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UNIVERSIDAD DE LEÓN

FACULTAD DE VETERINARIA

DEPARTAMENTO DE MEDICINA, CIRUGÍA Y ANATOMÍA VETERINARIA

**MEJORA Y EVOLUCIÓN DE LOS PROTOCOLOS
DE CONGELACIÓN DE EYACULADOS DE OSO**

PARDO (*URSUS ARCTOS*)

**Improvement and optimization of the freezing protocols of brown bear
ejaculated sperm samples (*Ursus arctos*)**

Memoria que presenta para optar al grado de Doctor el Licenciado en Biología.

D. MANUEL ÁLVAREZ RODRÍGUEZ

León, 2012

INFORME DE LOS DIRECTORES DE LA TESIS
(R.D. 99/2011, de 28 de enero y Normativa de la ULE)

Los Doctores D. Luis Anel Rodríguez y D. Paulino de Paz Cabello, como Directores de la Tesis Doctoral titulada *“Mejora y evolución de los protocolos de congelación de eyaculados de oso pardo (*Ursus arctos*)”* realizada por D. Manuel Álvarez Rodríguez en el programa de doctorado 099 Sanidad Animal y Reproducción, en el Departamento de Medicina, Cirugía y Anatomía Veterinaria, informan favorablemente el depósito de la misma, dado que reúne las condiciones necesarias para su defensa.

Lo que firmamos, en León a 14 de DICIEMBRE de 2012.

Fdo.: Dr. Luis Anel Rodríguez

Dr. Paulino de Paz

TESIS DOCTORAL COMO COMPENDIO DE ARTÍCULOS

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ISSN 0936-6768

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PLOS One: Enviado: 12 Diciembre 2012.

FINANCIACIÓN:

CICYT (CGL2007-63748/BOS y CGL 2010-19213/BOS) y CANTUR S.A.

CV MANUEL ÁLVAREZ-RODRÍGUEZ

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Ignorance more frequently begets confidence than does knowledge:
it is those who know Little, not those who know much, who so
positively assert that this or that problem will never be solved by
science.

Charles Darwin, *Introduction to The Descent of Man*, 1871.

A mis padres: Manuel y Emilia

A Laura

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CAPÍTULO 1

RESUMEN

RESUMEN

La situación crítica del oso pardo (*Ursus arctos*) en España plantea la necesidad de aplicar diferentes estrategias de conservación, dentro de las cuales tiene especial relevancia la creación de un banco de recursos genéticos. La eficacia de esta herramienta depende, entre otros factores, de la adaptación específica de los protocolos de congelación espermática ya existentes y que permitan el almacenamiento eficaz de los espermatozoides del oso pardo en un banco de germoplasma.

En la presente tesis doctoral se pretende optimizar un protocolo de congelación de espermatozoides de oso pardo valorando diferentes aspectos relativos al agente crioprotector (glicerol), al uso de alternativas a la yema de huevo (LDL y lecitina de soja), al empleo de la proteína de choque térmico A8 (HSPA8) y al uso de la recongelación como herramienta complementaria de la criopreservación espermática.

En el primer trabajo, estudiamos el efecto de la adición del glicerol (8 %) en diversos momentos del proceso de congelación: 1) antes de la refrigeración (todo el glicerol a temperatura ambiente), 2) después de la refrigeración (todo el glicerol cuando las muestras se encuentran a 5 °C) y 3) en dos pasos: la mitad antes de la refrigeración (se refrigera con 4 % de glicerol) y el resto en la dilución final, una vez alcanzados los 5 °C. No se encontraron diferencias en la calidad espermática entre estos protocolos de adición de glicerol, lo que podría indicar una buena tolerancia del semen de oso pardo al crioprotector. Esta flexibilidad en el manejo del glicerol puede tener su aplicación en la modificación de los protocolos de congelación y, de este modo, podría facilitar la preparación de muestras en el campo.

En el segundo trabajo, analizamos el efecto de diferentes concentraciones de glicerol y su interacción con las rampas de congelación empleadas en la criopreservación de espermatozoides de oso pardo. Las variables de este diseño experimental fueron: 1) concentración de glicerol (2%, 4%, 6%, 8% y 10%) y 2) rampas de congelación (-10 °C/min, -20 °C/min y -40 °C/min hasta -100°C). Los resultados pre-congelación no muestran diferencias en movilidad total y progresiva entre las concentraciones de glicerol empleadas. En cambio, la velocidad curvilínea (VCL) y la amplitud lateral del desplazamiento de la cabeza (ALH) tuvieron valores decrecientes a medida que aumentaba la concentración de glicerol ($p < 0,05$). Tras la descongelación, los parámetros de movilidad mostraron valores más altos para las concentraciones del 4 %, 6 % y 8 % de glicerol. En cambio, la viabilidad espermática y el porcentaje de espermatozoides

con acrosomas intactos presentaron valores superiores para los diluyentes con 4 % y 6 % de glicerol. Las rampas de congelación no tuvieron un efecto significativo para el 4 % y el 6 % de glicerol. El análisis de los datos mediante un modelo lineal nos permitió establecer el 6% como concentración óptima de glicerol, que combinada con una rampa de -20 °C/min, se postula como el protocolo más adecuado para la congelación de espermatozoides de oso pardo.

En el tercer trabajo, realizamos un estudio comparativo de estabilizadores de membrana con el objetivo de sustituir la yema de huevo en los diluyentes de congelación. Las alternativas planteadas fueron las lipoproteínas de baja densidad de yema de huevo -LDL- (5, 10 y 15%) y la lecitina de soja -S- (2, 3,5 y 5%) de dos orígenes (tipo A: L- α -fosfatidilcolina 24%, y tipo B: L- α -fosfatidilcolina 14-23%). Los parámetros de movilidad en la soja tipo A al 5% son similares al control (20% yema de huevo). En cambio, los valores de viabilidad y estatus acrosomal fueron inferiores en todos los tipos y concentraciones de soja estudiados, lo que puede deberse a que la soja no tiene la capacidad protectora que ofrece la yema de huevo durante la congelación o bien a que las concentraciones y/o tipos de lecitina de soja usadas en este estudio no son las más adecuadas. Los diluyentes que contienen 10% y 15% de LDL pueden ser buenos sustitutos de la yema de huevo ya que aumentan de manera significativa la viabilidad de las muestras. Sin embargo, la movilidad se ve reducida en los diluyentes con LDL lo que se interpreta como un efecto diferencial de las LDL sobre los distintos compartimentos del espermatozoides, lo que ya ha sido señalado por otros autores. Por otro lado, los resultados de viabilidad obtenidos concuerdan con el estudio de la capacidad antioxidante de los medios, en el cual encontramos los valores más altos en concentraciones crecientes de los diluyentes con LDL.

En el cuarto trabajo, evaluamos el uso de la proteína de choque térmico A8 (HSPA8) en refrigeración (0, 24, 48 h) y congelación de espermatozoides de oso pardo así como en un test de progresión espermática en ácido hialurónico. Las muestras espermáticas se diluyeron con tres concentraciones de HSPA8 (0,5, 1 y 5 μ g/mL) y un control (0 μ g/mL HSPA8). En el experimento de refrigeración, observamos que la viabilidad mejora respecto al control en el diluyente con 0,5 μ g/mL de HSPA8 para los tres tiempos de muestreo. Asimismo, el porcentaje de acrosomas dañados a las 48 h disminuye significativamente para todas las concentraciones de HSPA8. En el experimento de congelación no se encontraron diferencias significativas en la movilidad ni en la viabilidad espermática, para ninguno de los puntos de muestreo (precongelación,

descongelación e incubación 2 h). En cambio, la permeabilidad de membrana disminuyó en el diluyente con 1 µg/mL de HSPA8 y el porcentaje de acrosomas dañados bajó en presencia de 1 y 5 µg/mL de la proteína; ambos parámetros evaluados después de la incubación a 37 °C durante 2 h. Los resultados del test de migración espermática en ácido hialurónico, mostraron un descenso significativo del número de espermatozoides capaces de avanzar hasta la región del capilar comprendida entre 0,5 y 2 cm para todas las concentraciones de HSPA8. En cuanto a la distancia recorrida por el espermatozoide de vanguardia, se observa una relación lineal creciente dependiente de la dosis. En términos generales, la proteína reduce la motilidad de la población de espermatozoides, pero el espermatozoide de vanguardia parece representar una subpoblación particular cuya motilidad es similar al control, en el diluyente con la más alta concentración de HSPA8. En resumen, podemos recomendar la concentración de 0,5 µg/mL HSPA8 para la refrigeración a 5°C, y la de 1 µg/mL HSPA8 para la congelación de espermatozoides de oso pardo.

Finalmente, en el quinto trabajo analizamos la viabilidad de la recongelación de espermatozoides de oso pardo, procedimiento que permitiría aplicar determinadas biotecnologías (por ejemplo el sex-sorting) a estos biomateriales antes de ser almacenados en los bancos de gamoplasma. Se llevaron a cabo dos experimentos: 1) Valoración inicial de la recongelación espermática y evaluación de diferentes tiempos de espera entre ciclos (30, 90 y 180 min) y 2) Uso de un gradiente de separación espermática (PureSperm®) entre los dos ciclos de congelación-descongelación. En el primer experimento, de manera general, los resultados obtenidos establecen la necesidad de realizar futuros estudios para minimizar las pérdidas de calidad entre el primer y el segundo ciclo (de un 31% en viabilidad y de un 37% en movilidad entre ambas descongelaciones). Por otra parte, no se encontraron diferencias entre los tiempos de espera testados, lo que permitiría un margen para aplicar diferentes técnicas de mejora seminal entre ciclos. En el segundo experimento, las muestras sometidas al gradiente de selección espermática presentan un aumento en la viabilidad y un descenso en el porcentaje de acrosomas dañados con respecto al control. Este resultado nos permite concluir la utilidad de la selección entre ciclos con el fin de mejorar la calidad de los espermatozoides sometidos a recongelación.

CAPÍTULO 2

ABSTRACT

ABSTRACT

The critical situation of the brown bear (*Ursus arctos*) in Spain raises the need of application of different conservation strategies, being particularly relevant the creation of a genetic resource bank. Among other factors, the effectiveness of this tool depends on the specific adaptation of existing sperm freezing protocols, thus enabling an effective long-term storage of bear spermatozoa. Consequently, the present thesis was aimed at design an efficient sperm freezing protocol for brown bear, focusing on finding an optimal use of cryoprotectant (glycerol), the use of alternatives to egg yolk (LDL and soy lecithin), the use of heat shock protein A8 (HSPA8) as supplement and the application of sperm re-freezing as a complementary cryopreservation tool.

In the first study, we studied the effect of the addition of glycerol (8%) at different moments of the freezing process: 1) before cooling (full glycerol concentration at room temperature), 2) after cooling (full glycerol concentration after cooling samples at 5 °C) and 3) in two steps: half glycerol concentration before cooling (cooled with 4% glycerol), and the rest in the final dilution, once the samples reached 5 °C. No differences were found in sperm quality between these protocols, which could indicate good tolerance of brown bear semen to cryoprotectants. This flexibility in managing the glycerol has an immediate application, helping to the modification of freezing protocols, and facilitating sample preparation in the field.

