1: Effect of temperature on sperm during long-term storage

After centrifugation, the pellet was divided into three aliquots, diluted 1:1 with the TTF extender at 0% (15 °C and RT) and 6% (5 °C) glycerol and stored at different temperatures. Sperm samples at 15 °C and RT were diluted with TTF extender at 0% glycerol up to a final concentration of 100 × 10^6 sperm/mL. Sperm at 5 °C was 1:1 diluted using the TTF extender with 9% glycerol (to obtain a final glycerol concentration of 6%), and subsequently, TTF with 6% glycerol was added to obtain a final concentration of 100 × 10^6 sperm/mL. The samples were equilibrated for 1 hour, and one aliquot of sample at 5 °C was loaded into a 0.25-mL straw and frozen (standard freezing; control). At 24 and 48 hours, one aliquot of samples at 15 °C and one at RT, enough to prepare one 0.25-mL straw, were placed in another tube and 3% glycerol was added; then, they were cooled at 5 °C for 1 hour, diluted 1:1 using 9% glycerol (final 6%), and frozen. Nine electroejaculates from six brown bears were processed.

We analyzed the effect of long-term storage on post-thawing sperm quality and after of 2-hour incubation at 37 °C. Then, a comparison was carried out between the sperm cryopreservation of the standard freezing protocol and the long-term storage, both susceptible to be submitted to a biotechnology center. To do this, the postthawing values of standard freezing samples (1 hour, control) were compared to data corresponding to a sample in prefreezing storage (5 °C, 15 °C, and RT) for 24 and 48 hours. Finally, we evaluated postthawing recovery rates in comparison with data for prefreezing sperm (see Section 2.6).

2.3.2. Experiment 2: Effect of glycerol concentration on sperm in long-term storage

The first experiment indicated that storage at 5 °C before freezing provided the highest quality, so this temperature was selected for experiments 2 and 3. After centrifugation, the pellet was divided into three aliquots, one diluted 1:1 with the TTF extender at 0 (0% glycerol [0Gly]) and two diluted 1:1 with 6% glycerol (final 3% glycerol [3Gly]), and cooled at 5 °C [38]. Then one aliquot of 3Gly was diluted 1:1 with the TTF extender at 9% glycerol (final 6% glycerol [6Gly], control). Samples from 0Gly, 3Gly, and 6Gly were diluted with the TTF extender at 0%, 3%, and 6% glycerol, respectively, to obtain a final concentration of 100 × 10^6 sperm/mL. After 1 hour, one aliquot of 6Gly was frozen (used as the control to standard freezing). After 24 and 48 hours of storage, one aliquot of 0Gly final and one 3Gly were transferred to another tube and diluted 1:1 using 12% or 9% glycerol, respectively (final 6%); then, 0.25-mL straws were loaded and frozen. Ten electroejaculates from nine brown bears were used.

2.3.3. Experiment 3: Effect of sperm concentration in long-term storage (dilution rate)

Sperm was stored at 5 °C using the TTF extender with 6% glycerol according to our previous results. After centrifugation, the pellet was divided into two aliquots, diluted 1:1 using the TTF extender with 12% (low dilution) or 6% (high dilution, control) glycerol and cooled [38]. At 5 °C, the high-dilution group was 1:1 diluted using the TTF extender with 9% glycerol (final 6% glycerol); and TTF extender with 6% glycerol was added to obtain a final concentration of 100 × 10^6 sperm/mL (high dilution). After 1 hour, one 0.25-mL straw was loaded and frozen (standard freezing; control). During 24 and 48 hours of storage, the low-dilution sample presented a concentration of 1782 (±166) × 10^6 sperm/mL. After storage, one aliquot of low-dilution sample was transferred to another tube and diluted with TTF at 6% glycerol to obtain a final concentration of 100 × 10^6 sperm/mL; then, 0.25-mL straws were loaded and frozen. Ten electroejaculates from nine brown bears were processed.

2.4. Sperm cryopreservation

The 0.25-mL straws were frozen in a programmable biofreezer (Planer Kryo 560-16; Planer Plc., Sunbury-On-Thames, UK) at −20 °C/min down to −100 °C, transferred to liquid nitrogen containers, and stored for a minimum of 1 week. Thawing was performed by dropping straws in water at 65 °C for 6 seconds.

2.5. Sperm evaluation

The sperm were analyzed for motility, viability, acrosomal status, and apoptosis at three points of treatment: before freezing, after thawing (0 hours), and after incubation of thawed sperm for 2 hours at 37 °C (named "thermal stress test" [ThS Test]). The purpose of the ThS test

is to amplify the sublethal damage induced in cells by freezing. Sperm motility was evaluated according to Paz et al. [25], sperm viability and acrosomal status were analyzed according to Garcia-Macias et al. [37], and the apoptosis assessment according to Nicolas et al. [39].

2.5.1. Motility assessment

Motility and kinematic parameters were assessed using CASA. A 5-μL sperm sample was placed in a Makler counting cell chamber (10-μm depth; Sefi Medical Instruments, Haifa, Israel) and examined using an E400 phase-contrast microscope (Nikon, Tokyo, Japan) with a warmed stage (38 °C). The standard parameter settings were as follows: 25 frames/s; 5 to 50 μm^2 for head area; curvilinear velocity greater than 10 μm/s to classify a spermatozoon as motile. At least five fields or 200 spermatozoa were saved and analyzed afterward. Reported parameters were total motility (TM, %), progressive motility (PM, %; spermatozoa were considered progressive if curvilinear velocity > 25 μm/s, straightness > 80), and straight-line velocity (VSL, μm/s).

2.5.2. Viability assessment

To assess sperm viability, we applied a double staining of SYBR-14 (L-7011 LIVE/DEAD Sperm Viability Kit; Molecular Probes, Invitrogen, Barcelona, Spain) and propidium iodide (PI). Sperm samples were diluted to 5×10^6 sperm/mL with PBS, and 300 μL was transferred to a polypropylene tube, with added 3-μL PI (24 mM in water) and 1.5-μL SYBR-14 (1-mM in DMSO). The tubes were maintained at 37 °C for 20 minutes in the dark. In this study, we evaluated the percentage of viable sperm ($SYBR\text{-}14^+/IP^-$, named VIAB).

2.5.3. Acrosomal status assessment

For acrosomal status, we used the double stain of peanut agglutinin–fluorescein isothiocyanate (PNA-FITC) and PI. Sperm samples were diluted with PBS (5×10^6 sperm/mL), and 300 μL was transferred to a polypropylene tube to which we added 2.5-μL PI (1 mg/mL in water) and 2.5 μL PNA-FITC (0.2 mg/mL in water). The tubes were incubated for 10 minutes at RT until analysis. Flow cytometer rendered the percentage sperm with intact acrosomes (PNA^-, named iACR).

2.5.4. Apoptosis assessment

Changes in sperm membrane permeability were analyzed with double staining YO-PRO-1/PI. The YO-PRO-1 (Invitrogen) is a membrane-impermeable probe that can only penetrate in the sperm after the destabilization of the membrane [40]. Sperm samples were diluted with PBS down to 5×10^6 sperm/mL in polypropylene tubes (300 μL/tube), adding 2.5-μM YO-PRO-1 and 10-μM PI. The tubes were incubated at 37 °C for 10 minutes until analysis by flow cytometry. We used the percentage of viable and nonapoptotic sperm ($YO\text{-}PRO\text{-}1^-/PI^-$, named $YOPRO^-$).

2.5.5. Flow cytometry evaluation

Viability, acrosomal status, and apoptosis were analyzed using a FACScalibur flow cytometer (Becton Dickinson Immunochemistry Systems, San Jose, CA, USA), equipped with standard optics and an argon ion laser, tuned at 488 nm and running at 200 mW. Calibration was carried out periodically using standard beads (Calibrites; Becton Dickinson). Data corresponding to the red (FL3 photodetector) and green fluorescence (FL1 photodetector) of acquired particles were recorded. In all cases, we assessed 10,000 events per sample with a flow rate of 200 cells/s.

2.6. Statistical analysis

Data were analyzed using the SAS v.9.1 package (SAS Institute, Cary, NC, USA). Our hypotheses for temperature, glycerol concentration, and dilution for long-term storage before freezing were tested by using mixed-effects linear models after transforming the data (arc sine for percentages and natural logarithm for the rest of the analyzed

Table 1
Effect of three temperatures (5 °C, 15 °C, and room temperature [RT]) on brown bear sperm during long-term preefreezing storage (up to 48 hours) at 5 °C.

Variables	1 h	24 h			48 h		
	Control	5 °C	15 °C	RT	5 °C	15 °C	RT
Postthawing analysis							
VIAB	48.2 ± 3.6	42.4 ± 3.6^{a}	32.6 ± 2.4^{b*}	29.7 ± 3.7^{b*}	40.8 ± 3.1^{a*}	31.2 ± 2.8^{b*}	27 ± 3.9^{b*}
$YOPRO^-$	45.2 ± 3.2	37.9 ± 3.5^{a*}	32.6 ± 2.6^{a*}	27.2 ± 3.3^{b*}	36.5 ± 3.1^{a*}	29.3 ± 2.7^{b*}	26 ± 3.7^{b*}
iACR	83.5 ± 2.5	83.1 ± 1.8^{a}	77.8 ± 1.5^{b*}	77.3 ± 2.8^{b*}	82.9 ± 1.8^{a}	76.1 ± 2.1^{a,b*}	71.9 ± 5.1^{b*}
TM	47.4 ± 4.5	40.3 ± 3.7^{a}	31.7 ± 3.3^{a,b*}	25.3 ± 4.2^{b*}	38.9 ± 3.8^{a}	19.8 ± 5.2^{b*}	12.6 ± 2.8^{b*}
PM	24.2 ± 1.9	15.6 ± 1.8^{a*}	10.7 ± 1.3^{b*}	9 ± 1.7^{b*}	14 ± 1.9^{a*}	7.2 ± 2.2^{b*}	4.6 ± 1.3^{b*}
VSL	37.2 ± 2.9	29.9 ± 1.8^{a*}	26.3 ± 1.7^{a,b*}	25.6 ± 1.1^{b*}	27.3 ± 1.7^{a*}	20.4 ± 2.3^{a,b*}	17.7 ± 2.2^{b*}
Quality analysis after 2-hour incubation of postthawing sample (ThS test)							
VIAB	28.5 ± 4	26.9 ± 3.8	24.4 ± 2.3	21.2 ± 3.3*	28.5 ± 3.1^{a}	21.5 ± 2.4^{b*}	20.6 ± 4.1^{b*}
$YOPRO^-$	25.7 ± 3.6	25 ± 4.3	20.8 ± 2.6	21.1 ± 3.9	25.3 ± 3.3^{a}	20.7 ± 3^{a,b}	18.6 ± 3.1^{b}
iACR	76.5 ± 3.2	79.4 ± 2.7^{a}	73.2 ± 4.3^{b}	75.2 ± 3.1^{b}	79.8 ± 2.2^{a}	72.1 ± 3.8^{b}	70.8 ± 3.8^{b}
TM	27.6 ± 3.7	24.3 ± 5.6^{a}	20 ± 3.8^{a,b*}	14.2 ± 2.9^{b*}	17.2 ± 3.4^{a*}	6.4 ± 2.3^{b*}	2.8 ± 1.1^{b*}
PM	13.5 ± 1.3	11.2 ± 2.8^{a}	9.3 ± 2.1^{b}	6.1 ± 1.3^{c*}	5.8 ± 1.2^{a*}	2.3 ± 0.9^{b*}	0.9 ± 0.4^{b*}
VSL	27.8 ± 2.4	22.6 ± 2.6	24.5 ± 2.7	18.2 ± 3.4	17.2 ± 1.5*	13.3 ± 2.6*	9.9 ± 2.5*

The data represent the mean ± standard error of the mean (n = 9 electroejaculates from six bears).
^{a,b,c}Different letters indicate differences between temperatures during each storage time ($P < 0.05$).
Asterisks indicate that means (stored for 24 and 48 hours) differ from those of the control (standard freezing, 1 hour).
$YOPRO^-$ represents percentage viable and nonapoptotic spermatozoa.
Abbreviations: iACR, intact acrosomes (%); PM, progressive motility (%); ThS, thermal stress; TM, total motility (%); VIAB, viability (%); VSL, straight-line velocity (μm/s).

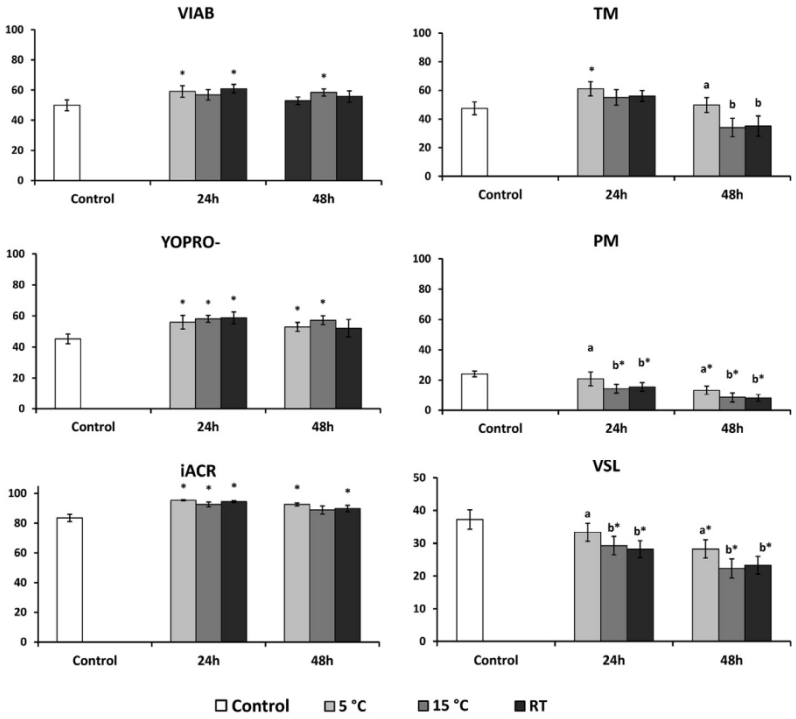

Fig. 1. Comparison of long-term storage results (for 24 and 48 hours, prefreezing data) at different temperatures (5 °C, 15 °C, and room temperature [RT]) versus postthawing values of standard freezing. The data represent the mean ± standard error of the mean (n = 9 electroejaculates from six bears). [a,b]Different letters indicate that means of temperature during each time of long-term storage differ for each parameter ($P < 0.05$). Asterisks indicate that means of each storage temperature (prefreezing data) differ from postthawing data for the control (standard freezing, 1 hour), for 24 and 48 hours. YOPRO$^-$ represents percentage viable and nonapoptotic spermatozoa. iACR, intact acrosomes (%); PM, progressive motility (%); TM, total motility (%); VIAB, viability (%); VSL, straight-line velocity (μm/s).

parameters). Male was considered a random effect. Least-square means were computed for each effect listed, and P values for differences in the multiple comparisons were calculated by Tukey's test. Values were considered to be statistically significant at $P < 0.05$. All results are presented as the mean ± standard error of the mean.