In the second study, we analyzed the effect of different concentrations of glycerol and their interaction with freezing rates used in brown bear sperm cryopreservation. The variables in this experimental design were: 1) concentration of glycerol (2 %, 4 %, 6 %, 8 % and 10 %) and 2) freezing rates (-10 °C/min, -20 °C/min and -40 °C/min) from 5 °C to -100 °C). Pre-freezing analyses showed no differences in total and progressive motility between glycerol concentrations. However, curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH) decreased with increasing glycerol concentration ($p < 0.05$). After thawing, the motility parameters showed higher values for 4%, 6% and 8% glycerol. In contrast, sperm viability and the percentage of spermatozoa with intact acrosomes showed higher values for extenders with 4% and 6% glycerol. Freezing rates were not significantly different for the 4% and 6% glycerol. The analysis of the data by fitting a quadratic model allowed us to establish the optimal concentration closely to 6% glycerol, possibly being better combined with a freezing

rate of $-20\text{ }^{\circ}\text{C/min}$. We propose this protocol as the best one among those tested for brown bear sperm freezing.

In the third study, we carried out a comparative study of membrane stabilizers in order to replace egg yolk in freezing extenders. The alternatives were low density lipoproteins from egg yolk —LDL— (5, 10 and 15%) and soybean lecithin —S— (2, 3.5 and 5%) of two different kinds (type A: L- α -phosphatidylcholine 24%, and type B: L- α -phosphatidylcholine 14-23%). Motility parameters of soybean type A (5%) were similar to the control (20% egg yolk). In contrast, viability and acrosomal status were lower for soybean, irrespective of kind or concentration, which may be caused by decreasing of the protective capacity of soybean during freezing, comparing to egg yolk. We cannot discard that the soybean lecithin —S1 and S2— used in this study were unfitted for brown bear sperm cryopreservation. Diluents containing 10% and 15% of LDL could be good substitutes for the egg yolk, since they increased significantly the viability of the samples. However, motility was reduced in the LDL extenders, which could be interpreted as a differential effect of LDL on the different compartments of the spermatozoon, which has already been noted by other authors. Moreover, the results obtained was consistent with the study of the antioxidant capacity of the extenders, in which we found the highest values in increasing concentrations of LDL.

In the fourth study, we evaluated the use of heat shock protein A8 (HSPA8) for refrigeration ($5\text{ }^{\circ}\text{C}$, 0, 24, 48 h) and freezing of brown bear sperm. We also used a progression test of sperm in hyaluronic acid. The sperm samples were diluted with three HSPA8 concentrations (0.5, 1 and $5\text{ }\mu\text{g/mL}$) and a control ($0\text{ }\mu\text{g/mL}$ HSPA8). In the refrigeration experiment, we observed a viability improvement comparing to the control in the extender with $0.5\text{ }\mu\text{g/mL}$ of HSPA8 for the three sampling times. Likewise, the percentage of damaged acrosomes at 48 h decreased significantly for all concentrations of HSPA8. In the freezing experiment we found no significant differences neither in motility nor sperm viability for any of the sampling points (before freezing, after thawing and 2 h of incubation at $37\text{ }^{\circ}\text{C}$). In contrast, membrane permeability decreased in the extender with $1\text{ }\mu\text{g/mL}$ of HSPA8 and the percentage of damaged acrosomes were lower in the presence of 1 and $5\text{ }\mu\text{g/mL}$ of protein, both parameters evaluated after incubation at $37\text{ }^{\circ}\text{C}$ for 2 h. Results of sperm migration test in hyaluronic acid showed a significant decrease in the number of sperm able to advance across the capillary region between 0.5 and 2 cm for all HSPA8 concentrations. Regarding the distance traveled by the vanguard sperm, there was a dose-response

linear relationship Overall, the protein decreased the population motility of sperm, but the vanguard sperm appears to represent a special sub-population which motility was similar to the control, in the extender with the highest concentration of HSPA8. In summary, we can recommend a concentration of 0.5 µg/mL HSPA8 for refrigeration at 5 ° C and 1 µg/mL HSPA8 for freezing brown bear sperm.

Finally, in the fifth study we analyzed the viability of brown bear sperm refreezing, a procedure that would allow us to apply certain biotechnologies (e.g., sex-sorting) to these biomaterials before being stored in genebanks. We carried out two experiments: 1) initial evaluation and assessment of sperm refreezing at different elapsed times between cycles (30, 90 and 180 min) and 2) use of a gradient for sperm selection (PureSperm®) between the two freeze-thaw cycles. In the first experiment, in general, the results established the need for future studies in order to minimize the loss in quality between the first and second cycle (31% in viability and 37% in motility between the two cycles). Moreover, no differences were found between elapsed times, allowing a margin for applying different techniques on spermatozoa between cycles. In the second experiment, the samples subjected to the selection gradient showed an increase in sperm viability and a decrease in the percentage of damaged acrosomes, respect to the unselected control. Therefore, we conclude that a selection step between cycles could be useful to improve the overall quality of sperm subjected to the refreezing process.

CAPÍTULO 3

INTRODUCCIÓN

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1.1 SITUACIÓN ACTUAL DEL OSO PARDO

El oso pardo (*Ursus arctos*) es una especie no catalogada como en peligro a nivel mundial (**IUCN 2008: Preocupación Menor LC (McLellan et al., 2008)**), debido a su amplia distribución geográfica, sobre todo con numerosa representación en el norte del continente americano y en Asia.

En cambio, la población situada en España, dentro de una pequeña región montañosa en la Cordillera Cantábrica, está dividida en dos subpoblaciones, estimándose un rango de 160 osos en el núcleo occidental y de 30 en el oriental (datos orientativos de la Fundación Oso Pardo (FOP), 2012). Por todo ello, el oso pardo cantábrico está catalogado como en Peligro Crítico (**Real Decreto 439/1990 de la Ley Española, regulada en el Catálogo Nacional de las especies amenazadas**). El ajuste del número anual de osas con crías utilizando un modelo de crecimiento exponencial (**Palomero et al., 2007**) establece una tasa de crecimiento en el núcleo occidental del 7,47% anual (entre 1994 y 2004) y en el oriental no se ha observado ningún crecimiento significativo (entre 1989 y 2004).

Los factores que intervienen negativamente en la conservación del oso pardo cantábrico han sido evaluados por diversos autores, existiendo una coincidencia básica sobre dos de ellos: la caza furtiva y la pérdida de hábitat (**Purroy, 2008**).

Para mejorar estos aspectos se ha desarrollado la Estrategia para la conservación del oso pardo cantábrico (**Comisión Nacional de Protección de la Naturaleza (2001)**), centrada en los siguientes objetivos: a) reducir el número de bajas generadas por el furtivismo; b) conservar y mejorar el hábitat; c) asegurar la conectividad entre poblaciones y núcleos de población; y, d) garantizar el apoyo público a la conservación de la especie.

Los bancos de recursos genéticos (BRG) (colección de espermatozoides, óvulos, embriones y células somáticas en suspensión biológica temporal) constituyen una herramienta importante en la conservación de especies amenazadas. Estos BRG permiten el establecimiento de un depósito de material biológico cuya gestión, manejo y estudio tiene como fin ayudar a la conservación de las especies.

La creación de BRG exige la optimización de protocolos específicos para esta especie, con el fin de preservar el material genético en las mejores condiciones. La conservación de este material genético, junto al desarrollo de los estudios en la hembra,

permitirían a medio plazo su utilización para la aplicación de técnicas de reproducción asistida *in vivo*. Por tanto, la adaptación de los protocolos de conservación del semen junto con el desarrollo y aplicación de técnicas de reproducción asistida, constituye una herramienta útil en el establecimiento de los BRG, que debe ser usada en la conservación de especies amenazadas (**Holt and Lloyd, 2009**).

Un aspecto importante en este apartado de la conservación *ex situ*, corresponde a la correcta evaluación de la calidad de las muestras de un BRG, con el fin de asegurar un alto rendimiento en los fines que persigue. Las técnicas desarrolladas para otras especies han permitido el análisis de múltiples parámetros. Así, los avances en citometría de flujo (**Martinez-Pastor et al., 2010**) han permitido la obtención de un número elevado de observaciones respecto a la microscopia de fluorescencia, así como la utilización de tinciones combinadas. De este modo, han sido evaluadas con éxito para el semen de oso varias tinciones, lo que ha permitido una valoración mucho más objetiva de los parámetros de calidad espermática como la viabilidad, el estatus acrosomal, cambios apoptóticos, etc. Esta valoración tiene especial relevancia en especies como el oso pardo, en las que la realización de pruebas de fertilidad *in vivo* son prácticamente inviables debido a su complejidad (manejo, nº de animales, etc.).

1.2 CRIOPRESERVACIÓN ESPERMÁTICA

Un paso obligado para establecer protocolos de conservación seminal en el oso sería la adaptación de los protocolos ya utilizados en especies cercanas, permitiendo el diseño de un protocolo óptimo para esta especie. Son muchos los puntos del proceso de conservación que se deben revisar y/o modificar para conseguir dicho fin, algunos de los cuales son abordados en esta memoria.

El objetivo final del proceso de adaptación, es conseguir protocolos de alta fiabilidad teniendo en cuenta el alto valor estratégico de todas y cada una de las muestras procesadas. En este sentido, la centrifugación de las muestras antes de ser criopreservadas permitiría recuperar el máximo posible de espermatozoides para su posterior utilización. **Nicolás et al. (2012a)** establecen, para oso pardo, unos parámetros óptimos de centrifugación de $2.400\times g$ durante 6 min, con el fin de evitar efectos dañinos del proceso sobre la calidad de las muestras descongeladas. Por otro lado, la calidad espermática del pellet obtenido tras este proceso de centrifugación puede ser incrementada mediante la dilución previa a la centrifugación de un hasta 1:8 con un diluyente Tris-cítrico-glucosa (**Nicolás et al., 2011**).

Uno de los aspectos más relevantes de un protocolo de criopreservación espermática es la elección del diluyente. Ésta ha de constituir, no sólo un entorno similar al del plasma seminal en cuanto a características físico-químicas (pH, presión osmótica, etc), sino que también ha de paliar el efecto perjudicial de los procesos a los que se ve sometido el espermatozoide durante su manipulación en las distintas etapas del protocolo de conservación espermática. Respecto a la presión osmótica del diluyente utilizado, tenemos que tener en cuenta además la procedencia de la muestra espermática, ya que nuestro grupo (**Anel et al., 2010**) ha definido 300 mOsm/kg como la presión osmótica adecuada a la congelación de muestras electroeyaculadas en esta especie. En cambio, en un estudio llevado a cabo con muestras epididimarias (**Anel et al., 2011**), la presión osmótica recomendada fue de 430 mOsm/kg, lo que concuerda con lo descrito para células epididimarias en otras especies (ovino: **Tamayo-Canul et al., 2011**; ciervo: **Martínez-Pastor et al., 2006**).

Los diluyentes de congelación que tienen que mantener una osmolaridad y un pH adecuados para la muestra tratada, también deben presentar la capacidad de proteger a las células de los daños inducidos por el frío. Por ello, es conveniente la realización de diseños experimentales que permitan evaluar cada componente del diluyente por separado, así como combinaciones específicas que permitan definir el protocolo óptimo para la especie. En este sentido, **Anel et al. (2010)** proponen específicamente para la congelación de espermatozoides oso pardo un diluyente básico definido en 300 mOsm/kg, con 20% de yema de huevo y un rango del 4-8% de glicerol.

Con el fin de desarrollar un diluyente óptimo, además de los aspectos generales ya mencionados hemos de tener en cuenta otros problemas específicos como por ejemplo el de la aglutinación espermática que se presenta en numerosas especies, incluido el oso pardo (**Anel et al., 2008**). Este problema aumenta la dificultad del manejo de la muestra y puede influir en la congelabilidad espermática. Una de las posibles opciones para minimizar este problema es la adición al medio de un agente surfactante (Equex paste) y un agente quelante de calcio (EDTA). Existen numerosos estudios en diversas especies en los que se demuestra una mejoría en la calidad de las muestras espermáticas con la incorporación de estos aditivos al diluyente de congelación: perro (**Rota et al., 1997**), gato (**Axner et al., 2004**), cerdo (**Pursel et al., 1978**), caballo (**Martin et al., 1979**), toro (**Arriola et al., 1987**), ciervo (**Cheng et al., 2004**) y oso pardo (**Anel et al., 2010**). **Anel et al. (2010)** estudiando el posible efecto de estos dos aditivos, comprueban un

efecto positivo a la descongelación por lo que proponen la utilización de 1% Equex paste y 2% EDTA en el diluyente de congelación de espermatozoides de oso pardo.

Otro punto fundamental en el proceso de criopreservación espermática, es el estudio de la velocidad de refrigeración y congelación espermática, debido al distinto comportamiento que pueden presentar las células espermáticas de especies diferentes. Por una parte, si el proceso de congelación es muy rápido, el agua no puede salir de la célula de forma adecuada como para mantener el equilibrio de solutos necesario para la supervivencia espermática. En cambio, la salida de agua en el caso de una rampa lenta de congelación es muy acusada, hecho que puede causar daño celular (**Mazur, 2010**). En cualquier caso, un análisis específico de especie sobre la naturaleza de los daños inducidos por el proceso de congelación en los espermatozoides debe ser realizado con el objetivo de definir el protocolo de congelación más adecuado (**Woods et al., 2004**). En este sentido, **Anel et al. (2010)** describen un enfriamiento lento (0,25 °C/min hasta 5 °C) y una rampa de congelación de -20 °C (hasta -100 °C) como un protocolo adecuado para los espermatozoides del oso pardo. Estos mismos autores sugieren un protocolo de descongelación durante 6 segundos a una temperatura de 65 °C.

1.2.1 CRIOPROTECTORES

Uno de los componentes más estudiados de los diluyentes de criopreservación es el crioprotector empleado. Si bien el glicerol ha sido a lo largo de los años el principal crioprotector utilizado en criopreservación espermática (**Watson, 1990**), la naturaleza permeable del mismo causa toxicidad a altas concentraciones, siendo este efecto citotóxico variable en función de la especie. Este hecho hace necesario el estudio específico, en espermatozoides de oso pardo, de diferentes concentraciones de glicerol, con el fin de establecer un equilibrio entre la mínima concentración útil para preservar la integridad espermática y la máxima concentración que produciría toxicidad celular.

En cuanto al momento de adición de glicerol al diluyente (antes o después del proceso de refrigeración o una combinación de ambos), este estudio permitiría definir, por un lado, el daño que es producido durante el proceso de refrigeración de las muestras y, por otro lado, el daño que sufren las células en el proceso de congelación, ambos procesos causados por el efecto tóxico intrínseco al glicerol. Existen numerosos estudios en otras especies y, si bien ciertos autores concluyen el beneficio de la adición del glicerol antes del proceso de refrigeración (caballo, **Vidament, 2005**; ciervo rojo, **Fernandez-Santos et al., 2005**; humano, **Deppe et al., 2004**), otros autores establecen que la adición del glicerol después de la refrigeración (ovino, **Gil et al., 2003**; cerdo,

Almlid et al., 1988; perro, **Peña et al., 1998**) es más beneficiosa para la criopreservación espermática, con lo que una vez más parece observarse un componente específico en la respuesta al manejo del agente crioprotector.

En la criopreservación de células espermáticas de úrsidos se han empleado distintas concentraciones de glicerol con escasos estudios comparativos: en panda gigante -*Ailuropoda melanoleuca*- el rango se encuentra entre el 5% (**Spindler et al., 2004**) y el 6% (**Perez-Garnelo et al., 2004**); en oso pardo de Hokkaido -*Ursus arctos yeoensis*- la concentración final utilizada fue del 4,7% (**Ishikawa et al., 2002**); y en oso negro japonés (*Ursus thibetanus japonicus*), 8% (**Okano et al., 2004, 2006 a**). En eyaculados de esta última especie y comparando un rango entre el 4 y el 12% de glicerol, **Okano et al. (2006 b)** definen el 8% como la concentración más adecuada. En oso pardo, **Anel et al. (2010)** no encontraron diferencias entre el 4 y el 8% de glicerol en cuanto a la calidad espermática post-descongelación.

Otro factor que influye sobre la congelación espermática es la velocidad de congelación. Además del propio efecto que el glicerol ejerce sobre la muestra, conviene estudiar las posibles interacciones de diferentes concentraciones del crioprotector combinadas con el empleo de diferentes velocidades de congelación. Una combinación efectiva de estos parámetros (concentración de glicerol y rampa de congelación) podría ayudar a minimizar los efectos negativos asociados a los diferentes tipos de estrés a los que se ven sometidas las células tanto en el proceso de refrigeración (**Muldrew et al., 2008**), como en el proceso de congelación espermática (**Salamon and Maxwell., 2000**).

1.2.2 ESTABILIZADORES DE MEMBRANA

El efecto general de estos componentes reside en la protección que ofrecen a las células durante el proceso de criopreservación espermática. Se piensa que actúan sobre la estructura o composición de las membranas celulares y que son capaces de mantener la movilidad, viabilidad e integridad acrosomal de los espermatozoides tras la descongelación (**Salamon and Maxwell, 2000**).

Uno de los estabilizadores de membrana más utilizados en diferentes especies es la yema de huevo, siendo mayoritario su uso en los trabajos publicados en diferentes especies de úrsidos: oso pardo de Hokkaido (**Okano et al., 2004, 2006a, 2006b**), oso panda gigante: (**Spindler et al., 2004**). En un estudio previo de nuestro grupo, **Anel et al. (2010)** establecen la concentración del 20% como la más adecuada para la congelación de espermatozoides de oso pardo obtenidos mediante electroeyaculación.

A pesar de sus efectos beneficiosos contrastados, la yema de huevo puede presentar una serie de desventajas como: a) la heterogeneidad de las diferentes partidas de yema, b) la dificultad para una correcta estandarización del método de obtención y c) el origen animal de este componente, relacionado con aspectos de bioseguridad. Además, la propia yema de huevo puede provocar efectos deletéreos en los espermatozoides tanto en la integridad acrosomal (**Holt et al., 1996**) como en la viabilidad espermática (**Ritar et al., 1991**). Estos efectos perjudiciales de la yema como componente de los diluyentes espermáticos han sido ampliamente estudiados, afectando no sólo a la inhibición de la respiración de los espermatozoides sino también en la reducción de su movilidad (**Pace and Graham, 1974; Watson and Martin, 1975; Almirat et al., 2004**)

Un primer posible sustituto para la yema de huevo completa, sería una fracción de la misma compuesta por las lipoproteínas de baja densidad (LDL), las cuales pueden facilitar la elaboración de diluyentes definidos químicamente, evitando de esta manera los efectos perjudiciales de ciertos componentes de la yema de huevo. Las LDL constituyen uno de los principales componentes de la acción crioprotectora de la yema de huevo según un estudio de **Pace y Graham (1974)** en el que aislaron LDL de la yema de huevo mediante ultracentrifugación y obtuvieron buenos resultados en la conservación de semen de toro. Otros estudios más recientes en diversas especies han confirmado esos mismos resultados. En ovino, **Moustacas et al. (2011)** obtuvieron mejoras significativas en las muestras congeladas con LDL en comparación a la yema de huevo. **Varela Junior et al. (2009)**, en un trabajo publicado sobre semen de perro, observaron una mejora significativa de los parámetros postdescongelación en las muestras criopreservadas con diluyentes de LDL. **Vera Munoz et al., 2009** compararon dos diluyentes comerciales (Triladyl® -yema de huevo- y Bioxcell® -lecitina de soja-) y otro con LDL en muestras espermáticas de toro, obteniendo valores de movilidad e integridad de membrana superiores en el diluyente con LDL respecto a los otros dos diluyentes. Asimismo, **Stradioli et al. (2007)** comprobaron la eficacia de las LDL en la reducción de los daños inducidos por la congelación en las muestras espermáticas de toro. **Hu et al., 2008** en un estudio realizado en cerdo, obtuvieron mejores valores de calidad espermática postdescongelación en las muestras conservadas en diluyentes con LDL con respecto al control. Posteriormente, estos mismos autores, (**Hu et al., 2011**), observaron que los diluyentes que contenían LDL, además de mostrar los mejores porcentajes post-descongelación de movilidad, daño acrosomal e integridad de membrana, eran capaces de mantener, de manera más eficaz, la actividad de algunas

enzimas antioxidantes (CAT, GSH-Px y GSH). Esta preservación de la actividad enzimática puede, indirectamente, permitir la reducción del impacto de las especies reactivas del oxígeno, formadas tanto al durante el proceso de refrigeración como en la congelación.

Un segundo estabilizador de membrana de origen vegetal, la lecitina de soja, ha sido estudiado en diversas especies: ovino (**de Paz et al., 2010**); toro (**Ricker et al., 2006**, **Stradioli et al., 2007**); equino (**Aurich et al., 2007**). **Ricker et al. (2006)** describieron el mecanismo de estabilización de la membrana espermática de toro. Estos autores observaron la presencia de agregados lipídicos en la membrana y el efecto protector en movilidad, viabilidad y fertilidad ejercido por los diluyentes con lecitina de soja como estabilizador de membrana. Actualmente existen varios diluyentes comerciales que incluyen en su composición la lecitina de soja y que han sido ensayados en distintas especies con resultados dispares (Biociphos Plus[®]: toro (**Muiño et al., 2007**), toro (**van Wagendonk-de Leeuw et al., 2000**) y Botu-Crio[®]: caballo (**Papa et al., 2011**), tapir (**Pukazhenthil et al. 2011**)).

1.3 ADICIÓN DE PROTEÍNAS OVIDUCTALES

La supervivencia de los espermatozoides en el tracto genital femenino ha sido objeto de numerosos estudios y se ha observado una interacción entre el espermatozoide y las células del oviducto que parece desencadenar la producción de una familia específica de proteínas presuntamente responsables del aumento de la supervivencia en el tracto femenino. Estas proteínas denominadas HSPA8 (Heat Shock Proteins A8) pertenecen a una gran familia de proteínas (HSP70) que ha sido bien conservada a través de la evolución y está presente en numerosos grupos animales (**Daugaard et al., 2007**).

La familia de las HSP70 (70kDa) presenta numerosas funciones entre las que se incluyen el transporte, la modificación e incluso la degradación de proteínas. La naturaleza conservada de esta proteína en mamíferos sugiere que puede estar involucrada en un mecanismo biológicamente común para el mantenimiento de la supervivencia espermática en el oviducto. No obstante, su origen no se limita exclusivamente al epitelio oviductal, sino que se ha descrito la presencia de proteínas de la familia HSP70 en localizaciones diferentes dentro del propio espermatozoide en diversas especies como el cerdo, el caballo, el perro y el carnero (**Volpe et al., 2008**). Estas proteínas pueden ser sintetizadas en respuesta a un aumento de temperatura (**Tsunekawa et al., 1999**) o, en general, a otras posibles situaciones de estrés a las que

se puede ver sometido el espermatozoide durante el período de almacenamiento en el tracto femenino. Algunas proteínas de esta familia (HSPA8) han sido analizadas para evaluar el efecto protector que producen en la conservación de espermatozoides de diversas especies como cerdo (39 °C/24 h) y toro (37 °C/48 h) (**Elliott et al., 2009**) y ovino (5 °C y 38 °C hasta 48 h) (**Lloyd et al., 2009**), comprobándose que la adición de dichas proteínas en los diluyentes espermáticos incrementaba el tiempo de supervivencia de los espermatozoides.