We calculated postthawing recovery rates to assess sperm freezability in each experimental group. These rates were calculated by dividing the postthawing value of each sperm parameter by its prefreezing value and multiplying by 100.

3. Results

Sperm concentration media of the ejaculate used was 323.6 ± 286.2 × 10^6/mL.

3.1. Experiment 1: Effect of temperature in long-term storage

Prefreezing storage time had a significant effect on postthawing sperm quality. Postthawing viability, acrosomal integrity, and PM of sperm stored at 5 °C for 24 or 48 hours were greater than those of sperm stored in other conditions (Table 1). Only sperm stored at 5 °C for 24 hours showed similar postthawing results for viability, acrosomal integrity, and TM to the control sample. After the ThS test (Table 1), sperm stored at 5 °C for 24 or 48 hours showed higher values of TM, PM, and acrosome integrity than sperm stored at 15 °C and RT. Parameters of spermatozoa quality were significantly different between sperm stored until 48 hours and the control sample at any temperature.

Sperm qualitative characteristics during prefreezing long-term storage change significantly when compared to data for postthaw sperm in the freezing control sample (Fig. 1). At 24 hours, sperm viability and acrosome and membrane integrity were greater in prefreezing stored sperm, but PM and VSL were similar (at 5 °C) or lower (15 °C and RT).

Recovery rates for VIAB and YOPRO$^-$ of sperm stored at 5 °C during 24 or 48 hours (Fig. 2) were higher than those of sperm stored at 15 °C and RT. Recovery rates of sperm motility did not change because of temperatures and times of storage.

3.2. Experiment 2: Effect of glycerol concentration during long-term storage

Glycerol concentration in the TTF extender during prefreezing storage at 5 °C for 24 and 48 hours had a significant

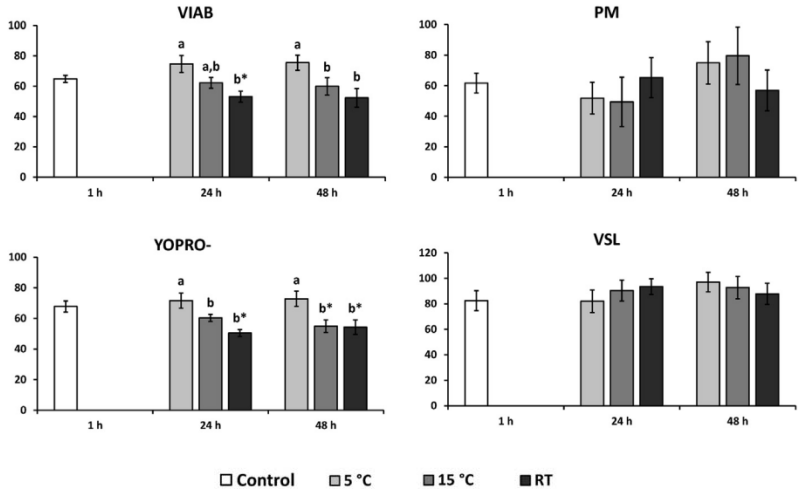

Fig. 2. Recovery rates (postthawing/prefreezing × 100) for sperm samples stored at three different temperatures (5 °C, 15 °C, and room temperature [RT]) during long-term storage (up to 48 hours) before cryopreservation. The data represent the mean ± standard error of the mean (n = 9 electroejaculates from six bears). [a,b]Different letters indicate that means of temperature during each time of long-term storage differ for each parameter (P < 0.05). Asterisks indicate that means of each storage temperature differ from postthawing data for the control (standard freezing, 1 hour), for 24 and 48 hours. YOPRO⁻ represents percentage viable and nonapoptotic spermatozoa. PM, progressive motility (%); VIAB, viability (%); VSL, straight-line velocity (μm/s).

effect on postthawing sperm parameters (Table 2). When the glycerol concentration increased from 0% to 6%, sperm viability, acrosome integrity, nonapoptotic cells, and sperm motility rose significantly. Sperm cooled for 48 hours had a lower percentage of nonapoptotic cells than the control sample. After the ThS test (Table 2), only 6Gly sperm stored at 5 °C for 24 hours yielded similar postthawing parameters to the control sample except sperm viability. The recovery rates showed that 6Gly sperm stored at 5 °C for 24 and 48 hours had the highest values when compared with the others treatments (Fig. 3), except VSL.

3.3. Experiment 3: Effect of dilution rate during long-term storage

At 24 hours, the dilution rate did not have a significant effect on postthawing sperm parameters except TM (Table 3), but after 48 hours of storage, all parameters were affected negatively by the low dilution rate except VSL. Almost all postthawing parameters of sperm stored were lower than those for the control sample. However, the ThS test (Table 3) revealed that samples of 24 or 48 hours did not show differences between rates of dilution, except VSL

Table 2
Effect of three glycerol concentrations (0%, 3%, and 6%: 0Gly, 3Gly and 6Gly, respectively) on brown bear sperm during long-term preefreezing storage (up to 48 hours) at 5 °C.

Variables	1 h	24 h			48 h		
	Control	0Gly	3Gly	6Gly	0Gly	3Gly	6Gly
Postthawing analysis							
VIAB	47.9 ± 3.3	17.5 ± 3.5^{a*}	29.9 ± 3.6^{b*}	42.7 ± 3.3^{c}	15.9 ± 5.7^{a*}	15.9 ± 2.6^{a*}	40.8 ± 5.2^{b}
YOPRO⁻	50.8 ± 3.1	15.2 ± 4.4^{a*}	26.1 ± 5.4^{b*}	45.7 ± 3.2^{c}	12.2 ± 3.5^{a*}	15.1 ± 3.6^{a*}	33.1 ± 5^{b*}
iACR	93.1 ± 1.7	90.3 ± 1.4	91.4 ± 0.7	92.8 ± 1.2	85.2 ± 1.8^{a*}	85.9 ± 1^{a,b*}	92.3 ± 1.4^{b}
TM	49.0 ± 5.4	20 ± 4.2^{a*}	29 ± 2.6^{a,b*}	44.1 ± 5.3^{b}	12.6 ± 3^{a*}	22.5 ± 2.3^{a*}	38.4 ± 2.8^{b}
PM	26.9 ± 3.2	6 ± 1^{a*}	10.1 ± 0.8^{b*}	17.1 ± 2.1^{c*}	4.2 ± 1.1^{a*}	7.4 ± 1.6^{a*}	16 ± 1.4^{b*}
VSL	42.3 ± 2.7	27.8 ± 2.6*	30.9 ± 1.9*	33.2 ± 2.3*	22.8 ± 3.2^{a*}	29.1 ± 1.9^{a*}	34.4 ± 1.2^{b*}
Quality analysis after 2-hour incubation of postthawing sample (ThS test)							
VIAB	21.8 ± 4.2	2.7 ± 0.8^{a*}	4.3 ± 1.1^{a*}	12.3 ± 2.4^{b*}	2.8 ± 1.6^{a*}	3.6 ± 1.5^{a*}	9.8 ± 1.8^{b*}
YOPRO⁻	22.9 ± 4	4.2 ± 1.1^{a*}	6.1 ± 1.5^{a*}	14.4 ± 2^{b}	6.1 ± 2.2^{a*}	5.8 ± 2.1^{a*}	11.6 ± 1.6^{b*}
iACR	87.5 ± 2.6	77.6 ± 2.9^{a*}	82.4 ± 2.4^{a,b}	88.2 ± 1.1^{b}	81.1 ± 3	79.2 ± 2.8*	84.3 ± 2.2
TM	33.4 ± 4.2	3.4 ± 1.4^{a*}	9.2 ± 1.7^{b*}	37.3 ± 6.1^{c}	1.3 ± 0.3^{a*}	2.1 ± 0.4^{a*}	20.5 ± 3.1^{b*}
PM	13.4 ± 1.9	1 ± 0.5^{a*}	2.9 ± 0.7^{a*}	15.8 ± 2.7^{b}	0.4 ± 0.2^{a*}	0.7 ± 0.2^{a*}	9.2 ± 1.7^{b*}
VSL	28.5 ± 1.8	10.4 ± 2.7^{a*}	16.4 ± 2.4^{a,b*}	30.2 ± 2.5^{b}	7.4 ± 1.6^{a*}	13.2 ± 2.8^{a*}	23.9 ± 1.4^{b*}

The data represent the mean ± standard error of the mean (n = 10 electroejaculates from nine bears).
[a,b,c]Different letters indicate that means of temperature during each time of long-term storage differ for each parameter (P < 0.05).
Asterisks indicate that means of each storage temperature differ from those of the control (standard freezing, 1 hour), during 24 and 48 hours.
Abbreviations: iACR, intact acrosomes (%); PM, progressive motility (%); ThS, thermal stress; TM, total motility (%); VIAB, viability (%); VSL, straight-line velocity (μm/s).

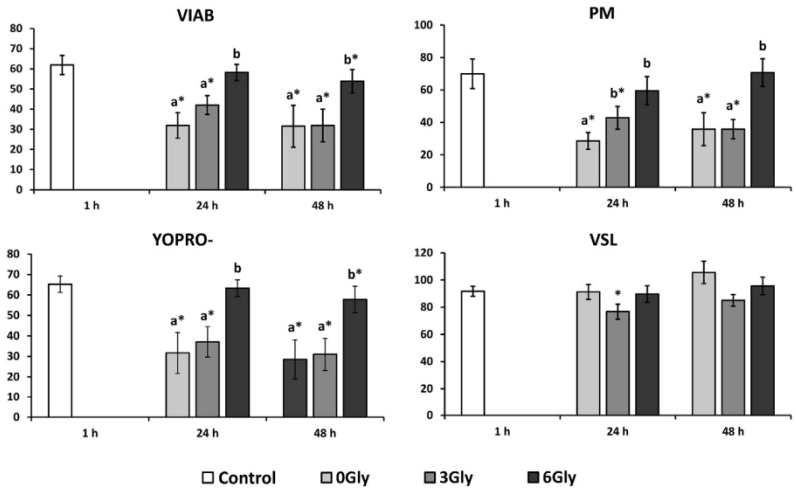

Fig. 3. Recovery rates (postthawing/prefreezing × 100) for sperm samples stored using three different glycerol concentrations (0%, 3%, and 6%) during long-term storage (up to 48 hours) before cryopreservation. The data represent the mean ± standard error of the mean (n = 10 electroejaculates from nine bears). [a,b]Different letters indicate that means of temperature during each time of long-term storage differ for each parameter ($P < 0.05$). Asterisks indicate that means of each storage temperature differ from postthawing data for the control (standard freezing, 1 hour), for 24 and 48 hours. YOPRO$^-$ represents percentage viable and nonapoptotic spermatozoa. PM, progressive motility (%); VIAB, viability (%); VSL, straight-line velocity (μm/s).

for 48 hours. The freezability evaluation by recovery rates showed that postthawing sperm motility of sperm samples stored for 24 and 48 hours at 5 °C were not significantly different to the control sample (Fig. 4), but sperm viability and nonapoptotic cells were significantly lower than in the control sample because of the dilution rate.

4. Discussion

In wild life species, sperm collection is usually carried out in the field and the laboratory for sex sorting before cryopreservation is not immediately accessible. In this situation, the development of sperm handling procedures to minimize the effects of shipping on postthawing sperm quality is important. Temperature, time, glycerol rate, and dilution were evaluated for the development of optimum conditions during shipping.

The integrity of sperm function during the prefreezing period depends on storage temperature, among others factors [3]. When sperm is transported, the storage temperature is defined depending on the distance between the collection center and the laboratory [41]. This distance was referenced in the present study by storage time. Our results indicated that brown bear sperm stored at 5 °C

Table 3
Effect of two dilution rates (low and high) on long-term storage up to 48 hours before cryopreservation of brown bear sperm on postthawing and ThS test quality.

Variables	1 h	24 h		48 h	
	Control	Low dilution	High dilution	Low dilution	High dilution
Postthawing					
VIAB	56.7 ± 3.4	37.9 ± 2.4*	39.8 ± 3.5*	21.6 ± 3.2[a*]	38.8 ± 4.6[b*]
YOPRO$^-$	57 ± 3.2	41.7 ± 2.1*	44.1 ± 1.9*	23.7 ± 3.5[a*]	35.2 ± 3[b*]
iACR	91.5 ± 2.4	91.0 ± 1.8	90.8 ± 2.6	88.2 ± 1.9[a]	92.4 ± 1.3[b]
TM	53 ± 5.9	27.1 ± 5[a*]	49.2 ± 7.2[b]	17.8 ± 4.5[a*]	37.1 ± 4.2[b*]
PM	26.1 ± 2.8	14.9 ± 2.4*	18.3 ± 2.6*	8 ± 2.2[a*]	14.2 ± 1.6[b*]
VSL	38.3 ± 3	34.1 ± 3.2	30.6 ± 1.8*	28 ± 2.6*	28.9 ± 1.7*
ThS test quality					
VIAB	25 ± 3.9	14.5 ± 1.8*	15.7 ± 2.4*	6.2 ± 1.4*	9.9 ± 1.6*
YOPRO$^-$	25.3 ± 3.9	16.9 ± 3*	14.3 ± 4.2*	8.5 ± 1.8*	11.6 ± 1.4*
iACR	84.5 ± 2.8	86.5 ± 2.3	88.9 ± 1.4	83.0 ± 3.2	86.5 ± 1.4
TM	36.6 ± 5.4	23.3 ± 5.2*	27.9 ± 4.6	7.7 ± 2.3*	16.4 ± 3.9*
PM	13.9 ± 2.7	10.6 ± 2.3	12.3 ± 2.2	3.5 ± 1.2*	7.1 ± 1.9
VSL	28.5 ± 1.6	25.7 ± 1.7	25 ± 1.6	14.7 ± 2.5[a*]	20.9 ± 2.1[b*]

The data represent the mean ± standard error of the mean (n = 10 electroejaculates from nine bears).
[a,b]Different letters indicate that means of temperature during each time of long-term storage differ for each parameter ($P < 0.05$).
Asterisks indicate that means of each storage temperature differ from those of the control (standard freezing 1 h) during 24 and 48 hours.
Abbreviations: iACR, intact acrosomes (%); PM, progressive motility (%); ThS, thermal stress; TM, total motility (%); VIAB, viability (%); VSL, straight-line velocity (μm/s).

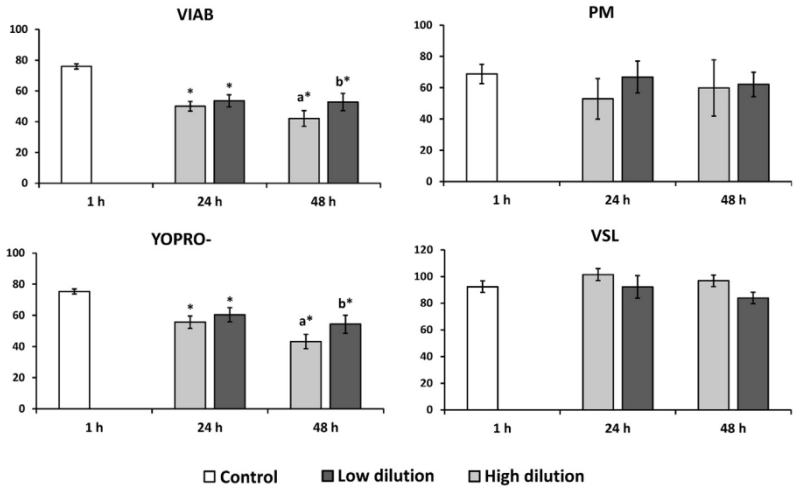

Fig. 4. Recovery rates (postthawing/prefreezing × 100) for sperm samples diluted at different dilution rates (1:1 or final dilution) during long-term storage (up to 48 hours) before cryopreservation. The data represent the mean ± standard error of the mean (n = 10 electroejaculates from nine bears). [a,b]Different letters indicate that means of temperature during each time of long-term storage differ for each parameter ($P < 0.05$). Asterisks indicate that means of each storage temperature differ from the postthawing data for the control (standard freezing, 1 hour), for 24 and 48 hours. YOPRO$^-$ represents percentage viable and non-apoptotic spermatozoa. PM, progressive motility (%); VIAB, viability (%); VSL, straight-line velocity (μm/s).

yielded better postthawing results comparable to those observed in the control sample not subjected to prolonged storage. In contrast, 15 °C storage showed a moderate decline in sperm quality and sperm stored at RT afforded the greatest drop in the evaluated parameters.

It is generally accepted that sperm stored at 4 °C reduces energy consumption and metabolism rate thus enabling sperm to live longer. A previous report showed that in bull sperm transported for over 24 hours at 4 °C, postthawing viability and motility were higher than in the control sperm [41]. However, other authors have reported that sperm may be preserved both at 20 °C and 4 °C until 24 hours with no differences in sperm quality (boars [13]; bucks [42]; elephant [43]). These facts reflect specific differences between the spermatozoa in susceptibility to damage during cooling, and therefore, the interspecies comparison is a little bit speculative.

For a short-term period, storage at 21 °C was optimal for transporting ram spermatozoa (6 hours, [18]) or red deer sperm (8 hours, [19]) to the sorting site. Other studies concluded that boar sperm may be stored at 20 °C or 15 °C for 48 hours [13] or 96 hours [12] without significant effects on sperm quality. This fact has been linked with cold shock phenomenon that occurs below 12 °C in boar sperm [11]. Cold shock has been related to the lipid composition of the plasma membrane [44]. To date, lipid composition of brown bear spermatozoa membrane has not been studied, but we have observed that cooling rates affected the refrigeration of brown bear sperm [38]. A slow cooling rate allowed brown bear sperm to better resist storage at 5 °C possibly because of the greater stability of their membrane [41]. These results allow us to assume that brown bear spermatozoa do not respond to cold shock in the same way as described in other species and that longevity of spermatozoa stored at 5 °C is influenced by specific differences in membrane properties.

The effects of sperm stored at 5 °C during long term on postthawing sperm quality have been reported in some studies. Backman et al. [15] described a procedure for cooled storage of stallion sperm for 18 hours before cryopreservation without any detrimental effects on fertility. A similar result was observed after 24 hours of storage of dolphin sperm at 5 °C; postthawing sperm characteristics were maintained at a level of initial ejaculate [45]. The protective effect of 5 °C storage could be due to a reduction of sperm metabolic activity [46]. These reports agree with the results of the present study on brown bear sperm where we suggest that the brown sperm shipped at 5 °C to the laboratory to the application of sperm-sorting technology was an option better than the shipment in a frozen state for periods up to 48 hours.

The optimal glycerol concentration for the cryopreservation of brown bear spermatozoa has been established at 4% to 8% (higher quality around 6% glycerol) [23–25]. However, the effects of glycerol concentration on bear sperm throughout prefreezing long-term storage have not been tested. In our study, glycerol is necessary to store brown bear sperm long term at 5 °C because the sperm stored in TTF with 6% glycerol during the prefreezing period (24 or 48 hours) yielded better results after the freezing–thawing process than sperm stored without glycerol. Sperm tolerance to glycerol in the cooling extenders differs between species (bear [21]; red deer [47]; dogs [48]; bulls [49]), and this would be due to the difference in the structure and composition of plasma membrane [49]. Province et al. [50] reported that canine sperm motility was depressed with the inclusion of 6% glycerol in extender at 5 °C, but glycerol concentration had no effect on equine

sperm motility. In brown bear sperm, Alvarez-Rodriguez et al. [21] evaluated prefreezing methods of glycerol addition and concluded that the sperm of this species seemed to possess good glycerol tolerance. Likewise, canine spermatozoa showed good resistance to hypertonic solutions of glycerol [48]. In some wild species, the addition of 6% glycerol had a positive effect on sperm stored for more than 24 hours (buffalo [31]; flying foxes [35]). Vera-Munoz et al. [49] did not find any significant differences in motility, membrane and acrosome integrity when they cooled bull semen up to 48 hours supplemented with 0% or 6% glycerol, although they observed a negative effect of glycerol beyond this time.

The exposure time of sperm to glycerol in prefreezing long-term storage before sperm cryopreservation has effects on their postthawing sperm quality [51]. We evaluated the effect of long-term storage up to 72 hours in freezing extender with 6% glycerol on cryopreserved brown bear sperm [22] and observed that sperm quality (motility and viability) dropped gradually after 48 hours and dramatically up to 72 hours. In other mammals, sperm diluted with extenders supplemented with glycerol and stored for a long-term period yielded good sperm quality (rams [27–29]; monkeys [30]). Anzar et al. [41] reported that bull sperm exposed to extenders with glycerol at 4 °C during an extended period of transport (24 hours) showed the improvement of postthaw sperm quality. Moreover, Gil et al. [52] noticed that the addition of egg yolk and glycerol to extenders had a positive effect on the quality of ram sperm preserved at 5 °C for 48-hour preservation. The "solution effects" are major problem in slow freezing and are directly linked with glycerol concentration. During long-term storage, the "solution effects" induced by glycerol could lead to sperm damage, but there is no information about biochemistry of bear sperm cells at 5 °C to evaluate this effect.

Semen dilution is other important parameter that requires controlling in sperm storage because of interactions between spermatozoa and extender components [49]. In the present study, the dilution rate had no effect on sperm quality after 24 hours of storage at 5 °C. However, negative effects were observed in low-dilution samples after 48 hours. Cell concentration during storage has been significantly associated with deterioration in structural, functional, and sperm motility parameters [53]. These authors observed that qualitative changes in sperm were less dramatic in the 200 × 10^6/mL concentration compared to 50 × 10^6/mL concentration and proposed that one possible physiological reason for this damage might be extracellular oxidative stress, effects of seminal plasma and endogenous free radical production. Miro et al. [32] diluted donkey semen (1:1, 1:5, and 1:10) and stored it at 5 °C up to 72 hours; they concluded sperm viability after storage increased with the dilution rate and only 1:10 dilution (24 and 48 hours) was similar to fresh samples. This improvement in semen quality by increasing the dilution rate may be a result of a reduction in metabolic products concentration [36]; a higher concentration of spermatozoa may imply a major oxidant activity which is related to dead sperm [54].

On the other hand, handling of semen in the laboratory may require high concentrations of spermatozoa or the elimination of extenders used for shipment, and this, in turn, requires centrifugation and washing, which can impair sperm quality or decrease the number of sperm recovered (donkeys [32]; bear [33]). In our study, we observed that brown bear sperm could be extended with a 1:1 dilution and stored up to 24 hours without a significant drop in quality in comparison with the final dilution. Kiso et al. [43] observed a negative effect of egg yolk which interferes at sex sorting; so, seminal samples before sex sorting should be centrifuged. However, Shannon and Curson [54] reported that the toxic effect of peroxide is enhanced by increasing egg yolk and inhibited by EDTA. Besides, recently, del Olmo et al. [55] revealed a synergistic effect of egg yolk and EDTA and concluded that these components should be a support for the fertility of sorted boar sperm.

In conclusion, our results suggested that better prefreezing conditions for long-term storage of brown bear electroejaculates (up to 48 hours) consist of a temperature of 5 °C, 6% glycerol concentration, and a final dilution rate throughout storage, even with 1:1 dilution only within 24 hours.

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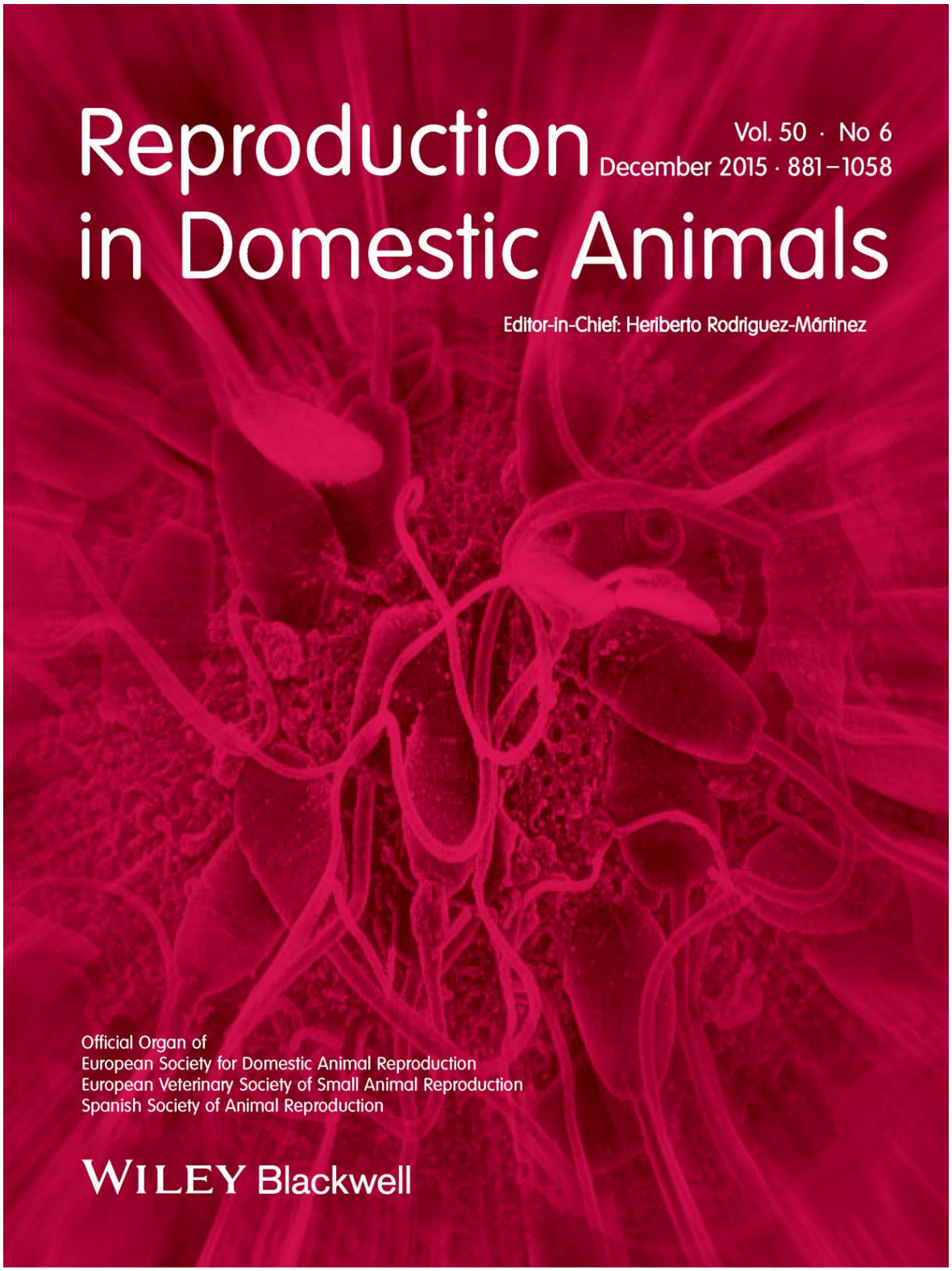
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Use of gelatine in long-term storage (up to 48 h) at 5 °C preserve the prefreezing and post-thawing quality from brown bear sperm

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Abstract

Solid storage could decrease deleterious changes of cells in extender which would optimize the sperm quality for a long-term storage necessary to ship brown bear sperm to laboratory to apply biotechnologies (e.g. sex-sorting) before freezing. We evaluate the handling of solid storage media during prefreezing, tested four models of storage using dilution in TTF-ULE-Bear extender (TesT-fructose-egg yolk-glycerol): 1) 1:1 dilution at RT (room temperature), cooling in tube, final dilution at 5 °C -Control-; 2) final dilution at RT, cooling in FD-Tube; 3) final dilution at RT, cooling in 0.25 mL plastic straw -FD-Straw- and 4) final dilution at RT in extender supplemented with 1.5 % gelatine, cooling in 0.25 mL plastic straw -Gelatine-. Sperm were stored at 5 °C for 1 (only Control1), 24 and 48 h before freezing. At prefreezing and post-thawing (65 °C, 6 s), quality of samples was assessed for motility by CASA, and viability (SYBR-14/propidium iodide-PI-; VIAB), acrosomal status (PNA-FITC/PI; iACR) and apoptotic status (YO-PRO-1/PI; YOPRO-) by flow cytometry. At prefreezing after 48 h, Gelatine had significantly higher viability (for VIAB and YOPRO-) and progressiveness (PM, LIN and STR), although lower VAP. Gelatine showed similar YOPRO-, iACR, LIN, STR and ALH respect to Control1. At post-thawing, in general, Gelatine reached higher VIAB, YOPRO-, iACR, medium PM and LIN, and lower VAP and ALH; independently time of storage. No differences were found among the rest of experimental groups (Control24/Control48, FD-Tube and FD-Straw). Compared to Control1, Gelatine had similar post-thawing scores for VIAB (24 h), YOPRO-, iACR, LIN, STR and WOB (24 and 48 h), and lower TM, PM, rapidPM, VAP and ALH. In

conclusion, gelatine in extender could be a suitable alternative to preserve the viability parameters and progressive motility of brown bear ejaculates for a long-term prefreezing storage at 5 °C.

Keywords: long-term storage, solid storage, gelatine, sperm cryopreservation, brown bear

1. Introduction

The Cantabrian brown bear (*Ursus arctos*) is an endangered species in Spain, distributed in two isolated subpopulations, with high priority to conservation of biological diversity and a Genetic Resource Banks (GRB) is the answer to this threatened populations (García-Macías et al. 2006; Anel et al. 2008). Bear sperm collection could be carried out in field so we need to adapt standard protocols for sperm cryopreservation (Elephant: (Graham et al. 2004); cheetah: (Crosier et al. 2006); buffalo: (Herold et al. 2006); bear: (de Paz et al. 2012). A new methodology would allow to improve storage conditions to lengthen the time that spermatozoa maintain their functional status (Rosato and Iaffaldano. 2011) and thus to transport the sample to distant laboratory to freeze or apply biotechnologies (e.g. sex sorting). So an artificial insemination could be carried out between these two subpopulations and to promote the genetic exchange between them

Sedimentation of sperm cells at the bottom is occurred during liquid long-term storage; therefore, this storage may produce deleterious changes in extender (i.e., pH fluctuations) and increases toxic metabolic products at this region (Rabbit: (Nagy et al. 2002); ram: (Paulenz et al. 2010)). Solid media, as gelatine, can be an alternative to prevent this problematic. Gelatine would allow sperm to be more uniformly distributed (Salvador et al. 2006; Paulenz et al. 2010), even it would minimize the metabolic demands of sperm motion (Rabbit: (Lopez-Gatius et al. 2005); ram: (Yaniz et al. 2005)).