Por lo tanto, las proteínas HSPA8, derivadas de las interacciones espermatozoide-oviducto, son capaces de mantener *in vitro* la viabilidad de las muestras espermáticas (**Elliott et al. 2009; Lloyd et al., 2009**). Estos resultados, bien debidos a una posible interacción con receptores de membrana o bien mediante la estabilización de ciertas estructuras de la membrana del espermatozoide, permiten pensar en la posible utilidad de su aplicación en los procesos de criopreservación espermática esperando obtener el mismo efecto protector que cuando actúan a temperaturas altas.

1.4 RECONGELACIÓN ESPERMÁTICA

El uso de dos o más ciclos de congelación-descongelación ha sido aplicado en varias especies de animales domésticos con el objetivo de optimizar los recursos genéticos disponibles, reduciendo costes de almacenamiento (**Saragusty et al., 2009**) o bien para permitir aplicar una clasificación X-Y de espermatozoides mediante citometría de flujo (**Maxwell et al., 2007**).

Existen diversos estudios de recongelación espermática en los que se demuestra la eficacia de los espermatozoides recongelados en cuanto a la fertilidad final obtenida (ovino: **Hollinnshead et al., 2004; de Graag et al., 2007; Morton et al., 2006**; toro: **Arav et al., 2002**; caballo: **McCue et al. (2004)**). En este sentido, **Choi et al. (2006)** llevaron a cabo un estudio de ICSI en caballo empleando semen recongelado y obtienen unos resultados de desarrollo embrionario temprano que no presentan diferencias respecto a un semen control sometido a un sólo ciclo de congelación.

Uno de los fines fundamentales de la recongelación consistiría en permitir el sexado de espermatozoides entre ciclos de congelación-descongelación (**Vazquez et al., 2009**). Esta técnica requiere un coste muy alto tanto de instalaciones como de mantenimiento y en consecuencia existen pocos laboratorios disponibles para su aplicación. Por ello es habitual que se deban enviar los biomateriales desde el punto de recogida en el campo a un laboratorio especializado que ofrezca este servicio. El envío de muestras

refrigeradas, desde la fuente de gametos hasta el laboratorio de referencia puede resultar muy perjudicial para los espermatozoides aún considerando un control exhaustivo de la temperatura de almacenamiento así como el máximo tiempo recomendado para el manejo de las muestras. Este proceso de refrigeración supone una gran limitación logística para el manejo de las muestras y por lo tanto, la recongelación espermática sería la respuesta lógica ya que permitiría resolver uno de los principales problemas de este tipo de transporte: la caducidad de las muestras espermáticas.

Por otro lado, debido a las pérdidas de calidad que se observan en el proceso de recongelación, sería conveniente la utilización de gradientes de selección espermática. Su uso entre dos ciclos de congelación consecutivos podría mejorar la calidad de las muestras, seleccionando de manera efectiva la población con mayor movilidad (**Nicolas et al., 2012b**). Esta selección permitiría el aumento de la eficacia del proceso de sexado espermático, ya que aseguraríamos una mayor proporción de espermatozoides vivos tras el proceso de separación de espermatozoides X e Y.

1.5 PROPUESTAS DEL PRESENTE TRABAJO DE TESIS

El presente trabajo de tesis se plantea optimizar un protocolo adaptado de criopreservación de espermatozoides de oso pardo, mediante el estudio del glicerol (porcentaje y modo de adicción y las rampas de congelación), el uso de sustitutivos de la yema de huevo en los diluyentes (LDL y soja), el empleo de la proteína de choque térmico HSPA8 en el mantenimiento o mejora de la calidad espermática, tanto en pre y post-descongelación así como en refrigeración, además de un primer acercamiento a al proceso de recongelación espermática como un procedimiento criobiológico que permita aplicar técnicas de sexado a las muestras de semen de oso pardo.

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CAPÍTULO 4

OBJECTIVOS

OBJETIVOS

El objetivo general del presente trabajo de tesis doctoral es la mejora y evolución de los protocolos existentes para la criopreservación espermática de muestras de oso pardo obtenidas mediante electroeyaculación.

Los objetivos específicos que se plantean en la presente tesis son los siguientes:

1. Evaluar el empleo del glicerol en los extender de criopreservación espermática.

1ª publicación: pautas de adición del glicerol.

2ª publicación: concentraciones de glicerol

2. Estudiar alternativas a los componentes clásicos de los extender de criopreservación espermática.

3ª publicación: empleo de LDL de yema de huevo y lecitina de soja como alternativas a la yema de huevo.

4ª publicación: adición de proteínas de choque térmico A8 (HSPA8) en la refrigeración y congelación de espermatozoides.

3. Valorar las posibilidades de uso de la re congelación espermática como modelo de conservación que permite la aplicación diferida de técnicas de mejora seminal.

5ª publicación: efecto de dos ciclos consecutivos de congelación sobre la calidad espermática.

CAPÍTULO 5

OBJECTIVES

OBJECTIVES

The general objective of this thesis project is the improvement and development of existing protocols for sperm cryopreservation of brown bear samples obtained by electroejaculation.

The specific objectives posed in this thesis are:

1. The evaluation of the use of glycerol in sperm cryopreservation extenders.

1st publication: patterns for the addition of glycerol.

2nd publication: glycerol concentrations

2. The evaluation of alternatives to classical components of sperm cryopreservation extenders.

3rd publication: LDL egg yolk and soybean lecithin as alternatives to egg yolk.

4th publication: addition of the heat shock proteins A8 (HSPA8) for the refrigeration and freezing of spermatozoa.

3. The evaluation of sperm refreezing as a conservation proposal for allowing the deferred application of sperm work techniques.

5th publication: effect of two consecutive freezing-thawing cycles on sperm quality.

CAPÍTULO 6

**PUBLICACIÓN 1. QUALITY OF FROZEN-THAWED SEMEN IN
BROWN BEAR IS NOT AFFECTED BY TIMING OF GLYCEROL
ADDITION**

Technical note

Quality of frozen-thawed semen in brown bear is not affected by timing of glycerol addition

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Received 10 September 2010; received in revised form 29 November 2010; accepted 6 December 2010

Abstract

We have tested several freezing protocols for brown bear semen, modifying the time when glycerol was added (before and after cooling to 5 °C). No differences were found among protocols, indicating a good tolerance of brown bear semen to glycerol. This finding indicates that freezing protocols for brown bear semen could be modified to fit practical solutions which would facilitate preparation of the seminal samples in the field with the addition of glycerol at ambient temperature.

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Keywords: Brown bear; Semen; Glycerol; Freezing

1. Introduction

Genome Resource Banks (GRB) are a valuable support for wild species conservation, but their application requires the optimization of cooling and freezing protocols. We are currently working on the conservation of Cantabrian brown bear (*Ursus arctos*) in Spain, which is seriously endangered due to habitat loss and population fragmentation, and is a suitable target for the application of GRBs [1].

One of the critical steps in the cryopreservation of a semen sample is the choice of an adequate cryoprotectant, its optimal concentration and the method of adding it during the freezing protocol to minimize the osmotic and toxic effects induced by cryoprotectant on

the cells. Glycerol has been one of the most widely applied cryoprotectant additives for mammalian spermatozoa and it has been used successfully to freeze bear spermatozoa (giant panda [2], Hokkaido brown bears [3] and Japanese black bears [4–6]). Different protocols of glycerol addition have been reported in these studies. Thus, giant panda spermatozoa were cryopreserved adding glycerol at room temperature, immediately before slow cooling [2]. Ishikawa et al [3] reported freezing semen from Hokkaido brown bears using serial dilutions, adding 1/10, 2/10, 3/10 and 4/10 of 14% extender at 4 °C, achieving a final concentration of 4.7%. Okano et al [4–6] cryopreserved semen from Japanese black bears at different final concentrations, diluting extended semen at 4 °C, adding the same volume of extender with twice the final glycerol concentration. However, these results do not show which is the most effective method of addition in the manage-

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ment of bear semen. To our knowledge, there are no previous studies on when or the method to be used for adding glycerol to the extended semen of brown bear.

In other species, many studies have been carried out on the protocol of addition of glycerol, some concluding that glycerol should be added after cooling (4–5 °C), for example in sheep [7], pig [8] and dog [9], whereas others recommend adding it at room temperature, before cooling the samples (stallion [10], red deer [11] and human [12]). Other authors have obtained the same quality regardless of when glycerol was added (stallion [13], red deer [14] and dog: [15]).

Work with endangered population often has to be carried out in the field. This includes collecting and freezing semen, therefore, the protocols are conditioned by this need. Thus, the aim of the present study was to find the most suitable protocol of glycerol addition at a final concentration of 8% for freezing brown bear semen to facilitate the preparation of the semen in the field, keeping the semen quality in these difficult conditions.

We tested three variants: 1) reaching half of the final concentration at ambient temperature, completing to final concentration after cooling to 5 °C; 2) adding all the glycerol at ambient temperature, reaching the final concentration [2]; and 3) adding all the glycerol after cooling to 5 °C [3–6]. Our starting hypothesis was that if the method of glycerol addition does not affect the sperm quality, it could be added at ambient temperature, thus making preparation of the seminal sample in the field easier.

2. Materials and methods

All the chemicals were obtained from Sigma (Madrid, Spain), except Equex STM Paste (Minitüb, Tiefenbach, Germany). Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD223/1998 (European Union Regulation 86/609).

2.1. Animals and sample collection

We used 17 fresh semen samples, collected from 16 adult brown bears (between 7 and 20 years old) in seventeen electroejaculation sessions, during the breeding season (late April to early July). The animals were housed in a half-freedom regimen in Cabárceno Park (Cantabria, Spain; 43° 21' N, 3° 50' W, altitude: 142 metres), and fed with a varied diet.

The animals were immobilized by intramuscular administration of Zolacepan HCl, Tiletamine HCl

(Zoletil1001; Virbac, Carros, France) 7 mg/kg and ketamine (Imalgene 10001; Rhone-Mérieux, Lyon, France) 2 mg/kg applied by teleanaesthesia and were monitored in order to control their general status under anaesthesia. The pubic region and the penis were cleaned, the rectum was emptied of stools and the bladder was emptied by catheterization and electroejaculation was carried out with a PT Electronics electroejaculator (PT Electronics, Boring, OR, USA) using a transrectal probe 320 mm in length and 26 mm in diameter. Electric stimuli were given until ejaculation (10 V and 250 mA, in average) and ejaculates were collected in 15-mL glass tubes at 30–32 °C.

2.2. Experimental design and semen evaluation

Samples obtained by electroejaculation were centrifuged immediately to remove seminal plasma (600×g, 6 min). The pellet was divided in three aliquots depending on the moment of glycerol addition: PRE (before cooling to 5 °C), POST (after cooling to 5 °C) and HALF (half of the final concentration at ambient temperature, completing to final concentration after cooling to 5 °C). First, each aliquot was diluted with the same volume of TTF extender (TES-Tris–Fructose 300 mOsm/kg, pH 7.1, with 20% egg yolk, 2% EDTA and 1% Equex STM paste) at ambient temperature with 16% glycerol (PRE), 8% glycerol (HALF) and 0% glycerol (POST) according to treatment. The tubes with extended sample were put in glass vessels containing 100 mL of water at ambient temperature and transferred to a 5 °C refrigerator. After reaching 5 °C, the same volume of extender was added to the POST and HALF tubes (16% and 8% glycerol, respectively) to reach the final glycerol concentration (8%). Final sperm concentration was achieved by adding the appropriate volume of extender to achieve a final glycerol content of 8%). After packaging into 0.25 mL plastic straws, the sample is immediately cooled for 1 h at 5 °C, samples were frozen in a programmable biofreezer (Kryo 560-16; PLANER plc, Sunbury, UK) at -20 °C/min down to -100 °C, and then transferred to liquid nitrogen containers. The cryopreserved samples remained in liquid nitrogen for a minimum of 1 week. Thawing was performed by plunging the straws in water at 65 °C for 6 s. After thawing, the samples were subjected to 4 h incubation at 37 °C. The semen samples were evaluated before freezing and after thawing (at 0 h and 4 h post-incubation to check for latent damage to the sperm).