Cryopreservation protocol for brown bear sperm developed by our group (Anel et al. 2008; Anel et al. 2010; de Paz et al. 2012; Lopez-Uruena et al. 2014a), involves the addition of glycerol to be carried out in two steps (an initial 1:1 dilution at room temperature (RT) and up to final concentration at 5 °C using a specific extender (TTF-

ULE-Bear). For handling of gelatine, final dilution (6 % glycerol, 100×10^6 sperm/mL) and packaged into plastic straws was performed at RT, because seminal samples with gelatine become solid state under 20 °C. Therefore, it would be desirable to study effects of dilution at RT and cooling into straw on long-term storage of brown bear spermatozoa. Some authors observed a drop of post-thawing quality when glycerol was added around RT respect to 4-5 °C (Ram: (Colas. 1975; Gil et al. 2011); boar: (Almlid and Johnson. 1988); cat: (Hermansson and Axner. 2007)). However, Alvarez-Rodriguez et al. (Alvarez-Rodríguez et al. 2011) suggested brown bear semen shown a good tolerance to glycerol because they did not find differences among protocols of glycerol addition (two steps at room temperature, one step at room temperature and other at 5 °C, or two steps at 5 °C). Crosier et al. (Crosier et al. 2006) observed, under field conditions, that changes in sample temperature would be prevented if glycerol addition was carried out at RT before cooling, instead of at 5 °C. Thereby, some authors in their studies diluted seminal samples with final glycerol at RT and packaged into straws at 5 °C (Cynomolgus monkey: (Li et al. 2005); Namibian cheetah: (Crosier et al. 2006); African buffalo: (Herold et al. 2006); ocelot and tigrina: (Baudi et al. 2008); brown bear: (Alvarez-Rodríguez et al. 2011)), even packaged into straws at RT and cooled into plastic straws (Namibian cheetah: (Crosier et al. 2006); ram: (Camara et al. 2011)) with an acceptable post-thawing quality.

Our aim was to improve prefreezing long-term storage conditions of brown bear spermatozoa applying a solid storage media that permit the sample shipment to reference laboratories or for artificial insemination. Moreover, we tested permissiveness to cryopreserve them by no habitual protocols with successful guaranties, as final dilution at RT (with the final percentage of glycerol) and cooling to 5 °C (in straws, instead of in tube). We tested four models of sperm dilution in TTF-ULE-Bear extender (TesT-fructose-egg yolk-glycerol): 1) 1:1 dilution at RT, cooling in tube, final dilution at 5 °C; 2) final dilution at RT, cooling in tube; 3) final dilution at RT, cooling in 0.25 mL plastic straw; 4) final dilution at RT with extender supplemented with 1.5 % gelatine, cooling in 0.25 mL plastic straw.

2. Materials and Methods

2.1. Materials

All chemicals were of at least reagent grade and were acquired from Sigma-Aldrich

(Madrid, Spain), unless otherwise stated.

2.2. Animals and sperm collection

Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. All experiments were performed after obtaining ethical committee approval from the Ethical Committee for Experimentation with Animals of León University, Spain (03-02/2010).

Ejaculates from 5 sexually mature males (≥ 6 years old) of brown bear (*Ursus arctos*) were obtained by electroejaculation in one or two sessions, spaced each at least three weeks, during the breeding season (end of April to early July). Animals were housed in a half freedom regimen in Cabarceno Park (Cantabria, Spain; 43° 21' N, 3° 50' W; altitude: 143 m), and fed with a varied diet.

The animals were immobilized by teleanaesthesia, using 750 mg of zolazepam HCl + tiletamine HCl (Zoletil 100®, 50 mg/mL both zolazepam and tiletamine; Virbac, Carros, France) and 6 mg of medetomidine (Zalopine® 10mg/mL; Orion Pharma Animal Health, Finland). After immobilization, the bears were weighed and monitored during anaesthesia (pulse, saturation of peripheral oxygen and respiration). The pubic region and the penis were cleaned and the rectum was emptied of faeces. The bladder was catheterized during sperm collection. Electroejaculation was carried out with a PT Electronics1® electroejaculator (PT Electronics, Boring, OR, USA) using a transrectal probe 420 mm in length and 39 mm in diameter. Electric stimuli were applied until ejaculation (6-10 V and 250-300 mA). Ejaculates were collected in a graduated glass tube.

2.3. Experimental design

The selected ejaculates (> 50% total motility and < 80 mg urea/dL) were centrifuged at 600×g during 6 min to remove seminal plasma. The pellet was divided in four aliquots one for each treatment and diluted using TTF-ULE-Bear extender (TES-Tris-Fructose, 300 mOsm/kg, pH 7.1; 20% egg yolk, 6 % glycerol, supplemented with 2% EDTA, 1% Equex -Equex STM Paste; Minitüb, Tiefenbach, Germany-, 0.302 mg penicillin G sodium salt/mL and 0.625 mg dihydrostreptomycin sesquisulfate/mL-TTF-ULE Bear; (de Paz et al. 2012)), according the following procedure : 1) 1:1 dilution at RT, cooling in tube, final dilution at 5 °C (Control); 2) final dilution at RT, cooling in tube (FD-

Tube); 3) final dilution at RT, cooling in 0.25 mL plastic straw (FD-Straw) and 4) final dilution at RT with extender supplemented with 1.5 % gelatine, cooling in 0.25 mL plastic straw (Gelatine).

Control samples were cooled to 5 °C, 1:1 diluted using the TTF extender at 9% glycerol (final glycerol concentration 6 %) and more TTF extender at 6% glycerol was added to obtain a final concentration of 100×10^6 sperm/mL; then seminal samples were packaged into 0.25 mL plastic straws after 1 h (Control1), 24 h and 48 h (Control24 and Control48, respectively). FD-Straw and Gelatine were final diluted (6 % glycerol and 100×10^6 sperm/mL) and packaged into 0.25 mL plastic straws at room temperature, then they were cooled in straws and equilibrated (up to 48 h); whereas, FD-Tube samples were final diluted at room temperature (6 % glycerol and 100×10^6 sperm/mL), cooled in a tube up to 5 °C and packaged into 0.25 mL plastic straws after 24 and 48 h. After 1 (only for Control1), 24 and 48 h, sperm samples were frozen in a programmable biofreezer (Kryo 560-16 Planer™, Planer plc., Sunbury-On-Thames, UK) at -20 °C/min down to -100 °C, and then transferred to liquid nitrogen containers. The frozen sperm samples were stored in liquid nitrogen for a minimum of one week. Thawing was performed by dropping straws in water at 65 °C for 6 s. For this experiment, 6 electroejaculates from 5 brown bears were processed.

We analyzed the effect of handling of solid state (Control, FD-Tube, FD-Straw and Gelatine) in long-term storage (24 and 48 h) on prefreezing and post-thawing sperm quality; standard freezing samples stored for 1 h was used as control (Control1).

2.4. Sperm evaluation

The sperm were analyzed for motility, viability and acrosomal and apoptotic status before freezing and after thawing.

2.4.1. Motility assessment

Motility and kinematic parameters were assessed using a computer assisted sperm analysis system (CASA) (ISAS v.1.2, Integrated Semen Analyser System; Proiser, Valencia, Spain). A 5-µl sperm sample was placed into a Makler counting cell chamber (10 µm depth; Sefi Medical Instruments, Haifa, Israel) and examined using an E200 phase contrast microscope (Nikon, Tokio, Japan) with a warmed (38 °C) stage. The standard parameter settings were as follows: 25 frames/s; 5 to 50 μm^2 for head area; curvilinear velocity > 10 µm/s to classify a spermatozoon as motile. At least 5 fields or

200 spermatozoa were saved and analysed afterwards. Reported parameters were total motility (TM, %), progressive motility (PM, %; spermatozoa were considered progressive if VCL > 25, STR > 80); this percentage was divided in velocity categories (named SlowPM -with VCL: 10-65 µm/s -, MediumPM -with VCL: 65-100 µm/s - and RapidPM -with VCL>100 µm/s -, %), average path velocity (VAP, µm/s), linearity (LIN, %), straightness (STR, %; VSL/VAP), wobble (WOB, %; VAP/VCL), amplitude of lateral head displacement (ALH, µm).

2.4.2. Assessment of viability and acrosomal and apoptotic status

A double staining of SYBR-14 and PI (Molecular Probes, L-7011 LIVE / DEAD ® Sperm Viability Kit; Invitrogen, Barcelona, Spain) were applied to assess sperm viability (García-Macías et al. 2006). Sperm samples were diluted to 5×10^6 sperm/mL with PBS, and 300 µL were transferred to a polypropylene tube, with added 3 µL PI (24 mM in water) and 1.5 µL SYBR-14 (100 nM in dimethyl sulfoxide (DMSO)). The tubes were maintained at 37 °C for 20 min in the dark. In this study we evaluated the percentage of viable sperm (SYBR-14+/PI-, named VIAB).

For acrosomal status, we used the double stain of PNA-FITC and PI (García-Macías et al. 2006). Sperm samples were diluted with PBS (5×10^6 sperm/mL), and 300 µL were transferred to a polypropylene tube to which we added 2.5 µL PI (1 mg/mL in water) and 2.5 µL PNA-FITC (0.2 mg/mL in water). The tubes were incubated for 10 min at room temperature until analysis. Flow cytometer rendered the percentage sperm with damaged acrosomes (PNA-, named iACR).

Changes in sperm membrane permeability were analyzed with double staining YO-PRO-1/PI (Nicolas et al. 2011). Sperm samples were diluted with PBS down to 5×10^6 sperm/mL in polypropylene tubes (300 µL / tube), adding 2.5 µM YO-PRO-1 and 10 µM PI. The tubes were incubated at 37°C for 10 min until analysis by flow cytometry. We used the percentage of viable and non-apoptotic sperm (YO-PRO-1-/IP-, named YOPRO-).

Viability, acrosomal status, and apoptosis were analyzed using a FACScalibur flow cytometer (Becton Dickinson Immunochemistry Systems, San Jose, CA, USA), equipped with standard optics and an argon ion laser, tuned at 488 nm, and running at 200 mW. Calibration was carried out periodically using standard beads (Calibrites; Becton Dickinson). Data corresponding to the red (FL3 photodetector) and green

fluorescence (FL1 photodetector) of acquired particles were recorded. In all the cases, we assessed 10,000 events per sample with a flow rate of 200 cells/sec.

2.5. Statistical analysis

Data were analyzed using the SAS ™ v.9.1 package. The effect of solid media for a long-term storage before freezing were tested by using mixed-effects linear models after transforming the data (arc sine for percentages and natural logarithm for the rest of the analysed parameters). Male was considered a random effect. Least-squares means were computed for each effect listed and p-values for differences in the multiple comparisons were calculated by Tukey's test. Values were considered to be statistically significant at $P < 0.05$. All results are presented as mean±SEM.

3. Results and discussion

The fresh sperm samples of the brown bears studied in this experiment had a volume of 2.5±0.7 mL and sperm concentration of 303.5±77.6 × 10^6 sperm/mL.

At prefreezing, all experimental groups had lower results for VIAB, motilities, VAP (Gelatine) and WOB than Control1 but differences only were found after 48 h storage at 5 °C (Table 1). Gelatine yielded higher viability values (VIAB and YOPRO-) than FD-Straw; for motility, Gelatine had generally higher progressiveness (PM, LIN and STR), although lower VAP. Moreover, Gelatine showed similar YOPRO-, iACR, LIN, STR and ALH respect to Control1.

At post-thawing, sperm stored previously with Gelatine at 5 °C during 24 h reached the highest viability (VIAB, YOPRO- and iACR), higher LIN than sperm samples final diluted at RT (FD-Straw and FD-Tube), but the lowest VAP and ALH. Gelatine had similar viability (VIAB, YOPRO- and iACR) and kinetic parameters (LIN, STR and WOB), but lower motility and velocity than Control1. Besides, Control1 yielded higher VIAB (FD-groups), YOPRO- or iACR (FD-Straw), and some differences with motility and kinetic parameters, except for VAP and ALH, respect to the other experimental groups (Control24, FD-Tube, FD-Straw). It is due to the Control1 was processed under optimal conditions since collection of seminal sample (standard protocol).

After 48 h storage, although differences were not always statistically significant for the sperm characteristics studied at post-thawing, all parameters were better where adding

gelatine for 48 h at 5 °C; except for TM and VAP. Gelatine supplementation showed a clear improvement respect to Control48 (VIAB, YOPRO-, iACR, LIN, STR and ALH); respect Control1, Gelatine kept similar scores for YOPRO-, iACR, LIN, STR and WOB, although afforded lower motility and velocity; the rest of experimental groups dropped for most of post-thawing quality values.

After freezing-thawing process, diluted samples with TTF-ULE-Bear extender yielded higher RapidPM (about 70%) than the other categories (SlowPM and MediumPM) (Fig. 1); while, Gelatine showed a more homogeneous distribution of the three categories (about 50% RapidPM). Gelatine afforded higher MediumPM than the other experimental groups. Control1 yielded higher RapidPM (18.7) than Control48 (5.7), FD-Straw (48h: 11.2) and Gelatine (24h: 9.1, and 48h: 8.0).

Some authors studied the effect of solid storage in ram (Meque et al. 2005; Yaniz et al. 2005; López-Urueña et al. 2012), goat (Salvador et al. 2005; Salvador et al. 2006) and rabbit (Nagy et al. 2002; Lopez-Gatius et al. 2005; Rosato and Iaffaldano. 2011) spermatozoa with a successful storage, but none of them froze sperm samples after long-term storage. Thus, the effect of gelatine on sperm freezing is little known. At prefreezing evaluation, we did not find differences for 24 h storage, these results were consistent with those with Paulenz et al. (Paulenz et al. 2010); other authors only assessed sperm characteristics after 48 h (Rosato and Iaffaldano. 2011) or 72 h (Nagy et al. 2002). Paulenz et al. (Paulenz et al. 2010) tested the effect of gelatine on ram semen, diluted with milk-based extender (M) or with M supplemented with 0.5 % gelatine for 12 or 24 h. They did not find significant differences in fertility between M12 (69.6 %) and G12 (69.4 %) or M24 (63.6 %) and G24 (58.3 %). However, Lopez-Gatius et al. (Lopez-Gatius et al. 2005) reported motility differences from 0 to 96 h; gelatine supplementation (0.7 and 1.4 %) increased % motile cells and velocities, but no motion quality parameters (LIN, STR and WOB). These results disagree with those of this study (prefreezing values, after storage for 48 h) and a previous study with ram semen (López-Urueña et al. 2012), where we observed extender supplemented with 1.5 % gelatine reached superior progressive movement (higher PM, LIN, WOB and STR, lower ALH), but lower velocity (VAP). It could be due to differences on species spermatozoa. Elzanaty et al. (ELzanaty et al. 2004) studied the visco-elasticity of seminal fluid and its impact on human sperm motility; they noted that hyper-visco-

elasticity was associated with lower percentage of velocities (VAP, VCL) and ALH; similar to our results.