Motility parameters were evaluated using a com-

Table 1

Pre-freezing (PRE-F) and post-thawing parameters (Sperm motility and viability) of seventeen ejaculates from Cantabrian Brown Bears for the three groups of glycerol dilution PRE, HALF and POST (mean $\pm$ standard error). PRE (at room temperature before refrigeration), HALF (up to half the final concentration at room temperature and the final concentration at 5 °C) and POST (5 °C, after refrigeration). Frozen-thawed sample was assessed immediately after thawing (POST 0 h) and after 4 h of incubation (POST 4 h).

Parameter	Sample	Post	Half	Pre
TM (%)	PRE-F	77.4 $\pm$ 3.8	79.4 $\pm$ 3.7	77.4 $\pm$ 4.1
	POST 0 h	53.0 $\pm$ 3.2	49.9 $\pm$ 4.2	48.0 $\pm$ 4.4
	POST 4 h	27.4 $\pm$ 7.3	23.1 $\pm$ 8.0	25.6 $\pm$ 4.8
PM (%)	PRE-F	38.2 $\pm$ 3.2	32.8 $\pm$ 3.6	36.2 $\pm$ 4.2
	POST 0 h	22.1 $\pm$ 2.5	18.0 $\pm$ 3.3	18.7 $\pm$ 3.4
	POST 4 h	9.1 $\pm$ 2.3	7.8 $\pm$ 3.0	9.8 $\pm$ 2.0
VAP (μ m/s)	PRE-F	77.0 $\pm$ 2.7	70.8 $\pm$ 2.8	76.6 $\pm$ 1.8
	POST 0 h	63.8 $\pm$ 2.5	57.4 $\pm$ 4.0	60.3 $\pm$ 5.2
	POST 4 h	30.2 $\pm$ 3.0 ^a	27.6 $\pm$ 3.5 ^{a,b}	26.5 $\pm$ 3.4 ^b
LIN (%)	PRE-F	46.8 $\pm$ 2.5	45.9 $\pm$ 2.2	46.8 $\pm$ 2.1
	POST 0 h	37.4 $\pm$ 0.9	36.8 $\pm$ 1.2	36.0 $\pm$ 1.4
	POST 4 h	31.8 $\pm$ 1.3	33.8 $\pm$ 1.4	35.8 $\pm$ 2.6
iACR (%)	PRE-F	74.3 $\pm$ 5.6	77.3 $\pm$ 5.1	74.3 $\pm$ 5.3
	POST 0 h	62.9 $\pm$ 3.0	63.5 $\pm$ 3.3	64.2 $\pm$ 3.4
	POST 4 h	49.1 $\pm$ 8.9	43.8 $\pm$ 9.9	44.9 $\pm$ 9.7
dACR (%)	PRE-F	3.0 $\pm$ 0.6	3.3 $\pm$ 0.9	4.2 $\pm$ 1.1
	POST 0 h	10.3 $\pm$ 2.3	10.2 $\pm$ 1.8	10.5 $\pm$ 2.1
	POST 4 h	19.2 $\pm$ 3.9	17.5 $\pm$ 3.2	18.8 $\pm$ 2.9

CASA Analyses: Total motility (TM; %), Progressive motility (PM; %), Average velocity (VAP; μ m/s), Linearity (LIN; %).

Flow Cytometric Analyses: Viable spermatozoa with intact acrosomes (iACR: PNA-/PI-) and spermatozoa with damaged acrosomes (dACR: PNA+/PI- and PNA+/PI+).

^{a,b} Superscript letters indicate significant differences among groups of glycerol dilution ($P < 0.05$).

puter assisted sperm analysis system (CASA) (ISAS (Integrated Semen Analyser System), Proiser, Valencia, Spain). Samples were diluted ($10\text{--}20 \times 10^6$ cells/mL) in buffered medium (HEPES 20 mm/L, 197 mmol/L NaCl, 2.5 mmol/L KOH, 10 mmol/L glucose, 1% egg yolk; pH 7; 300 mOsm/kg), and warmed on a 37 °C plate for 5 min. Then, 5 μ L were placed in a Makler counting cell chamber (10 μ m deep; Sefi Medical Instruments, Haifa, Israel). The sample was examined at $\times 10$ (negative phase contrast) in a microscope with a warmed stage (38 °C). The standard parameter settings were set at 25 frames/s, 20 to 90 μ m² for head area and VCL > 10 μ m/s to classify a spermatozoon as motile. At least five sequences or 200 spermatozoa were saved and analyzed afterwards.

Sperm viability and acrosomal status were assessed using the double stain PNA-FITC/PI (peanut agglutinin/propidium iodide) and flow cytometry. Sperm samples were diluted with PBS down to 5×10^6 spermatozoa/mL in conical polypropylene tubes (300 μ L/tube), staining with PI and 0.8 μ g/mL PNA-FITC. Samples were read with 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, obtaining the percentage of viable spermatozoa with intact acrosomes (PI-

and PNA-, termed iACR) and the percentage of spermatozoa with damaged acrosomes (PNA+, termed dACR).

2.4. Statistical analysis

Data were analyzed using SASTM v. 9.1 (SAS Institute Inc., Cary, NC, USA), by linear mixed-effects models (MIXED procedure) considering samples as a random effect. Results were expressed as the mean $\pm$ SEM (standard error of the mean).

3. Results and discussion

The fresh semen samples of the brown bears studied in this experiment had a volume of 1.6 ± 0.8 mL (mean $\pm$ SD), a pH of 8.5 ± 0.5 , an osmotic pressure of 315.7 ± 86.3 and a sperm concentration of $296.3 \pm 76.4 \times 10^6$ spermatozoa/mL.

Studies in dog, a carnivore like the brown bear, have indicated that glycerol should be added at 4 °C after cooling [16,17]. This procedure avoids the exposure of sperm to the cryoprotectant at ambient temperature, decreasing the damage sperm undergo before freezing. Conversely, Silva et al [15] concluded that glycerol could be added both at room temperature (27 °C) and at

4 °C, without detrimental effects. These results are supported by studies showing that, although canine spermatozoa are sensitive to dehydration, they were able to tolerate shrinking and swelling (osmotic stress) during exposure to hypertonic solutions of glycerol [18].

The results of our study showed no significant differences between the three protocols at 0 h, both pre-freezing and post-thawing (Table 1). The incubation of the samples at 37 °C post-thawing for 4 h did not show any differences among protocols, except for sperm velocity (VAP), which was decreased when the final concentration of glycerol was achieved before refrigeration (PRE), in comparison with POST treatment, but no differences were observed between PRE and HALF. This finding suggests a possible negative effect of glycerol, due to a longer exposure of cells to the cryoprotectants during post-thaw incubation.

Most spermatozoa freezing protocols that have been tested for sperm of Ursidae mammalian family, glycerol has been added after cooling and thus, spermatozoa are in contact with glycerol only during the equilibration time at 4 °C [5–8]. The implications of species variability in both the beneficial and detrimental effects of glycerol are worth considering in this respect [19]. The cryoprotective action of glycerol depends on its permeability coefficient that is likely to be different among species, since it depends on the structure and composition of the membrane. No studies have been carried out to evaluate the permeability of glycerol in brown bear spermatozoa and therefore we can not assess the extent of osmotic stress in these cells. In this sense, we speculate that low VAP of spermatozoa exposed to glycerol at room temperature may be due to toxic effects rather than osmotic effects.

In conclusion, we did not detect any significant effect on sperm motility or viability in post-thawing analysis regarding the protocol of glycerol addition, suggesting that bear spermatozoa are resistant to the addition of glycerol at different temperatures. Therefore, freezing protocols for brown bear semen may be flexible in this step, which may be convenient in field conditions, when appropriate infrastructure might not be available.

Acknowledgments

This work was supported in part by CICYT (CGL 2007-63748/BOS) and CANTUR S.A.

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CAPÍTULO 7

**PUBLICACIÓN 2. OPTIMIZATION OF GLYCEROL
CONCENTRATION AND FREEZING RATE IN THE
CRYOPRESERVATION OF EJACULATE FROM BROWN BEAR
(URSUS ARCTOS)**

Optimization of Glycerol Concentration and Freezing Rate in the Cryopreservation of Ejaculate From Brown Bear (*Ursus arctos*)

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Contents

In order to establish a semen bank for the endangered Cantabrian brown bear, we tested five glycerol concentrations and three freezing rates for electroejaculated semen. Electroejaculation was performed on nine males. Semen was diluted in TES–Tris–Fructose (20% egg yolk, 2% EDTA, 1% Equex) with 2%, 4%, 6%, 8% or 10% glycerol and frozen at –10, –20 or –40°C/min. Before and after cryopreservation, samples were analysed for motility (CASA), viability and acrosomal status (flow cytometry). Pre-freezing results showed that glycerol concentration had no significant effect on total motility or progressive motility, but it significantly decreased VCL, ALH, viability and acrosomal status ($p < 0.05$). After thawing, sperm motility was higher at extender with 4%, 6% and 8% glycerol, but only at 4% and 6% glycerol for viability and acrosomal status. For 4% and 6% glycerol, freezing rates did not have significant effects. The curve fitting gave an estimate of the optimal glycerol concentration, with all the optimal values for every parameter between 6% and 7% glycerol falling. We propose using 6% glycerol and a freezing velocity of –20°C/min for freezing brown bear ejaculated spermatozoa.

Introduction

Specific adaptation of freezing protocols in wild species could improve strategies to establish and manage Genome Resource Banks (GRB). GRBs, in combination with assisted reproductive techniques, can be used to support the conservation of endangered species (Holt and Lloyd 2009). This is the case of the Cantabrian brown bear (*Ursus arctos*). The species is not catalogued as threatened worldwide (Least Concern, IUCN 2008), because of large populations in Russia, Canada and some regions in Europe. However, the Iberian population (currently limited to Northern Spain) is seriously endangered because of isolation and high vulnerability (Anel et al. 2008) and is considered to be at risk of extinction (Real Decreto 439/1990 of the Spanish law, regulation of the National Catalogue of Endangered Species).

A critical step in the cryopreservation of a sample is the choice of an adequate cryoprotectant and its optimal concentration. Glycerol has been one of the most widely applied cryoprotectant additives for spermatozoa (Watson 1990), being successfully used in mammals (Pegg 2002). With regard to Ursidae, Giant panda (*Ailuropoda melanoleuca*) spermatozoa have been cryopreserved using 5% (Spindler et al. 2004) and 6% (Perez-Garnelo et al. 2004) glycerol. Ishikawa et al. (2002) reported using 4.7% glycerol for freezing ejaculated semen from Hokkaido brown bears (*Ursus arctos yezoensis*). Okano et al. (2004, 2006a) cryopreserved semen from Japanese black bears (*Ursus thibetanus japonicus*) at a final glycerol concentration of 8%. However, in other

experiments with this species, Okano et al. (2006b) tested different concentrations of glycerol at between 4% and 12%, to evaluate its effects on semen cryopreservation, obtaining the best results [viability (VIAB) and intact acrosomes] using 4% and 6% glycerol. In a preliminary study on brown bear semen (*U. arctos*), Anel et al. (2010) did not detect any differences among samples cryopreserved using 4% or 8% glycerol.

Freezing rate is another relevant aspect in semen cryopreservation. Cells are subjected to different kinds of stress depending on the freezing rate at which they are cooled Muldrew (2008). The optimal freezing rate depends on many factors affecting the osmotic behaviour of the cell, the most important being the cryoprotectant media and the pre-freezing protocol. Thus, an optimal freezing method must define the appropriate combination of these parameters (Woelders et al. 1997; Gao and Critser 2000; Fernández-Santos et al. 2006). If cells are cooled too rapidly, water cannot exit from the cell fast enough to maintain a balance of solutes. Conversely, if the cells are cooled too slowly, most of the water inside them exits and this may cause cell injury. The optimal freezing rate would dehydrate cells with minimal impact, while preventing intracellular ice growth (Woods et al. 2004). Studies on bears usually report freezing rates as height above liquid nitrogen (LN), being difficult to compare results. Thus, Okano et al. (2004, 2006a,b) reported freezing Japanese black bear semen by placing straws 6 cm above LN, not reporting freezing rates. Ishikawa et al. (2002) used a variable height system (30, 4 and 2 cm above LN, at 2 min intervals) for freezing Hokkaido brown bear sperm. These authors measured the freezing rate provided by that system, being –9.8°C/min from 4 to –7.5°C, and –46.6°C/min from –7.5 to –100°C. Although sperm VIAB was good after thawing, motility was only 21% compared with 55% of the pre-freezing value.

In this study, we implemented five different glycerol concentrations (2%, 4%, 6%, 8% and 10% v/v) and three different freezing rates (–10, –20 and –40°C/min) to define the optimal values for the species. Moreover, as the factors involved in a successful freezing protocol usually interact, we aimed at seeking the best combination of both factors for freezing brown bear semen.

Materials and Methods

All the products were obtained from Sigma (Madrid, Spain), except Equex STM Paste (Minitüb, Tiefenbach, Germany).

Animals and sample collection

Fifteen ejaculates from nine brown bears were obtained by electroejaculation during the breeding season (late April to early July, 2008–2009). Animal age was estimated, so all the captured males were adults (between 7 and 20 years). The animals were housed in a half-freedom regime in Cabárceno Park (Cantabria, Spain; 43°21'N, 3°50'W, altitude: 142 m) and fed with a diet based on chicken meat, bread and fruits; feeding followed the hierarchical order, with the dominant bears taking most food. Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65.

The males were immobilized by teleanaesthesia, using zolazepam HCl, tiletamine HCl (Zoletil100®; Virbac, Carros, France) 7 mg/kg, and ketamine (Imalgene 1000®; Rhone-Mérieux, Lyon, France) 2 mg/kg. After immobilization, they were weighed and monitored (pulse, oxygen saturation and respiration). Prior to electroejaculation, the prepuce area was shaved and washed with sterile physiological saline, and the rectum was emptied of faeces. The bladder was catheterized during semen collection to prevent urine contamination. Electroejaculation was carried out with a PT Electronics® electroejaculator (PT Electronics, Boring, OR, USA). The transrectal probe was 320 mm long with a diameter of 26 mm. Electric stimuli were given until ejaculation (10 V and 250 mA, on average). The average duration of the procedure of electroejaculation is 10 min 34 s (5 min 49 s from the start of the process until ejaculation begins + 4 min 45 s of ejaculation). The ejaculates were collected as isolated fractions in graduated glass tubes to prevent urine contamination or low cellular concentration. Fractions of reduced concentration ($< 200 \times 10^6$ cell/ml), low motility ($< 50\%$) or urine contaminated (> 80 mg urea/dl) were rejected as explained in the following paragraphs. Two ejaculates were obtained by bear (one per year). Of the 18 ejaculates obtained from nine bears, three have been eliminated for failing to provide the minimum quality. All valid fractions of the same ejaculate were mixed (fifteen ejaculates in experiments).

Experimental design

The selected ejaculates were centrifuged at $600 \times g$ for 6 min for reaching an adequate sperm concentration and cleaning urine-contaminated samples (Nicolas et al. 2011). The supernatant was discarded, and each pellet was diluted with an equal volume of TTF cryopreservation extender (TES–Tris–Fructose, supplemented with 4% glycerol, 20% egg yolk, 2% EDTA and 1% Equex STM paste; 300 mOsm/kg, pH 7.1) at room temperature. Tubes with the diluted semen (at 2% glycerol) were put in glasses containing 100 ml of water at room temperature and transferred to refrigerated containers at 5°C, so the temperature decreased slowly to 5°C (70–80 min). Then, the samples were split among five tubes, and each tube was diluted 1 : 1 with an extender containing 2%, 6%, 10%, 14% or 18% glycerol to achieve the final glycerol concentrations (2%, 4%, 6%, 8% and 10%, respectively). Final sperm

concentration (100×10^6 cell/ml) was achieved by adding the appropriate volume of the extender at the same glycerol concentration.

An equilibration period of 1 h at 5°C was applied, during which the semen was packaged into 0.25-ml plastic straws. The straws were frozen in a programmable biofreezer (Kryo 560-16 Planer™, Middlesex, UK) at each of the three ramps tested (–10, –20 or –40°C/min), down to –100°C, and then transferred to LN containers. The cryopreserved samples were stored in LN for a minimum of 1 week. The straws were thawed by plunging them into water at 65°C for 6 s. Sperm quality parameters were evaluated before freezing and after thawing at room temperature.

Semen evaluation

Immediately after collection, for each fraction, volume was recorded, osmolality was measured using a cryoscopic osmometer Osmomat-030 (Gonotec™, Berlin, Germany) and the pH value was determined by a CG 837 pH meter (Schott Instruments, Main, Germany). Sperm concentration was assessed (Bürker hemocytometer, Marienfeld GmbH, Marienfeld, Germany) using Computer Assisted Sperm Analysis (CASA) (ISAS, Integrated Semen Analyser System; Proiser, Valencia, Spain). Subjective total motility was assessed with a phase contrast microscope ($\times 100$) and urospermia was evaluated by means of a Merckgnost Urea Rapid Screening test (Merck, Barcelona, Spain).

The motility and kinematics parameters were evaluated using a computer-assisted semen motility analysis system (ISAS, Integrated Semen Analyser System; Proiser). The standard settings of the CASA used correspond to the values of dog spermatozoa defined by ISAS. The settings used to define progressive motility are specific to bears and are defined by our previous experience (Anel et al. 2010). Samples were diluted ($10\text{--}20 \times 10^6$ cells/ml) in a buffer (HEPES 20, 197 mM NaCl, 2.5 mM KOH, 10 mM glucose; pH 7; 300 mOsm/kg) with 1% egg yolk, and warmed on a 37.5°C plate for 5 min. Then, a 5 μ l sperm sample was placed in a Makler counting cell chamber (10 μ m depth; Sefi Medical Instruments, Haifa, Israel) and examined using a negative phase contrast microscope ($\times 10$) with a warmed stage (38°C). The standard settings were 25 frames/s, 5–80 μm^2 for head area and curvilinear velocity > 10 $\mu\text{m/s}$ to classify a spermatozoon as motile. At least five fields or 200 spermatozoa were saved and analysed afterwards. Reported parameters were total motility (TM, %; spermatozoa with VCL > 10 $\mu\text{m/s}$), progressive motility (PM, %; spermatozoa were considered progressive if VCL > 25 and STR > 80), average path velocity (VAP, $\mu\text{m/s}$), curvilinear velocity (VCL, $\mu\text{m/s}$), straight-line velocity ($\mu\text{m/s}$), linearity (%) and amplitude of lateral head displacement (ALH, μm).

Sperm viability and acrosomal status were assessed using the double stain PNA-FITC/PI and flow cytometry. Stock solutions of the fluorochromes were prepared in PBS at 1 $\mu\text{g/ml}$ PNA-FITC and 24 μM PI. Sperm samples were diluted with PBS down to 5×10^6 spermatozoa/ml in polypropylene tubes (300 $\mu\text{l/tube}$),

adding 2.5 μ l PI and 2.5 μ l PNA-FITC to the diluted samples. PNA is a lectin from the peanut plant that binds to beta-galactose moieties associated with the outer acrosomal membrane of fixed spermatozoa, indicating acrosome-damaged cells. The flow cytometry analysis yielded the percentage of viable spermatozoa with intact acrosomes (PI- and PNA-, named VIAB) and the percentage of those with damaged acrosomes (PNA+, named dACR).

Flow cytometry evaluations were carried out using a FACScalibur flow cytometer (Becton Dickinson Bio-Sciences, 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 (Calibres; 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.

Statistical analysis

Statistical analysis was performed in the R statistical environment (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0). Results are shown as means and standard errors, unless otherwise stated. Analyses of the pre-freezing data (only glycerol effects) and post-thawing data (glycerol and freezing rate effects) were carried out using linear mixed-effects models. Models were built with glycerol, freezing rate and their interaction as fixed effects (for pre-freezing data, only glycerol), and sample (with interactions) in the random part of the model. Significant fixed effects were further analysed using multiple comparisons of means with Tukey contrasts; when interactions between glycerol and the freezing rate were significant, these factors were assessed separately by grouping the data by the other factor.

As we expected to find an optimal concentration of glycerol within the range tried, we tested whether the parameters could fit a quadratic linear model, yielding a theoretical maximum for a given glycerol concentration. This test was carried out using linear models with glycerol as factor.

We calculated post-thawing recovery rates as a way of assessing sample freezability in each glycerol/freezing rate combination. These rates were calculated by dividing the post-thawing value of each parameter by its pre-freezing value and multiplying by 100.

Results

The pre-freezing evaluation of the samples showed some differences among glycerol concentrations (Fig. 1). The glycerol concentration did not have a significant effect on total or progressive motility, but it did affect VCL, ALH, VIAB and dACR ($p < 0.05$). Increasing the glycerol concentration from 2% to 10% in the equilibration phase caused the kinematic parameters VCL and ALH to drop by almost 25%. Glycerol also affected sperm VIAB and acrosomal status, and 10% glycerol caused a significant decrease in VIAB in comparison with 6% glycerol.

After thawing (Fig. 2), the effect of glycerol was highly significant for the motility parameters ($p < 0.001$). In general, the lower concentration of 2% yielded lower motility values ($p < 0.05$). The 6% concentration gave higher average results for motility, being significantly higher than 2% and 10%. Curve fitting analyses (Fig. 3) showed that the data for all motility parameters could be fitted to a quadratic curve, showing a maximum of between 6% and 7% glycerol. Also, VIAB was fitted to a quadratic linear model (maximum at 6%), while dACR showed a minimum value at 2.9% glycerol.