Salvador et al. (Salvador et al. 2005; Salvador et al. 2006) observed differences on goat sperm supplemented with gelatine for motility but no for acrosomal status or HOST; while Nagy et al. (Nagy et al. 2002) did find differences for acrosomal status from rabbit spermatozoa. Both researcher group yielded better quality results with the supplementation with gelatine, but its effect was different about acrosomal status; this could be due to differences in the percentage of gelatine or the species sperm. Salvador et al. (Salvador et al. 2005; Salvador et al. 2006) did not afforded a protection of acrosomes with 1.5 % gelatine (the same percentage as our study), while Nagy et al. (Nagy et al. 2002) afforded, as our results, higher spermatozoa with intact acrosome; so differences appear to be related species, but no with percentage. For ram sperm, 1.5 % gelatine resulted adequate for in vitro fertilization, motility and integrity membrane (Yaniz et al. 2005).

On the other hand, with prefreezing results, we observed that if we need to store sperm into straw up to 48 h (interesting conservation strategy from a practical point of view), samples supplemented with gelatine are much more suitable because of higher viability (VIAB and YOPRO-) and PM than when extender without gelatine, but also loaded into a straw.

At post-thawing evaluation, we observed a clear positive tendency for the effect of solid long-term storage at 5 °C for both 24 and 48 hours. Gelatine showed an improvement in viability and progressiveness versus the remain experimental groups (included Control24 and Control48), although seminal samples stored in extender with 1.5 % gelatine had similar motility (TM and PM) and lower velocity (VAP) and RapidPM. Furthermore, gelatine supplementation yielded similar results than Control1 for viability and progressiveness, but no for motility, velocity and RapidPM. It is important that gelatine yielded similar scores for some parameters than Control1, because this fact implies a little loss of quality with storage time. Moreover, for fertilization process, it could be interesting to achieve higher progressive spermatozoa although slow ones.

These findings could be due to positive effects of gelatine by solid state; first, gelatine seem to distribute uniformly seminal samples, thus, solid media would avoid sedimentation of spermatozoa, so deleterious changes in extender (Nagy et al. 2002; Paulenz et al. 2010); this fact could explain the maintenance of viability even after 48

hours respect to standard control (Control1) or the improvement versus the other handling conditions (Control24/Control48, FD-Tube and FD-Straw). On the other hand, this solid state may immobilize sperm cells reducing the metabolic demands of motion (Lopez-Gatius et al. 2005); the differences among motility patterns in gelatine supplemented samples (a decline of velocity although a good progressiveness) versus the other handling conditions, seem related to this possible immobilization. Shahiduzzaman & Linde-Forsberg (Shahiduzzaman and Linde-Forsberg. 2007) assessed immotility induced by the CLONE chilled semen extender (viscosus media) versus Tris-egg yolk-glucose extender (liquid media) for long-term storage at 5 °C. The immotility by CLONE did not prolong the lifespan of canine sperm and Tris-egg yolk-glucose extender maintained good quality for longer period. So, the improvement in our results was not only for immobilization but also to avoid sedimentation. Moreover, Nagy et al. (Nagy et al. 2002) concluded that gelatine had a further advantage through to be solid, which made handling and shipment samples easier and safer.

To our knowledge, there are no studies about post-thawing quality after long-term solid storage, except a previous work of our researcher group (López-Urueña et al. 2011) which showed that pre-freezing washing to remove the viscous media was detrimental for motility and viability; we did not observe differences in post-thawing quality of ram sperm between extender alone or supplemented with gelatine, but time of incubation at 5 °C was short (3 h).

Standard cryopreservation protocols might be adapted to variable working conditions (Lopez-Uruena et al. 2014a); for this reason, it is necessary to know the permissiveness of spermatozoa in order to cryopreserve them by non-standard protocols reaching an acceptable level of post-thawing quality (Herold et al. 2006). In the case of gelatine, dilution was carried out in a single step at room temperature and cooling from RT to 5 °C inside straws. In this work, among Control, FD-Tube and FD-Straw groups no differences were found, except for YOPRO- between FD-Straw and Control at prefreezing analysis for 48 h. So, brown bear spermatozoa have good tolerance in dilution and cooling methods.

Similarly, Dong et al. (Dong et al. 2008) studied effect of equilibration methods from rhesus monkey semen, they compared two methods similar to our Control (named, equilibration with pre-cooling) and FD-Straw (named, equilibration without pre-cooling). Although seminal samples equilibrated without pre-cooling had slightly higher

327 motility, there were no significant differences. Even, there are laboratories that
328 recommended filling the final diluted sperm in 0.5 ml straws at ambient temperature for
329 canine sperm cryopreservation (CaniPlus Freeze, Minitüb, Tiefenbach, Germany).

330 There are other compounds in addition of gelatine which have been used to increase the
331 viscosity of the media. In two previous studies with ram semen, we tested some of them
332 (Alvarez et al. 2012; López-Urueña et al. 2012). Lopez-Urueña et al. (López-Urueña et
333 al. 2011) evaluated the effect of extender (TRES-Tris-Fructose, egg yolk and glycerol -
334 TTFYG-) alone (control), or supplemented with 1.5 % gelatine (G), 1 % guar gum
335 (GG), or 2 % methylcellulose (MC) at 5 °C for 3 h, on post-thawing quality. GG and
336 MC yielded lower results (motility and viability) than control, whereas G was similar to
337 TTFYG. Alvarez et al. (Alvarez et al. 2012) assessed the effect of TTFY alone (control)
338 or supplemented with 0.5 % hyaluronic acid (HA), 3 % gelatine (G), 5 %
339 polyvinylpyrrolidone (PVP) or 1 % carrageenan (C). C yielded lower TM at all times 0,
340 24 and 72 h); for PM, in general, Control were similar to HA and G, but higher than C
341 and PVP. Probably, gelatine did not reach higher results (as our study) because the
342 percentage was high (3 vs. 1.5 %); the main problem for HA was that afforded higher
343 agglutination at all times.

344 Hyaluronic acid have been widely used in spermatology, mainly for ICSI (Barak et al.
345 2001; Balaban et al. 2003) as PVP (Balaban et al. 2003). Hyaluronic acid increased
346 retention (reversible) of spermatic motility (Human: (Sbracia et al. 1997), delay
347 capacitation for three days at 15 °C, and increased percentages of spermatozoa with
348 reactive acrosome (boar: (Yeste et al. 2008). PVP was observed to diminish even arrest
349 motility (Bull: (Hammerstedt et al. 1988); Opposum: (Moore and Taggart. 1995)).
350 Moore et al. (Moore and Taggart. 1995) found motility dropped when viscosity
351 increased, this effect disappeared when PVP was removed. The main problem of the use
352 of PVP is that at high concentration (10 %) resulted in a damage on acrosome, nucleus,
353 mitochondria, etc (Strehler et al. 1998). To our knowledge, the effects of carrageenan on
354 the sperm have not been studied; but carrageenan has been used in bacterial cultures of
355 *Bacillus spp* for immobilization (Baudet et al. 1983; Moon and Parulekar. 1991; Castet
356 et al. 1994). Coy et al. (Coy et al. 2009) evaluated the use of colloidal preparations for
357 boar sperm; they concluded that in vitro this viscous media can improvement motility,
358 viability, ability of sperm to bind and penetrate the oocyte, but this effect may be

masked for in vivo study because of high viscosity in the oviductal fluid during ovulation.

Other authors tested microencapsulated boar (Huang et al. 2005) and canine spermatozoa (Shah et al. 2010) on preservation at 4-5 °C for long-term storage. Huang et al. (Huang et al. 2005) found higher motility after three day storage. Shah et al. (Shah et al. 2010) found that microcapsules in gel (alginate only) yielded lower results than polycation (poly-L-lysine membrane bound) ones during 48 h storage, and polycation microcapsulation at 1 % alginate afforded higher motility and viability for up to 7 days. This technique could be applied in further studies of brown bear sperm for long-term chilled storage, although loss of spermatozoa released (recovery rate) must be taken in account.

In conclusion, our findings suggested that if a long-term storage at 5 °C of brown bear sperm was required, gelatine supplementation in TTF-ULE-Bear extender had a beneficial effect in post-thawing quality. On the other hand, brown bear electroejaculates appeared to have a good tolerance to cryopreserve them by no habitual protocols with successful guaranties, as final dilution (full glycerol) at RT and cooling in straws.

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523 Fig 1. Effect of handling of solid storage (Control, FD-Tube, FD-Straw, and Gelatine)
524 in progressive motility (PM) categories by velocity for long-term storage (24 and 48 h)
525 of brown bear sperm, prior to cryopreservation and respect to standard freezing
526 (Control1), on post-thawing evaluation (PM: % progressive motility; SlowPM: % PM
527 with with VCL: 25-65 µm/s; MediumPM: % PM with with VCL: 65-100 µm/s;
528 RapidPM: % PM with with VCL: >100 µm/s)
529
530

Table 1.- Effect of handling of solid storage (Control, FD-Tube, FD-Straw, and Gelatine) in long-term storage for 24 h and 48 h prior to cryopreservation of brown bear sperm and respect to standard freezing (Control1), on prefreezing and post-thawing quality (VIAB: % viability; YOPRO-: % viable and non-apoptotic spermatozoa; iACR: % intact acrosomes; TM: % total motility; PM: % progressive motility; VAP: average path velocity -µm/s-; LIN: linearity -%-; STR: straightness -%-; WOB: wobble -%-; ALH: amplitude of lateral head displacement -µm-).

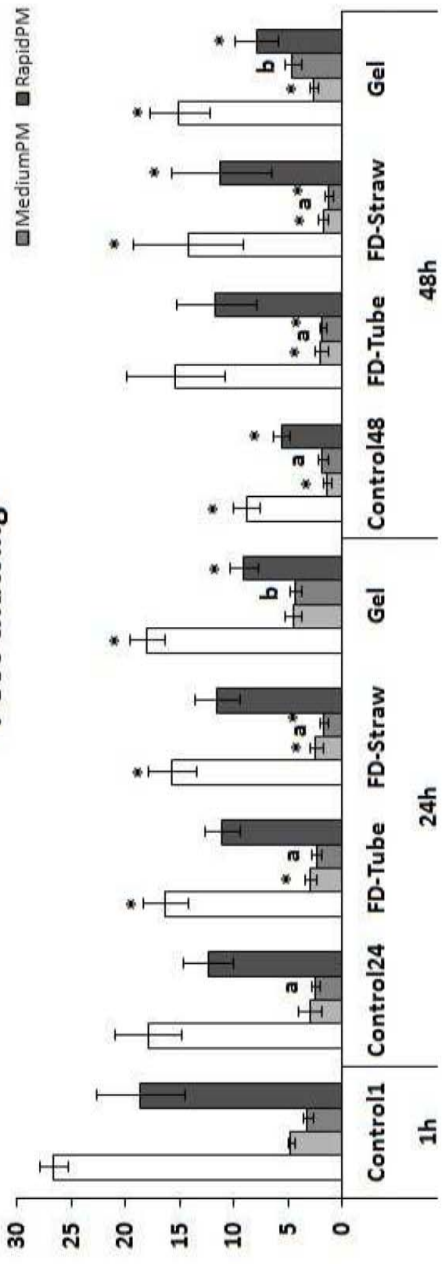
	PREFREEZING					POST-THAWING				
24 h	**Control1**	**Control24**	**FD-Tube**	**FD-Straw**	**Gelatine**	**Control1**	**Control24**	**FD-Tube**	**FD-Straw**	**Gelatine**
VIAB	73.0±1.3	48.6±3.5	48.4±6.0	45.0±7.6	53.5±5.3	52.5±1.0	42.4±3.5 ^{a}	41.7±6.0 ^{a}*	42.5±7.6 ^{a}*	55.5±5.3 ^{b}
YOPRO-	71.3±1.0	52.8±3.0	50.6±5.6	48.2±5.2	57.3±5.8	56.3±0.9	40.2±3.0 ^{a}*	40.1±5.6 ^{a}*	43.4±5.2 ^{a}*	54.6±5.8 ^{b}
iACR	98.4±4.8	97.1±0.9	97.3±0.7	97.2±1.5	97.7±0.5	95.5±2.4	94.4±0.9 ^{a}	95.2±0.7 ^{ab}	94.2±1.5 ^{a}*	96.9±0.5 ^{b}
TM	71.7±1.0	47.9±6.5 *	46.8±5.0	53.1±5.1	50.5±2.6	50.4±1.1	43.2±6.5	41.2±5.0	37.6±5.1 *	38.6±2.6 *
PM	36.3±1.3	28.0±3.1	26.1±2.1	25.3±2.2	30.3±1.6	26.7±1.3	17.9±3.1	16.4±2.1*	15.7±2.2 *	18.0±1.6 *
VAP	72.0±1.6	69.9±4.1	62.4±2.0	63.6±2.4	60.0±2.4 *	66.4±1.1	64.9±4.1 ^{a}	63.8±2.0 ^{a}	63.2±2.4 ^{a}	51.8±2.4 ^{b}*
LIN	36.5±2.1	32.4±1.5	31.9±1.7	31±2	34.2±1.0	35.0±2.4	30.2±1.5 ^{ab}*	29.5±1.7 ^{a}*	29.8±2.0 ^{a}	33.8±1.0 ^{b}
STR	60.1±2.6	61.6±2.2	61.6±2.7	58,7±3	64.4±1.1 *	62.1±2.8	57.1±2.2 *	56.1±2.7	57.3±3.0	60.6±1.1
WOB	60.1±1.7	53.0±0.9	51.6±1.3 *	52.7±1.0	53±0.7	56.1±2.1	52.7±0.9 ^{ab}*	52.5±1.3 ^{ab}*	51.6±1.0 ^{a}*	55.3±0.7 ^{b}
ALH	5.1±5.0	5.9±0.2	5.8±0.2	5.6±0.2 *	5.4±0.1	5.4±7.0	5.7±0.2 ^{a}	5.8±0.2 ^{a}	5.8±0.2 ^{a}	4.6±0.1 ^{b}*