In contrast to the clear effects of glycerol concentration, the freezing rate had little effect on post-thawing

Fig. 1. Boxplot of pre-freezing sperm parameters for the five glycerol concentrations. TM, total motility; PM, progressive motility; VCL, curvilinear velocity; ALH, mean amplitude of the lateral head displacement; VIAB, viable spermatozoa with intact acrosomes (PNA-/PI-), dACR: damaged acrosomes (PNA+). (—) Median and (●) Mean; (□) interquartile range; (┘) non-outlier range and (○) outliers. Different letters indicate that means differ between glycerol concentrations ($p < 0.05$)

Fig. 2. Boxplot of post-thaw sperm parameters for the five glycerol concentrations. TM, total motility; PM, progressive motility; VCL, curvilinear velocity; ALH, mean amplitude of the lateral head displacement; VIAB, viable spermatozoa with intact acrosomes (PNA-/PI-), dACR, damaged acrosomes (PNA+). (—) Median; (•) Mean; (□) interquartile range; (▮) non-outlier range and (○) outliers. Different letters indicate that means differ between glycerol concentrations ($p < 0.05$)

Fig. 3. Curve fitting for total motility (TM), curvilinear velocity (VCL), viable spermatozoa with intact acrosomes (VIAB) and spermatozoa with damaged acrosomes (dACR), with glycerol concentrations (quadratic model). The plots show data points with the model curve overlotted. The maximum values for each parameter corresponded to glycerol concentrations of 6.88% (TM), 6.21% (VCL), 6.19% (VIAB), and a minimum value for dACR at 2.9% glycerol

motility (Fig. 4). Only $-10^{\circ}\text{C}/\text{min}$ yielded significantly lower results on progressive motility in comparison with $-20^{\circ}\text{C}/\text{min}$.

For VIAB and dACR, the effect of glycerol depended on the freezing rate used as can be seen in Table 1. Glycerol at 2% rendered the lowest VIAB values for the three freezing ramps, whereas 6% glycerol showed the highest values, although these differences were not significant. Using 8% or 10% glycerol, $-10^{\circ}\text{C}/\text{min}$ yielded significantly higher VIAB than $-40^{\circ}\text{C}/\text{min}$. The interaction between glycerol and the freezing rate was clearly evident in the dACR parameter, where the increasingly lower performance of the highest concentrations, especially 10%, when the freezing velocity is increasing can be observed. Thus, for 2% and 4% glycerol, we observed greater damage to the acrosome at $-10^{\circ}\text{C}/\text{min}$ than at $-20^{\circ}\text{C}/\text{min}$ ($p < 0.05$), whereas for 8% and 10% $-40^{\circ}\text{C}/\text{min}$ yielded higher acrosomal damage. In all cases, 8% and 10% glycerol rendered significantly higher percentages of dACR than the other glycerol concentrations.

Analysis of recovery rates (Table 2) showed that only glycerol had an effect on these rates, thus

samples frozen with 2% glycerol had poorer freezability in terms of maintaining motility parameters or cell VIAB during the freezing/thawing process. In fact, freezing with 6% glycerol yielded the higher mean freezability in motility and VIAB, although the recovery rates of 4% and 8% were not significantly different in any case. Only freezability for dACR showed an interaction between the effects of glycerol and the freezing speed. The recovery rates of dACR (Table 3) were higher (increased acrosomal damage during freezing) with glycerol at 8% and 10% only when the freezing ramp was $-40^{\circ}\text{C}/\text{min}$. In general, for any freezing ramp, the recovery rates of dACR were higher with glycerol at 8% and 10%, although these differences did not reach significance for -10 and $-20^{\circ}\text{C}/\text{min}$.

Discussion

We have evaluated five glycerol concentrations and three freezing ramps for improving the cryopreservation of brown bear semen. Our results seek to optimize the protocol for spermatozoa cryopreservation of this

Fig. 4. Boxplot of post-thaw sperm parameters for the three freezing rates (–10; –20; –40°C/min). TM, total motility; PM, progressive motility; VCL, curvilinear velocity; ALH, mean amplitude of the lateral head displacement; VIAB, viable spermatozoa with intact acrosomes (PNA–/PI–), dACR, damaged acrosomes (PNA+). (—) Median; (●) Mean; (□) interquartile range; (⊠) non-outlier range and (○) outliers. Different letters indicate that means differ between freezing ramps ($p < 0.05$)

Table 1. Effects of interactions between glycerol concentration (GLY) and the freezing rates (RATE) on post-thaw spermatozoa quality (Means $\pm$ SEM): viability (VIAB, PI–/PNA–) and damaged acrosomes (dACR, PNA+)

GLY	RATE	VIAB	dACR
2	10	35.5 $\pm$ 3.5 ^A	6.8 $\pm$ 0.6 ^{Aa}
2	20	33.9 $\pm$ 4.4 ^A	5.6 $\pm$ 0.6 ^{Ab}
2	40	42.3 $\pm$ 2.8 ^A	4.7 $\pm$ 0.3 ^{Ab}
4	10	58.9 $\pm$ 3.9 ^{BC}	8.4 $\pm$ 0.7 ^{Aa}
4	20	60.3 $\pm$ 4.1 ^{BD}	6.3 $\pm$ 0.6 ^{Ab}
4	40	62.3 $\pm$ 4.1 ^B	5.6 $\pm$ 0.4 ^{Ab}
6	10	66.7 $\pm$ 2.8 ^B	8.3 $\pm$ 0.7 ^A
6	20	64.5 $\pm$ 3.8 ^B	7.9 $\pm$ 0.8 ^A
6	40	65.8 $\pm$ 3.8 ^B	7.9 $\pm$ 0.8 ^A
8	10	58.1 $\pm$ 3.1 ^{BCa}	11.9 $\pm$ 0.8 ^{Bab}
8	20	54.4 $\pm$ 3.8 ^{CDab}	11.9 $\pm$ 1.1 ^{Ba}
8	40	49.5 $\pm$ 3.8 ^{Ab}	14.6 $\pm$ 1.1 ^{Bb}
10	10	51.1 $\pm$ 3.8 ^{Ca}	18.5 $\pm$ 1.6 ^{Ca}
10	20	50.8 $\pm$ 3.5 ^{Ca}	16.4 $\pm$ 1.6 ^{Ba}
10	40	39.8 $\pm$ 4.1 ^{Ab}	23.9 $\pm$ 1.7 ^{Cb}

Capital letters indicate significant differences among glycerol concentrations for each freezing rate ($p < 0.05$) and lower-case letters indicate significant differences among freezing ramps for each glycerol concentration ($p < 0.05$).

species tested in previous studies (Okano et al. 2004, 2006a,b; Anel et al. 2010).

One of the objectives of this study was to analyse the differential effect of extreme glycerol concentrations (2% and 10%), in comparison with the optimal range of 4–6% suggested by other authors (Okano et al. 2006b). Cryoprotectants must be used balancing their cryoprotective action with their toxicity (Pegg 2002). Previous studies in wildlife by our group suggested that red deer spermatozoa might tolerate a wide range of glycerol concentrations (Martinez-Pastor et al. (2006). Pukazhenthi et al. (2007) reported that Eld's deer showed good acrosomal resistance, whereas cheetah or black-footed ferret showed high acrosomal sensitivity to the freezing/thawing process. Our pre-freezing results showed a damaging effect of 10% glycerol on acrosomes

Table 2. Recovery rates [(Post-thaw/pre-freeze value) $\times$ 100] of the samples cryopreserved with the five glycerol concentrations (GLY) and the three freezing rates (RATE), showed as mean $\pm$ SEM

	TM	PM	VCL	ALH	VIAB
GLY*					
2	56.1 $\pm$ 4.3 ^a	52.1 $\pm$ 4.9 ^a	66.8 $\pm$ 3.3 ^a	81.6 $\pm$ 5.5 ^a	39.1 $\pm$ 2.3 ^a
4	79.9 $\pm$ 3.3 ^b	72.4 $\pm$ 6.2 ^b	81.4 $\pm$ 3.9 ^b	97.1 $\pm$ 6.9 ^a	67.6 $\pm$ 2.7 ^b
6	87.8 $\pm$ 3.5 ^b	85.3 $\pm$ 7.5 ^b	86.5 $\pm$ 3.5 ^{bc}	105.4 $\pm$ 6.4 ^b	77.4 $\pm$ 1.9 ^c
8	83.7 $\pm$ 3.4 ^b	75.5 $\pm$ 5.7 ^b	92.4 $\pm$ 3.9 ^c	117.8 $\pm$ 7.7 ^b	66.8 $\pm$ 16.5 ^{bd}
10	77.4 $\pm$ 3.6 ^b	63.8 $\pm$ 4.1 ^{ab}	93.5 $\pm$ 3.6 ^c	116.9 $\pm$ 6.2 ^b	55.3 $\pm$ 3.4 ^d
Rate*					
10	74.1 $\pm$ 2.9	64.4 $\pm$ 4.3	82.2 $\pm$ 3.3	102.6 $\pm$ 5.6	63.4 $\pm$ 2.7
20	78.7 $\pm$ 3.3	77.8 $\pm$ 5.4	84.9 $\pm$ 3.2	104.8 $\pm$ 5.6	63.1 $\pm$ 2.7
40	78.3 $\pm$ 2.9	67.6 $\pm$ 4.1	85.4 $\pm$ 2.7	104.2 $\pm$ 4.8	57.6 $\pm$ 2.4

*There were no interactions between the GLY and RATE factors.

TM, total motility; PM, progressive motility; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; VIAB, viable spermatozoa with intact acrosomes.

Lower-case letters indicate significant differences among glycerol concentrations ($p < 0.05$), and capital letters indicate significant differences among freezing rates ($p < 0.05$).

and plasmalemma, possibly because of osmotic effects, which are also reflected in diminished kinetic parameters. Although glycerol protects the spermatozoa during the freezing/thawing process, the osmotic stress associated with glycerol addition could be the cause of the decreasing quality in the pre-freezing analysis for the highest glycerol concentrations (Gao et al. 1995). Province et al. (1984) reported that 6% glycerol, not 3%, depressed sperm motility during long storage of canine spermatozoa at 5°C, whereas this effect was not observed for equine semen. These results resemble ours in bear, because VCL and ALH were higher in 2% glycerol, decreasing when the glycerol concentration was increased, and showing some kind of depressing effect on the motility. Moreover, we have to take into account that our freezing protocol included two dilution steps involving increasing the glycerol concentration. The first one occurred at ambient temperature after

RATE	GLY				
	2	4	6	8	10
10	417.7 ± 50.7 ^A	389.6 ± 77.1 ^A	442.2 ± 87.4 ^A	447.2 ± 71.2 ^{Bab}	593.6 ± 116.9 ^{Ca}
20	312.3 ± 56.6 ^A	305.2 ± 69.4 ^A	384.1 ± 77.7 ^{AB}	476.2 ± 128.1 ^{BCa}	521.4 ± 101.9 ^{Ca}
40	311.2 ± 51.3 ^A	327.8 ± 64.8 ^A	458.9 ± 116.8 ^A	687.4 ± 170.3 ^{Bb}	919.2 ± 181.2 ^{Cb}

*(Post-thaw/pre-freeze value) × 100.

Capital letters indicate significant differences among glycerol concentrations ($p < 0.05$), and lower-case letters indicate significant differences among freezing ramps ($p < 0.05$).

Table 3. Recovery rates* for damaged acrosomes (dACR, PNA+) of the samples cryopreserved with the five glycerol concentrations (GLY) and the three freezing rates (RATE), shown as mean ± SEM

centrifugation of the samples, achieving a 2% glycerol concentration. The second step occurred after cooling at 5°C, achieving the final glycerol concentration. Therefore, samples with 2% glycerol actually avoided a second osmotic challenge when adding higher concentrations of glycerol. However, as equilibration only lasted for approximately 1 h, it is possible that glycerol-induced irreversible effects may not have taken place yet, except for the highest concentration of 10%. This potential damaging effect of glycerol at 5°C should be taken into account in future studies, especially dealing with longer equilibration times or extended cooled storage; this may be necessary because the sampling area in wildlife is often far from the laboratories where the ejaculates are processed. The addition of glycerol in various steps has been applied by Ishikawa et al. (2002). These authors froze semen from Hokkaido brown bears using a final glycerol concentration of 4.7% (adding extender with 14% glycerol in various steps every 20 min during cooling) and using a multi-step freezing procedure. They obtained a post-thaw mean total motility of above 35% (90% in fresh spermatozoa), much lower than those obtained in the present study.