	PREFREEZING					POST-THAWING				
48 h	**Control1**	**Control48**	**FD-Tube**	**FD-Straw**	**Gelatine**	**Control1**	**Control48**	**FD-Tube**	**FD-Straw**	**Gelatine**
VIAB	73.0±1.3	57.9±8.2 ^{ab}*	54.9±9 ^{ab}*	48.7±8.2 ^{a}*	62.8±7.9 ^{b}*	52.5±1.0	28.8±4.8 ^{a}*	32.9±5.9 ^{ab}*	36.1±7.8 ^{ab}*	42.0±6.9 ^{b}*
YOPRO-	71.3±1.0	58.7±6.8 ^{b}*	55.0±9.3 ^{ab}*	43.1±9.3 ^{a}*	61.4±4.9 ^{b}	56.3±0.9	29.1±5.4 ^{a}*	32.1±4.9 ^{a}*	33.4±6.5 ^{a}*	47.2±7.8 ^{b}
iACR	98.4±4.8	97.4±1.1	98.3±0.5	97.5±0.5	98.4±0.2	95.5±2.4	91.5±0.9 ^{a}*	93.0±1.0 ^{a}*	92.1±2.0 ^{a}*	96.2±0.9 ^{b}
TM	71.7±1.0	54.4±6.9	52.6±0.8 *	47.2±4.2 *	55.4±4.1 *	50.4±1.1	30.5±3.0 *	37.8±8.3	35.7±7.8 *	34.8±3.9 *
PM	36.3±1.3	17.5±2.6 ^{a}*	21.1±3.5 ^{ab}*	16.9±2.0 ^{a}*	30.0±4.7 ^{b}*	26.7±1.3	8.9±1.3 *	15.5±4.6	14.3±5.1 *	15.1±2.8 *
VAP	72.0±1.6	66.7±2.8 ^{ab}	70.4±3.6 ^{a}	66.7±3.9 ^{ab}	57.5±1.6 ^{b}*	66.4±1.1	65.1±2.4 ^{a}	62.3±2.8 ^{a}	61.5±2.1 ^{a}	50.2±2.5 ^{b}*
LIN	36.5±2.1	25.9±1.6 ^{a}*	28.3±1.9 ^{a}*	28.0±1.4 ^{a}*	33.6±1.8 ^{b}	35.0±2.4	25.2±1.6 ^{a}*	27.4±1.4 ^{ab}*	28.2±2.4 ^{ab}*	31.2±1.7 ^{b}
STR	60.1±2.6	50.9±2.9 ^{a}*	53.6±3.7 ^{ab}	54.3±2.7 ^{ab}	61.6±2.5 ^{b}	62.1±2.8	49.7±2.6 ^{a}*	55.0±2.0 ^{ab}*	55.2±3.4 ^{ab}	58.1±1.9 ^{b}
WOB	60.1±1.7	51.1±2.8 *	52.7±1.9 *	51.3±0.3 *	54.2±0.9 *	56.1±2.1	50.6±1.2 *	49.6±0.8 *	50.8±1.2 *	53.3±1.4
ALH	5.1±5.0	6.0±0.4 ^{ab}*	6.0±0.3 ^{ab}*	6.4±0.4 ^{a}*	5.2±0.1 ^{b}	5.4±7.0	6.2±0.3 ^{a}*	6.0±0.2 ^{a}	5.8±0.2 ^{a}	4.7±0.1 ^{b}*

^{a,b} Within each sampling point, different letters indicate that means of handling models differ for each parameter ($P < 0.05$).

* Asterisks indicate that means of handling models in each time of long-term storage differ from standard freezing (Control1).

Figure 1



[a,b] Within each storage time, different letters indicate that means of handling models differ for each parameter ($P < 0.05$).

[a,b] Different letters indicate that means of handling of solid storage in each time of long-term storage differ for each parameter ($P < 0.05$).

* Asterisks indicate that means of handling models in each time of long-term storage differ from standard freezing (Control1).

Reproduction in Domestic Animals

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DISCUSIÓN

La creación de los Bancos de Recursos Genéticos es una estrategia fundamental para la recuperación de poblaciones en peligro de extinción, como es el caso de la población del oso pardo en la Cordillera Cantábrica. Para mejorar la eficacia de los bancos de germoplasma, hay que prestar especial atención a la calidad de los espermatozoides almacenados así como a determinadas características de específicas, como puede ser el caso del almacenaje de espermatozoides sexados. En muchas ocasiones, para la aplicación de las técnicas de mejoramiento espermático es necesario el envío de las muestras desde el lugar de recogida (condiciones de campo) hasta centros biotecnológicos de referencia; en este punto se pone de manifiesto la necesidad de disponer de tiempo extra en los protocolos estándar de crioconservación para realizar el envío y procesado de las muestras seminales antes de su congelación. En consecuencia, los protocolos clásicos de crioconservación de espermatozoides de oso pardo desarrollados hasta el momento, han de ser redefinidos. En el presente trabajo de tesis doctoral, se han evaluado diferentes alternativas en el diseño del periodo de refrigeración precongelación de semen de oso pardo obtenido mediante electroeyaculación, tales como: 1) diferentes rampas de refrigeración y la simplificación de los protocolos mediante la eliminación del tiempo de equilibrado sólo o junto con el periodo de refrigeración; 2) el almacenamiento a corto o largo plazo a 5 °C de las muestras espermáticas previamente a la congelación; 3) el control de parámetros como la temperatura, el porcentaje de glicerol o la tasa de dilución, en el almacenamiento precongelacion a largo plazo o congelación diferida; y 4) el uso de un medio sólido para la mejora de la calidad durante el almacenamiento a largo plazo.

En ocasiones, los protocolos de crioconservación seminal de las especies amenazadas, requieren ser simplificados, para adaptarlos a las condiciones de campo. El primer estudio de la presente tesis doctoral se ha diseñado para proporcionar un protocolo óptimo de crioconservación del semen de oso pardo, estudiando la velocidad de refrigeración y en el tiempo de equilibrado, pasos previos a la congelación. Para ello, evaluamos tres rampas de refrigeración (0,25, 1 y 4 °C/min), la eliminación del tiempo de equilibrado estándar (1 h) o la omisión conjunta de los periodos de refrigeración y equilibrado (denominada congelación directa). El shock térmico que se produce durante la refrigeración espermática afecta a un gran número de especies (Amann & Pickett, 1984; Watson, 2000) con distinto grado de sensibilidad de unas especies a otras (Althouse et al., 1998; Gao & Critser, 2000; Aurich et al., 2008). En nuestro estudio, observamos una caída en la calidad post-descongelación de la viabilidad, estado acrosomal y movilidad al incrementar la tasa de refrigeración. Los espermatozoides de carnívoros parecen ser menos susceptibles a la refrigeración que los de otras especies (Hay et al., 1997). Bouchard et al. (1990) estudiaron el efecto de dos rampas de refrigeración (0,3 y 1 °C/min) en el semen de perro, sin observar diferencias significativas entre

ambas. Hermansson & Axner (2007) concluyeron que los espermatozoides de gato no son susceptibles al shock térmico, ya que no encontraron diferencias entre las rampas de refrigeración de 0,5 y 3 °C/min; mientras que Pukazhenthi et al. (1999) sí observaron mayor daño en los acrosomas de los espermatozoides de gato con las rampas rápidas (4 °C/min) o ultrarápida (14 °C/min), en comparación con la rampa lenta (0,5 °C/min). Las rampas de refrigeración se han estudiado, también, en protocolos de congelación espermática de otras especies distintas a los carnívoros, con resultados variables; hay autores que han encontrado buenos resultados con una rampa de refrigeración rápida (Ciervo rojo: Fernández-Santos et al., 2006b; toro: Januskauskas et al., 1999) mientras que otros autores, como en nuestro estudio, encontraron mejor calidad tras una rampa lenta (Toro: Dhami et al., 1992; caballo: Varner et al., 1988).

Las diferencias de estos resultados podrían ser explicadas por el origen de las muestras espermáticas o la composición de los diluyentes utilizados, así como por la especie. En este sentido, en un estudio realizado en mamíferos africanos, Gilmore et al. (1998) observaron que los espermatozoides epididimarios eran claramente más resistentes al shock térmico que los de eyaculados; aunque en otras especies, como el gato, no se han observado estas diferencias (Hermansson & Axner, 2007). En cuanto a la composición de los diluyentes, el efecto protector de la yema de huevo ha sido ampliamente descrita (Fernández-Santos et al., 2006b; Akhter et al., 2011); en este estudio se ha usado un 20 % de yema de huevo en el diluyente, lo cual no evitó el efecto deletéreo de la rampa rápida en los espermatozoides de oso pardo, aunque sí se ha visto en el caso del ciervo rojo (Fernandez-Santos et al., 2006b). Parece ser que la respuesta del espermatozoide al shock térmico, está relacionada con la composición lipídica de su membrana plasmática; pero hasta la fecha, se desconoce la composición de la membrana en el caso de espermatozoides de oso pardo (Alvarez-Rodríguez et al., 2011). En nuestro estudio, el daño (reflejado en datos de viabilidad y estado acrosomal) no se observa hasta el análisis postdescongelación, por lo que se puede pensar que según la velocidad de refrigeración que aplicamos se produce distinto grado de cambio subletal probablemente ligado a una reorganización de la membrana, pero que no se hace evidente hasta no sufrir los cambios inducidos por la congelación.

Otro factor importante que afecta a la calidad post-descongelación es el tiempo de equilibrado (Berndtson & Foote, 1972; Almlid & Johnson, 1988). En referencia a los escasos estudios realizados en el oso, algunos trabajos aplican una hora de equilibrado (Ishikawa et al., 2002; Alvarez et al., 2008) o tres horas pero contabilizando el tiempo desde la adición del primer extender que no lleva glicerol (Okano et al., 2004a). En nuestro estudio, no se observaron claras diferencias hasta el análisis tras la incubación a 37 °C (ThS test), donde el equilibrado de 1 hora obtuvo mejores resultados en términos de movilidad (MT y MP), viabilidad y

estado acrosomal (respecto a la omisión de este paso -0 h-). En nuestro caso, a diferencia de otras especies, un periodo de equilibrado con glicerol durante 1 hora a 5 °C conserva mejores características de calidad post-descongelación que la omisión del mismo en el proceso/protocolo.

Con el propósito de optimizar los protocolos de manejo en diferentes situaciones que se pueden presentar en la recogida seminal bajo condiciones de campo, en una tercera experiencia, evaluamos el efecto de la congelación directa, es decir, la eliminación de los pasos de refrigeración y equilibrado, frente a una congelación estándar (refrigeración con rampa lenta -0,25 °C/min- y 1 hora de equilibrado a 5 °C). Las muestras congeladas mediante congelación directa mostraron una drástica caída de los valores de viabilidad, estado acrosomal y movilidad, en comparación con el protocolo de congelación convencional; el efecto deletéreo de la congelación directa es parcialmente visible a la descongelación, pero el test de incubación (ThS test) revela el grave daño sufrido. En este sentido, se ha investigado muy poco a cerca de la eliminación de ambos periodos, habitualmente en todos los trabajos de crioconservación de semen se realiza un periodo de refrigeración y de equilibrado previo a la congelación, pudiendo omitir en algunos estudios uno de los dos pasos (Okano et al., 2006a). Sin embargo, Kundu et al. (2000) testaron diferentes métodos para simplificar el modelo de crioconservación de la papilla epididimaria de macho cabrío. En dicho estudio, hubo una pérdida total de la movilidad en los dos protocolos que se detallan a continuación: 1) dilución (a temperatura ambiente) e inmersión en nitrógeno líquido; 2) dilución (a temperatura ambiente), refrigeración (a 0,25 °C/min) e inmersión directamente en nitrógeno líquido. A diferencia de este estudio, aunque nosotros eliminamos los pasos de refrigeración y equilibrado, realizamos una rampa de congelación controlada mediante un biocongelador que minimiza, en parte, los daños de la congelación directa.

Para el análisis del estado de la cromatina (% DFIt, mediante SCSA), no encontramos diferencias significativas en ninguna de las tres experiencias. Similares resultados fueron obtenidos por Kmenta et al. (2011), donde no varió el estado de la cromatina de las células espermáticas de perro, durante los 8 días que estuvieron almacenadas. Sin embargo, en un estudio previo de nuestro grupo de investigación (García-Macías et al., 2006) sobre el semen de oso pardo, sí se encontraron diferencias, observándose una fragmentación mayor de cromatina en la estación reproductiva (variaciones estacionales) en semen recién obtenido. Este estudio apoya este hecho, ya que todas las muestras fueron recogidas durante la época reproductiva, pero no se encuentran diferencias entre los distintos métodos de manejo de las muestras espermáticas.

Tras una primera aproximación, llevada a cabo en el primer trabajo, sobre el tiempo de equilibrado en los protocolos de congelación de semen de oso pardo,

en el segundo artículo de la tesis doctoral realizamos un estudio en profundidad de este paso. Por ello, evaluamos el efecto del tiempo de equilibrado a corto-medio plazo (con un amplio rango de horarios) y el almacenamiento a 5 °C durante un largo plazo de las muestras seminales (congelación diferida), con el fin de conocer la permisividad de los espermatozoides de oso pardo a variaciones substanciales de los protocolos estándar de criopreservación espermática y así poder definir un adecuado protocolo de congelación para los eyaculados de esta especie que son recogidos e inicialmente procesados bajo condiciones de campo. Llevamos a cabo dos experiencias en las cuales los eyaculados de oso pardo fueron 1) equilibrados hasta 10-12 h a 5 °C (tiempos de muestreo: 0, 0,5, 1 –control-, 4-5, 7-8 y 10-12 h), o 2) almacenados a 5 °C hasta las 72 h (tiempos de muestreo: 1 –control-, 24, 48 y 72 h), antes de congelar.

En la primera experiencia, coincidiendo con el estudio previo/preliminar, observamos una significativa reducción de la calidad post-descongelación (movilidad y estado acrosomal) para las muestras congeladas tras una refrigeración lenta sin equilibrado (0 h). En general, los mejores resultados se alcanzaron con un tiempo de equilibrado de 1 h, y se produjo un descenso gradual en la calidad a medida que aumentamos el tiempo de equilibrado, pero sin encontrar diferencias significativas. Con semen de oso panda, Olson et al. (2003) estudiaron cuatro métodos de refrigeración (0,5 o 2,5 h de refrigeración combinadas con 0,5 y 2,5 h de equilibrado) y no encontraron diferencias (movilidad y viabilidad), pero sólo analizaron datos precongelación. Además, los datos sólo tienen un valor descriptivo y carecen de validez estadística, por lo que no es comparable con nuestros resultados. Existe otro estudio con semen de oso japonés (Okano et al., 2006a), en el que los autores evaluaron el efecto del tiempo de equilibrado, testando 1 h de refrigeración seguida de 1 h de equilibrado (protocolo 1) frente a 3 h de refrigeración sin equilibrado (protocolo 2); a diferencia de nuestros resultados, ellos no observaron diferencias para los parámetros evaluados (movilidad, viabilidad, estado acrosomal y morfología), sugiriendo que el periodo de equilibrado puede ser omitido. Sus resultados tampoco coinciden con los de nuestro estudio, lo cual podría ser en parte explicado porque nosotros aplicamos un tiempo fijo de refrigeración (1 h) y ellos usan dos tiempos (3 o 1 h) que combinan con las 0 o 1 h de equilibrado, respectivamente. Okano et al. (2004b), en semen de perro, sugirieron que con un tiempo de refrigeración óptimo de 3 h, el tiempo específico destinado para el equilibrado podía ser omitido. Puede ser el motivo de las diferencias con nuestro trabajo, ya que las muestras en las que eliminan el equilibrado, refrigeran durante un tiempo más prolongado que el protocolo 1.

La elección de un tiempo concreto de equilibrado parece estar influenciado por diversos factores como la refrigeración (Toro: Dhami y Sahni,

1993; perro: Okano et al., 2004b), el porcentaje de glicerol (Cerdo: Corcuera et al., 2007; macho cabrío: Deka & Rao, 1986) y el parámetro analizado (Deka & Rao, 1986) o la especie (Li et al., 2005; Gao & Critser, 2000). Dhami & Sahni (1993) estudiaron cuatro rampas de refrigeración con dos tiempos de equilibrado (0 y 2 h), coincidiendo con nuestros resultados, concluyeron que una rampa de refrigeración lenta y al menos 2 horas de equilibrado eran esenciales para obtener unos resultados óptimos. Corcuera et al. (2007) vieron una correlación entre el porcentaje de glicerol y el tiempo de equilibrado en la calidad post-descongelación de semen de porcino, ya que con un tiempo de equilibrado mayor (30 vs 0 min) se alcanzó mayor movilidad, para el 4 % de glicerol, pero tuvo un efecto deletéreo para el 8 %. Deka & Rao (1986) evaluaron tres tiempos de equilibrado (1, 3 y 5 h) y obtuvieron los mejores resultados post-descongelación con un tiempo de equilibrado corto (1 h) para el estado del acrosoma o uno largo (5 h) para movilidad, dependiendo también del porcentaje de glicerol utilizado. La permeabilidad de la membrana al glicerol parece ser específica para cada especie, de esta forma, el glicerol puede penetrar rápidamente en los espermatozoides de unas especies pero lentamente en las de otras (Li et al., 2005); sin embargo, no hay estudios acerca de la penetración del glicerol al interior de los espermatozoides de oso pardo. Nuestros resultados mostraron que se produce una reducción de la calidad post-descongelación si el semen es congelado sin el periodo de equilibrado (0 h); mientras que un tiempo corto (0,5 h) parece ser suficiente para mejorar la calidad post-descongelación. Este hecho nos permite pensar que el glicerol podría necesitar un periodo corto para estabilizar las membranas celulares, como se describe por otros autores (Gañan et al., 2009). No hay que olvidar que, además del efecto protector del glicerol frente al shock térmico, la exposición a dicho crioprotector presenta cierta toxicidad (Holt, 2000; Li et al., 2005). En nuestro estudio, las muestras que fueron sometidas a un tiempo de equilibrado más prolongado (10-12 h) no mostraron ninguna diferencia significativa con respecto a los tiempos cortos, aunque se observa un descenso gradual de 1 a 10-12 h. En concordancia con nuestros resultados, Herold et al., (2006) testaron tiempos de equilibrado comprendidos entre 2 y 9 h para congelar espermatozoides obtenidos de epidídimos de búfalo, y no encontraron diferencias para movilidad ni para estado acrosomal. En este sentido, también algunos autores obtuvieron buenos resultados con tiempos de equilibrado largos (Macho cabrío: Deka & Rao, 1986, 5 h; búfalo: Tuli et al., 1981, 4 h; Fabrocini et al., 2000, 5 h; toro: Leite et al., 2010, 4 h; cerdo: Yi et al., 2002, 5 h).

En la segunda experiencia, obtuvimos un descenso de la calidad espermática post-descongelación después de las 24 h de almacenamiento a 5 °C en los resultados de movilidad, viabilidad y estado acrosomal, aunque las tasas de recuperación (descongelación/precongelación) estuvieron cercanas al 100% para la mayoría de los parámetros. Y el almacenamiento durante 72 h presentó una

grave caída en la calidad tanto en la precongelación como en la descongelación. Estos resultados discrepan con los de otros autores que mantuvieron una movilidad ≥ 50 % después de tres o más días de almacenamiento a refrigeración (Perro: Iguer-Ouada et al., 2001; Ponglowhapan et al., 2004; Sahashi et al., 2011; Shahiduzzaman et al., 2007; carnero: López-Sáez et al., 2000; Tamayo-Canul et al., 2011; búfalo: Akhter et al., 2011). Estas diferencias podrían ser explicadas por diferencias específicas, por el método de recogida o por el tipo de diluyente (% de glicerol) utilizado. En nuestro estudio, las muestras fueron recogidas por electroeyaculación mientras que en los trabajos citados anteriormente fueron obtenidas mediante vagina artificial, recogida post-mortem (epidídimo) o manipulación digital; y además procedían de otros mamíferos diferentes al oso pardo.

Por otro lado, algunos autores estudiaron la congelabilidad de las muestras espermáticas tras un almacenamiento a largo plazo, como en nuestro estudio, y prácticamente no encontraron diferencias entre los tiempos testados (Purdy et al., 2006: 0, 24 y 48 h, en carnero; Guthrie & Welch, 2005: 3 y 24 h, en cerdo. Hermanssoin & Forsberg, 2006: 24 y 48 h, en perro. Ponglowhapan et al., 2006: 0 y 48 h, en perro). Incluso, algunos autores (Toro: Anzar et al., 2011; caballo: Crockett et al., 2001) lograron mejores resultados tras 24 h de almacenamiento previo a la congelación que en el control (0 y 2,5 h, respectivamente). En nuestro estudio, la calidad post-descongelación de las muestras de 24 h fue similar a la del control de congelación estándar (1 h), por lo que se deduce que los espermatozoides de oso pardo se podrían congelar 24 horas tras su recogida si el lugar de recogida seminal y el laboratorio están distantes. Verstegen et al. (2005) almacenaron eyaculados de perro a 4 °C durante varios días y observaron que pese a que la movilidad no cambió significativamente durante los 10 primeros días y la fertilidad se mantuvo durante 6 días, la concentración de glucosa se desplomó al segundo día.

Como hemos comentando anteriormente, en especies amenazadas como el oso pardo, adquiere vital importancia aplicar técnicas como el sex-sorting, técnicas que se llevan a cabo en centros biotecnólogicos de referencia, los cuales están alejados del lugar de recogida seminal. Existen principalmente dos vías de actuación: 1) refrigeración, almacenamiento refrigerado a largo plazo, envío, sex-sorting y posterior congelación, frente a 2) refrigeración, congelación-descongelación, envío, sex-sorting y recongelación de las muestras seminales. Algunos autores (Perro: England & Ponzio, 1996; panga gigante: Olson et al., 2003; carnero: Kasimanickman et al., 2007; toro: Anzar et al., 2011) lograron mejores resultados tras el almacenamiento líquido durante 48 h que en la congelación estándar; posiblemente el daño espermático se atribuye al proceso de congelación/descongelación (England & Ponzio, 1996; Kasimanickman et al.,

2007). Cuando comparamos los datos de descongelación del control de congelación estándar frente a distintos tiempos de almacenamiento a largo plazo a 5 °C, nos encontramos con que la calidad (medida por viabilidad, espermatozoides viables y no apoptóticos, y estado acrosomal) del control estándar de congelación fue menor que las muestras almacenadas a refrigeración durante 24 h, y similar a las 48 h. En consecuencia, nosotros recomendamos el envío de las muestras a 5 °C, si el procesado en un centro biotecnológico se hace dentro de las 24 h. Como el control de congelación estándar y las muestras almacenadas a 5 °C durante 48 h alcanzaron similares resultados, la elección dependerá de la posibilidad de envío al laboratorio de referencia y de la congelabilidad del semen. Si el centro biotecnológico de referencia está aún más lejos (transporte de ≥ 72 h) se recomienda el envío de muestras congeladas. Las tasas de recuperación del control de congelación estándar fueron del 75 % de la viabilidad inicial y el 79 % de la movilidad inicial, pero desconocemos qué congelabilidad alcanzaría tras un segundo ciclo.

Considerando la alternativa de la recongelación, en un estudio previo de nuestro grupo de investigación, recongelamos semen de oso pardo y observamos un efecto perjudicial en los parámetros evaluados; notando que la movilidad y el estado acrosomal fueron los más negativamente afectados (tasas de recuperación entre las dos descongelaciones en torno al 50 %). Varios autores han reportado una clara caída de la calidad entre dos ciclos de congelación-descongelación en espermatozoides de humana (Polcz et al. 33, MT: 24,4 vs. 8,0 %, viabilidad: 39,1 vs 25,3; Verza & Esteves 43, MT: 42,0 vs.22,5, MP: 34 vs. 9,5, viabilidad: 45 vs. 20; Verza et al., 44, MT: 50,6 vs.26,9), caballo (Choi et al., 9, MT: 36 vs. 16), y toro (Underwood et al., 41, MT: 70 vs. 55, MP: 50 vs. 30, viabilidad: 50 vs. 40). En nuestro experimento la comparación no es completa porque no aplicamos una recongelación de semen congelado mediante la congelación estándar (1 h), pero es una aproximación inicial para establecer guías de manejo para las muestras espermáticas de oso pardo.

Por otro lado, aunque se ha comprobado que la congelación diferida es una posibilidad real y útil, sería importante encontrar las condiciones de manejo adecuadas en el semen de oso pardo óptimas para mitigar los efectos negativos del envío (en refrigeración) de las muestras y así mejorar su calidad post-descongelación. Entre estas condiciones, en el tercer trabajo de la tesis doctoral, testamos tres temperaturas (temperatura ambiente, 15 y 5 °C), tres porcentajes de glicerol (0, 3 y 6 %) y dos tasas de dilución (dilución 1:1 y final) para optimizar un protocolo de conservación adecuado durante el envío de las muestras seminales. Bouchard et al. (1990) sugirieron que la fertilidad dependía principalmente de las bajas temperaturas para disminuir el metabolismo, un diluyente adecuado y la

resistencia propia de los espermatozoides, en este caso de perro, frente al shock térmico.

La funcionalidad espermática en el periodo antes de la congelación, depende, entre otros factores, de la temperatura (Vishwanath & Shannon, 2000). En esta primera experiencia, observamos que la temperatura óptima de almacenamiento a largo plazo de los espermatozoides de oso pardo fueron los 5 °C, con los que conseguimos los mayores resultados post-descongelación, comparables a los obtenidos por el control de congelación estándar. La temperatura intermedia (15 °C) mostró un descenso moderado, mientras que la temperatura ambiente presentó la mayor caída de la calidad. Además, encontramos que para periodos de hasta 48 h, el transporte de las muestras seminales refrigeradas a 5 °C (incluso 15 °C) es mejor opción (en términos de viabilidad y estado acrosomal) que el envío de las muestras seminales congeladas. Estos resultados se explican porque con el almacenamiento a 5 °C se reduce el consumo de la energía y la tasa metabólica (disminución del pH -Jeyendran & Graham, 1982- y producción de radicales libres, al aumentar la temperatura de almacenamiento). A diferencia de nuestros resultados, algunos autores no han encontrado diferencias entre el almacenamiento a 20 y a 4 °C hasta las 24 h (Cerdo: Zou & Yang, 2000; macho cabrío: Batista et al., 2011; elefante: Kiso et al., 2011). Además, para un periodo corto (6-8 h), el almacenamiento a 21 °C representó una temperatura de elección para el transporte seminal al lugar donde se lleva a cabo el sexaje de los espermatozoides de carnero (Hollinshead et al., 2004) o de ciervo rojo (Parilla et al., 2010). Para el ajuste de la temperatura hay que tener en cuenta el shock térmico, al que no todas las especies responden de igual forma, por ejemplo, en el caso de los espermatozoides de cerdo se ha descrito que este fenómeno se produce por debajo de los 12 °C (Althouse et al., 1998), lo que explica que algunos autores concluyeran que el semen de cerdo podría ser almacenado a 20 o 15 °C durante 48 (Zou & Yang, 2000) o 96 h (Paulenz et al., 2000) sin efectos deletéreos en su calidad. Los resultados de nuestro estudio nos permiten pensar que los espermatozoides de oso pardo no responden al shock térmico de la misma forma que lo descrito en otras especies (como el cerdo) y que la longevidad de los espermatozoides almacenados a 5 °C está influenciado por diferencias específicas en la composición de la membrana (White, 1993; Parks et al., 1997; Varisli et al., 2009).

En el estudio del almacenamiento a largo plazo (pero sin congelación), para diferentes especies de mamíferos, se observó que la temperatura óptima estaba entre los 4-5 °C (Toro: Vishwanath et al., 2000; conejo: Rosato & Iaffaldano, 2011; carnero: O'Hara et al., 2010; Paulenz et al., 2002; caballo: Love et al., 2002; gato: Hermansson & Axner, 2007; perro: Bouchard et al., 1990). En algunos estudios, se investigó el almacenamiento espermático a 4-5 °C durante un tiempo

prolongado y su efecto en la calidad post-descongelación. Estos autores observaron resultados, de calidad y/ o fertilidad, similares a los datos de partida en semen de caballo (Backman et al., 2004: 18 h), toro (Anzar et al., 2011: 24 h) y delfín (Robeck & Obrien, 2004: 24 h). Estos estudios están de acuerdo con nuestros resultados, donde sugerimos que, cuando se trata de periodos de hasta las 48 h, es mejor opción el envío de semen a los centros biotecnológicos de referencia para su procesado (por ejemplo para aplicar un sex-sorting) a 5 °C en lugar de su envío en estado congelado.

La concentración de glicerol adecuada para la congelación de semen de oso se ha establecido en un rango entre el 4-8 %, alcanzando una mayor calidad en torno al 6 % (Okano et al., 2006a; Anel et al., 2010; de Paz et al., 2012); sin embargo, no se ha estudiado el efecto del porcentaje de glicerol durante el almacenamiento a largo plazo precongelación en el semen de osos. Nuestros resultados indican la necesidad de incorporar el glicerol en el diluyente para el almacenamiento prolongado a 5 °C, desde que las muestras seminales almacenadas en TTFY con el 6 % glicerol durante el periodo precongelación (24 y 48 h) alcanzaron los mejores resultados post-descongelación. La tolerancia al glicerol durante la refrigeración también difiere entre especies (Oso pardo: Alvarez-Rodríguez et al., 2011; ciervo rojo: Fernández-Santos et al., 2006; perro: Songsasen et al., 2002; toro: Vera-Muñoz et al., 2011). Algunos autores (Hermansson & Axner, 2007; Gil et al., 2011) consideran la adición de glicerol a temperatura ambiente como una praxis no conveniente, aunque Alvarez-Rodríguez et al. (2011) sugirieron que el semen de oso pardo parece poseer una buena tolerancia al glicerol, ya que no observaron diferencias en la calidad post-descongelación entre tres métodos de adición del glicerol: 1) todo a temperatura ambiente, 2) la mitad de la concentración final a temperatura ambiente y el resto a 5 °C, y 3) todo a 5 °C. Sin embargo, Providence et al. (1984) no encontraron ningún efecto del porcentaje de glicerol sobre la movilidad para el semen de caballo, pero observaron un efecto negativo en la movilidad de espermatozoides de perro tras el almacenamiento con el diluyente que contenía 6 % de glicerol; pese a que Songsasen et al. (2002) encontraron que los espermatozoides de perro presentaban una buena resistencia a soluciones hipertónicas de glicerol. Además, Vera-Muñoz et al. (2011) tampoco encontraron efectos para los porcentajes de glicerol testados (0 vs. 6 %) para el semen de toro refrigerado hasta las 48 h, aunque sí encontraron un efecto negativo de la adición de glicerol, pasado ese tiempo. En algunas especies salvajes, coincidiendo con nuestros resultados, la adición del 6 % glicerol tuvo un efecto positivo en el semen almacenado durante más de 24 h (Búfalo: Kumar et al., 1992; zorro volador: de Jong et al., 2005). En estudios preliminares, nosotros encontramos que los electroeyaculados de oso pardo diluidos 1:1 con diluyente suplementado con el 3 % glicerol (durante condiciones precongelación) alcanzaron mayores resultados en el almacenamiento

a largo plazo (48h) a 5 °C tanto en el análisis de precongelación (López-Urueña et al., 2014b) como postdescongelación (López-Urueña et al., 2014c) que los que no llevaban glicerol.

El tiempo de exposición de los espermatozoides al glicerol en el almacenamiento a largo plazo precongelación tiene efectos en su calidad post-descongelación (Herold et al., 2006). Algunos autores diluyeron las muestras seminales con medios que contenían glicerol y las almacenaron en refrigeración durante largos periodos con buenos resultados post-descongelación (Carnero: Purdy et al., 2006; Tamayo-Canul et al., 2011a,b; macaco Rhesus: Dong et al., 2008; perro: Hermansson & Axner, 2006); incluso sugirieron el efecto positivo del glicerol sólo (Anzar et al., 2011) o combinado con la yema de huevo (Gil et al., 2011) en la calidad post-descongelación de muestras espermáticas almacenadas a 5 °C durante 24 o 48 h, respectivamente.

La dilución del semen es otro parámetro importante que se debe controlar, a causa de las interacciones existentes entre los espermatozoides y los componentes del diluyente (Vera-Muñoz et al., 2011). En esta tercera experiencia, los resultados sugirieron que la tasa de dilución no tuvo efecto significativo en la calidad de semen de oso pardo tras las 24 h de almacenamiento a 5 °C; pero sí encontramos efectos negativos en la dilución baja (dilución 1:1) tras las 48 h. Verstegen et al. (2005) observaron un descenso grande de la glucosa del primer-segundo días debido a una alta actividad de los espermatozoides de perro a 5 °C durante las primeras 24 h; en nuestro estudio, las muestras seminales diluidas con la tasa 1:1 tendrían mayor consumo de glucosa en estas 24 h, lo que podría explicar parcialmente las diferencias observadas a las 48 h.

La concentración celular durante el almacenamiento se ha relacionado con un deterioro en parámetros estructurales, funcionales y de movilidad espermática; posiblemente causado por un estrés oxidativo extracelular, efectos del plasma seminal y producción de radicales libres endógenos (Carnero: Kasimanickman et al., 2011). Miro et al. (2009) concluyeron que la viabilidad de semen de burro tras el almacenamiento (72 h, 5 °C) se incrementó con la tasa de dilución (1:1 > 1:5 > 1:10) y sólo la tasa 1:10 tras el almacenamiento hasta 48 h obtuvo similares resultados a la muestra fresca. Esta mejora de la calidad por incremento de la tasa de dilución, como en nuestro estudio, podría deberse al resultado de una reducción en la concentración de metabolitos (Prathalingam et al., 2006); una mayor concentración celular podría implicar una mayor actividad oxidante, relacionada con la muerte celular (Shannon & Curson, 1971).

Por otra parte, el manejo de la muestra seminal en el laboratorio de referencia podría requerir concentraciones altas de espermatozoides o la eliminación de los diluyentes usados para el transporte, por lo que sería necesario

la centrifugación o lavado, lo cual perjudica la calidad de la muestra o disminuye el número de espermatozoides recuperados (Burro: Miro et al., 2009; oso pardo: Alvarez et al., 2012). En nuestro estudio, observamos que el semen de oso pardo se podría diluir con una tasa 1:1 y almacenar hasta 24 h sin una significativa caída de la calidad, en comparación con una dilución final. Kiso et al. (2011) encontraron un efecto negativo de la yema de huevo que interfería en el sex-sorting (Johnson & Welch, 1999); por lo tanto, las muestras seminales antes del proceso de selección deberían ser centrifugadas. Sin embargo, Shannon & Curson (1971) reportaron que el efecto tóxico del peróxido producido por la muerte celular y la enzima AAAO (enzima amino-ácido oxidasa aromatica) es mitigado por el incremento de la yema de huevo e inhibido por el EDTA (compuestos principales de nuestro diluyente elaborado para oso pardo -Anel et al., 2010-). Además, recientemente, del Olmo et al. (2013) revelaron un efecto sinérgico de la yema de huevo y el EDTA, y concluyeron que estos componentes deberían ser un sostén para la fertilidad de los espermatozoides sexados de cerdo.

Durante el almacenamiento líquido se produce una sedimentación celular en el fondo del tubo, que deriva en cambios deletéreos en el diluente (por ejemplo, fluctuaciones en el pH) e incrementa los productos metabólicos tóxicos en esta región (Conejo: Nagy et al., 2002; carnero: Paulenz et al., 2010). En el cuarto artículo que conforma esta tesis doctoral, evaluamos los efectos del uso de gelatina como medio sólido. El principal problema de manejo que presenta la gelatina es que es sólida en torno a los 20 °C, lo que supone que la dilución final de los electroeyaculados y el envasado en pajuela se deben hacer a temperatura ambiente, modificando el protocolo estándar desarrollado por nuestro grupo (Anel et al., 2008, 2010; de Paz et al., 2012; López-Urueña et al., 2014a); por este motivo evaluamos cuatro grupos experimentales: 1) dilución 1:1 con el diluyente estándar a temperatura ambiente, refrigeración en tubo, dilución final a 5 °C (denominad Control); 2) dilución final con el diluyente estándar a temperatura ambiente, refrigeración en tubo (denominado FD-Tube); 3) dilución final con el diluyente estándar a temperatura ambiente, refrigeración en pajuela (denominado FD-Straw); y 4) dilución final con el diluyente estándar suplementado con un 1,5 % de gelatina, refrigeración en pajuela (denominado Gel). Nuestros resultados mostraron los efectos beneficiosos del uso de gelatina con el tiempo de almacenamiento de espermatozoides de oso pardo; marcando el grupo experimental de gelatina diferencias tanto en precongelación (48 h) como post-descongelación (24 y 48 h), mayor viabilidad y progresividad aunque más baja velocidad que los otros grupos y similar al grupo control de congelación estándar.

Algunos autores han estudiado el efecto del almacenamiento sólido con semen de carnero (Yaniz et al., 2005; Meque et al., 2005; López-Urueña et al., 2012), macho cabrío (Salvador et al., 2005, 2006) y conejo (Nagy et al., 2002;

López-Gatius et al., 2005; Rosato & Iaffaldano, 2011) obteniendo muy buenos resultados, pero en ninguno de esos estudios congelaron las muestras seminales después del almacenamiento a largo plazo; por lo que, se desconoce el efecto de la gelatina en la congelación espermática. Para el análisis precongelación, no encontramos diferencias tras las 24 h de almacenamiento en gelatina, lo que coincide con los resultados de Paulenz et al. (2010) y López-Urueña et al. (2011); otros autores sólo evaluaron características tras las 48 (Rosato & Iaffaldano, 2011) o 72 h (Nagy et al., 2002). Sin embargo, López-Gatius et al. (2005) observaron diferencias en movilidad entre las 0 y las 96 h, donde la suplementación con gelatina incrementó la movilidad y velocidad pero no la calidad de la movilidad (LIN, STR y WOB). Estos resultados no coinciden con los nuestros en semen de oso (valores precongelación, tras 48 h) y en semen de carnero (López-Urueña et al., 2012), ya que nosotros observamos que las muestras suplementadas con gelatina alcanzaron superior movimiento progresivo pero menor velocidad. No existen estudios de congelación tras el almacenamiento sólido, excepto un estudio previo de nuestro grupo de investigación (López-Urueña et al., 2011) que mostró que el lavado, previo a la congelación, para eliminar la gelatina resultó perjudicial para la movilidad y viabilidad, y que no existían diferencias en la calidad post-descongelación de semen de carnero con la suplementación de gelatina, pero el tiempo de incubación a 5 °C fue corto (3 h).

Los efectos positivos de las muestras suplementadas con gelatina puede ser debidos a: 1) la gelatina distribuye uniformemente las muestras seminales, evitando la sedimentación, y así los cambios deletéreos en el diluyente (Nagy et al., 2002; Paulenz et al., 2010); este hecho podría explicar el mantenimiento de la viabilidad incluso tras 48 h respecto al control de congelación estándar o la mejora frente a los otros grupos experimentales. 2) Este estado sólido podría inmovilizar los espermatozoides, reduciendo las demandas metabólicas (López-Gatius et al., 2005); las diferencias entre los patrones de movilidad en las muestras suplementadas con gelatina (descenso de la velocidad aunque buena progresividad) frente a las otras condiciones de manejo o grupos experimentales, parece estar relacionado con esta posible inmovilización. 3) Nagy et al. (2002) concluyeron que la gelatina tenía una ventaja añadida por ser sólida, lo que hace un manejo y transporte de las muestras espermáticas más fácil y seguro.

El principal problema de manejo que presenta la gelatina es que es sólida en torno a los 20 °C, lo que supone que la dilución final de los electroeyaculados y el envasado en pajuela se deben hacer a temperatura ambiente, modificando el protocolo estándar desarrollado por nuestro grupo (Anel et al., 2008, 2010; de Paz et al., 2012; López-Urueña et al., 2014a). En este trabajo, entre los grupos experimentales (quitando el de la gelatina) no se encontraron prácticamente ninguna diferencia, por lo que el oso pardo parece tener una buena tolerancia de

los métodos de dilución (a temperatura ambiente) y refrigeración (en pajuela). Varios autores recomendaron o llevaron a cabo la adición de glicerol a temperatura ambiente (Caballo: Vidament et al., 2005; mono Cynomolgus: Li et al., 2005; guepardo de Namibia: Crosier et al., 2006; búfalo africano: Herold et al., 2006; ocelote y tigrina: Baudi et al., 2008; ciervo rojo: Fernández-Santos et al., 2005; toro: Leite et al., 2010) o tuvieron similares resultados independientemente de la temperatura de adición del glicerol (Caballo: Knop et al., 2005; ciervo rojo: Fernández-Santos et al., 2006; perro: Silva et al., 2006; oso pardo: Alvarez-Rodríguez et al., 2011). Bajo las condiciones de campo, si la adición del glicerol se lleva a cado a temperatura ambiente antes de la refrigeración, en lugar de a 5 °C, se previenen los efectos negativos de los cambios en la temperatura de la muestra durante el transporte (Crosier et al., 2006). En este último trabajo, se evaluaron los efectos de la adición del glicerol en un único o múltiples pasos, la adición del glicerol a temperatura ambiente ó a 5 °C, y la refrigeración en pajuelas o en tubos, en electroeyaculados de guepardo de Namibia. Estas pruebas fueron similares a las nuestras (excepto el grupo experimental de gelatina), y de acuerdo con nuestros resultados, ellos no observaron diferencias entre los grupos testados. De forma similar, Dong et al. (2008) estudiaron el efecto de los métodos de equilibrado en semen de macacos Rhesus, ellos compararon dos grupos experimentales similares a nuestro control (que denominaron "equilibrado con pre-enfriamiento") y el de "FD-Straw" (denominado, "equilibrado sin pre-enfriamiento"). Aunque las muestras seminales equilibradas sin pre-enfriamiento tuvieron ligeramente mayor movilidad, no hubo diferencias significativas. Incluso, hay laboratorios que comercializan diluyentes que recomiendan la dilución final y el envasado en pajuelas a temperatura ambiente, para la crioconservación de semen canino (CaniPlus Freeze, Minitüb, Tiefenbach, Alemania).

En esta tesis doctoral, se han estudiado de forma seriada las condiciones de manejo (aplicación del frío) de muestras seminales de oso en el periodo previo a la congelación (refrigeración y equilibrado), considerando diferentes supuestos prácticos, como son: la recogida en campo (medio silvestre), la conservación seminal hasta la congelación (almacenamiento a corto y largo plazo en diferentes condiciones de refrigeración) y el envío de dichas muestras a centros de referencia para su mejoramiento (sexado o fecundación in vitro) o para inseminación artificial (conexión de las dos subpoblaciones de osos de la Cordillera Cantábrica). El conocimiento exhaustivo de estos pasos nos permite redefinir y optimizar el proceso de congelación en un marco de trabajo no siempre ideal. En este sentido, la refrigeración debe realizarse lentamente (0,25 °C/min); no se puede eliminar la refrigeración ni el tiempo de equilibrado, aunque en este caso existe una amplia flexibilidad (de 0,5 h-mínimo- a 12 h). Consecuentemente, si se elimina el periodo de refrigeración y de equilibrado (muy atractivo para trabajar en campo), aunque

se congele de forma controlada, la calidad espermática postdescongelación será muy baja (inasumible).

El alargamiento del periodo de refrigeración-equilibrado previo a la congelación es posible, observándose una buena calidad post-descongelación hasta las 24 h y un disminución de la misma a las 48 y 72 h. En este nuevo modelo de criopreservación, ideado fundamentalmente para el envío de muestras a centros de referencia, se recomienda almacenar las muestras a 5 °C (sería posible a 15 °C hasta las 24h), con 6 % de glicerol (la tasa de glicerol final a la que se van a congelar) y diluidas (mejor a dilución final cuando el tiempo se alarga más de las 24 h). Finalmente, el uso de un medio sólido (gelatina al 1,5 %) mejora la calidad de las muestras almacenadas a 5 °C respecto al almacenamiento en medio líquido, observándose también una calidad postdescongelación similar entre la congelación estándar (1h-control) y las muestras congeladas después de un almacenamiento en gelatina durante 48 h.

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CONCLUSIONES

1.- La congelación del semen de oso pardo requiere una rampa lenta de refrigeración hasta los 5 °C (máxima de 1 °C/min); la congelación directa (eliminación de los pasos de refrigeración y equilibrado) afecta negativamente a la calidad postdescongelación.

2.- La eliminación del tiempo de equilibrado (0 h) produce una reducción significativa en la calidad postdescongelación de los espermatozoides de oso pardo. La duración de dicho periodo de equilibrado ofrece un amplio margen de tiempo efectivo (entre 0,5 y 12 horas), lo que confiere una alta versatilidad al protocolo de criopreservación espermática en esta especie.

3.- Los eyaculados de oso pardo pueden ser almacenados en precongelacion durante 24 horas a 5 °C sin reducción de la calidad postdescongelación, y con una merma asumible hasta 48 h, hecho que permite su envío a centros tecnológicos de referencia para la aplicación de protocolos de mejora espermática antes de la congelación.

4.- Las mejores condiciones para el almacenamiento precongelación a largo plazo (hasta 48 h) del semen de oso pardo son una temperatura de 5 °C, una concentración de glicerol del 6 % y una tasa de dilución final. La tasa de dilución 1:1 es aceptable si el envío y procesado de la muestra se realiza dentro de las 24 h posteriores a la recogida seminal.

5.- El almacenamiento sólido, mediante la suplementación del diluyente con el 1,5 % de gelatina, tiene un efecto beneficioso en la calidad postdescongelación de espermatozoides de oso pardo almacenados previamente a la congelación hasta 48 horas a 5 °C.

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