Despite the good pre-freezing results achieved by the samples with 2% glycerol, post-thawing analysis showed that its cryoprotecting ability was too low. The effects of cryo-injuries on spermatozoa are complex and may result from two main factors: osmotic stress and intracellular ice formation. Cells cannot survive these impacts without special measures of protection (Holt (2000a)). As glycerol prevents the formation of intracellular ice, it would be logical that 2% glycerol showed better results combined with the slower freezing rate (−10°C/min), as a slow freezing rate allows cells to achieve greater dehydration before reaching the ice nucleation temperature, thus reducing the risk of intracellular ice formation, therefore compensating the lack of cryoprotectant. However, our results showed that the −40°C/min freezing rate improved the freezability of samples frozen in 2% glycerol. We hypothesize that with a low glycerol concentration, damage resulting from osmotic stress or salt precipitation would be more important than damage from intracellular ice Muldrew (2008), thus achieving better results from faster freezing ramps.

Conversely, results obtained with 10% glycerol were worsened by fast freezing (−40°C/min). As the observed effects were increasing membrane and acrosomal damage, fast freezing may have created the conditions for enhanced cellular osmotic stress during the fast thawing procedure (Curry and Watson 1994; Woods et al. 2004).

Another possibility is that high glycerol concentrations could have caused sub-lethal damage during the equilibration phase, not observed in the pre-freezing analysis, but which could have prevented a subpopulation of spermatozoa from enduring the freezing/thawing process, especially when applying a faster freezing rate (greater chance for intracellular ice formation).

Curve fitting gave an estimate of the optimal glycerol concentration, and all of the optimal values for every parameter were between 6% and 7% glycerol. Nevertheless, we observed that semen motility does not vary significantly in the range of 4–8% for the three freezing rates analysed, coinciding with those observed by Okano et al. (2006b) and Anel et al. (2008).

Nevertheless, although 8% glycerol was found to render good results regarding motility, this glycerol concentration induces significant damage to the acrosome, and therefore, it seems that osmotic stress, a likely explanation for the acrosomal damage, exceeds other cryoprotective benefits. The optimal glycerol concentration might vary depending on the experimental protocol and species. In giant panda (*A. melanoleuca*), Spindler et al. (2004) used 5% glycerol (added before cooling), obtaining a total motility of 55% after thawing (from above 70% in fresh spermatozoa), which is more similar to our results. In fact, these authors tested a fast freezing rate (−40°C during the first minute and −100°C/min afterwards), reporting that neither acrosomal status nor capacitation status was affected. Thus, if glycerol concentration is almost optimal, the freezing rate might be a secondary factor in post-thawing quality.

Nevertheless, we have to take into account that we have used only one thawing rate in our experiment. Because a successful cryopreservation protocol depends as much on the thawing method as on the freezing method (Hammadeh et al. 2001), we cannot discard that different results could appear with a different thawing rate.

In any case, it must be highlighted that our curve fitting analyses did not consider the male factor. Thus, future studies should be aimed at analysing within and between male variability regarding glycerol tolerance. In fact, customized freezing protocols are beginning to be considered in domestic species, in order to cryopreserve spermatozoa from valuable males whose freezability differs from the general population, or in species with a high male-to-male variability in terms of sperm freezability (Chaveiro et al. 2006; Loomis and Graham 2008). Male-to-male variability in wildlife is possibly higher than in most farm animals, and therefore, specific studies are needed to establish standardized protocols

for those species (Holt 2000b; Estes et al. 2006; Zee et al. 2009). Therefore, the study of such variability and the adjustment of protocols to individual males must not be forgotten in the search for adequate cryopreservation variables for each species.

In conclusion, the optimal glycerol concentration for freezing brown bear spermatozoa obtained by electroejaculation was between 4% and 8%. Concentrations outside that range are either not enough to protect spermatozoa in the freezing/thawing process (2%) or toxic (10%). Freezing ramps in the range studied here (−10 to −40°C/min) might be of lesser importance for the outcome of sperm cryopreservation if glycerol is used within the aforementioned optimal range, but the rate of −10°C/min have negative results. Future studies should refine these results considering male-to-male variability in the response to freezing variables, in order to define the most adequate protocols for cryopreserving brown bear electroejaculated spermatozoa. We therefore propose a glycerol concentration of 6% glycerol and a freezing speed of −20°C/min for freezing brown bear spermatozoa.

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Submitted: 22 Feb 2011; Accepted: 21 Apr 2011

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CAPÍTULO 8

PUBLICACIÓN 3. THE ANTIOXIDANT EFFECTS OF SOYBEAN LECITHIN- OR LOW-DENSITY LIPOPROTEIN-BASED EXTENDERS FOR THE CRYOPRESERVATION OF BROWN BEAR (URSUS ARCTOS) SPERMATOZOA

The antioxidant effects of soybean lecithin- or low-density lipoprotein-based extenders for the cryopreservation of brown-bear (*Ursus arctos*) spermatozoa

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Abstract. Egg yolk low-density lipoproteins (LDL) and soybean lecithin were evaluated as replacements for egg yolk in extenders used for the cryopreservation of brown-bear spermatozoa. The motility, viability and acrosomal status of post-thawed spermatozoa were analysed, and an egg-yolk extender was used as a control. The total antioxidant capacity of these extenders was tested. Soybean lecithin showed an effect that was dependent on the soybean concentration (2%, 3.5% and 5%) and source (Type A: 24% L- α -phosphatidylcholine, and Type B: 14–23% L- α -phosphatidylcholine). Only semen cryopreserved with 5% Type A soybean exhibited a sperm motility similar to that of semen cryopreserved in egg-yolk-based extender after thawing, although the sperm viability and acrosome status were not as high. Semen frozen in an extender containing LDL (10–15%) exhibited improved sperm viability in comparison with the control, but sperm motility was lower. The LDL-based extender exhibited a higher anti-oxidant activity than the egg-yolk extender and soy lecithin-based extenders. The extenders with higher anti-oxidant activity showed improvements in frozen sperm viability but lower semen motility. These results indicate that soybean lecithin did not have the same protective effect as egg yolk during the freezing of brown-bear spermatozoa but suggest that LDL (10–15%) could be a useful substitute for egg yolk in these extenders.

Additional keywords: anti-oxidant activity, egg-yolk LDL, sperm motility, viability.

Received 8 June 2012, accepted 16 November 2012, published online xx xxxxx xxxx

Introduction

The Cantabrian brown bear (*Ursus arctos*) in Spain is seriously endangered (Anel *et al.* 2008) and it is considered to be at risk of extinction (Real Decreto 439/1990 of the Spanish law, regulation of the National Catalogue of Endangered Species). The optimisation of freezing protocols for the spermatozoa of wild species would help to improve strategies to establish and manage Genome Resource Banks (GRB) that support efforts to conserve endangered species (Holt and Lloyd 2009).

Egg yolk is commonly used in sperm cryopreservation media because of its protective effects against cold shock, particularly with respect to the preservation of sperm motility and the maintenance of acrosomal integrity (Salamon and Maxwell 2000). Egg yolk has routinely been included, albeit at different concentrations (e.g. from 5% to 20% for Cuvier's gazelle semen; Garde *et al.* 2008), in the majority of extenders for frozen semen from domestic animals and wild species. The

cryopreservation of bear spermatozoa has always been carried out with egg-yolk-containing extenders. Ishikawa *et al.* (2002) reported the use of 15% egg yolk for freezing semen from Hokkaido brown bears. Okano *et al.* (2004, 2006a, 2006b) cryopreserved semen from Japanese black bears at a final egg-yolk concentration of 20%. Giant panda spermatozoa have been cryopreserved using 20% egg yolk (Spindler *et al.* 2004). In a study of brown-bear semen, Anel *et al.* (2010) found that sperm motility and the percentages of viable spermatozoa and spermatozoa with intact acrosomes were higher in extenders containing 20% egg yolk than in those containing 10% egg yolk.

Several studies have shown that high concentrations of egg yolk have a negative effect on motility and acrosome integrity after thawing (Mohor gazelle, Holt *et al.* 1996) and on post-thawing viability (goat, Ritar and Salamon 1991). Other disadvantages of using egg yolk in freezing extenders are that: (1) the animal origin of egg yolk introduces a potential risk of

bacterial contamination, which may reduce the fertilisation potential of the spermatozoa (Bousseau *et al.* 1998), (2) the egg-yolk composition is variable and may differ between batches and (3) certain components of egg yolk have been reported to inhibit sperm respiration or reduce their motility (Pace and Graham 1974; Watson and Martin 1975; Amirat *et al.* 2004). Therefore egg yolk should be replaced by another material with similar activity.

Soybean lecithin, the main component of which is phosphatidylcholine, has been used to replace egg yolk with good results (ram, de Paz *et al.* 2010; bull, Aires *et al.* 2003; Stradaoli *et al.* 2007; horse, Ricker *et al.* 2006; Aurich *et al.* 2007). Ricker *et al.* (2006) described the mechanism of membrane stabilisation in spermatozoa that were cryopreserved in soy phosphatidylcholine-based diluent; they observed lipid aggregates on the membrane surface and protective effects on motility, viability and fertility.

Low-density lipoproteins (LDL) are another alternative to egg yolk. LDL are now considered to be the main factor responsible for the cryoprotective properties of egg yolk. Pace and Graham (1974) purified egg yolk using ultracentrifugation and observed that LDL had a cryoprotective effect on bull spermatozoa. The use of LDL may avoid the issues caused by the heterogeneity of the extenders prepared with whole egg yolk, facilitating extender standardisation. The suitability of LDL as a cryoprotectant has been confirmed in the bull (Hu JH *et al.* 2008, 2011; Vera-Munoz *et al.* 2009), stallion (Pillet *et al.* 2011), ram (Moustakas *et al.* 2011) and dog (Varela Junior *et al.* 2009).

Cooling and freezing-thawing cycles cause physical and chemical stresses on the sperm membrane that reduce sperm viability and fertilisation capability. Both cold shock and freezing damage are associated with reactive oxygen species (ROS) and oxidative stress generation (Stradaoli *et al.* 2007). Hu *et al.* (2011) stated that an extender containing LDL reduced the negative impact of ROS on bull spermatozoa. Thus, we carried out a total antioxidant capacity (TAC) assay on the extenders used in the present study to evaluate the relationship between sperm viability and exogenous antioxidant capacity.

The aim of the present study was to analyse the use of LDL or soybean lecithin in place of egg yolk in extenders for the cryopreservation of brown-bear semen. We evaluated the effects of these extenders on sperm quality (motility, membrane and acrosome integrity), and we determined the antioxidant capacity of the tested extenders.

Materials and methods

Reagents

All products were obtained from Sigma (Madrid, Spain) unless otherwise specified. The Equex STM Paste was obtained from Minitüb (Tiefenbach, Germany).

Animals and sample collection

A total of 31 ejaculate samples from 23 sexually mature male brown bears were obtained by electroejaculation during the breeding season (late April to early July). The animals were housed in a half-freedom regime in Cabárceno Park (Cantabria,

Spain; 43°21' N, 3°50' W, altitude 142 m) and fed a diet based on chicken meat, bread and fruits. Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65.

The male bears were immobilised by teleanaesthesia using 750 mg of zolazepam HCl + tiletamine HCl (Zoetel 100; Virbac, Carros, France) and 6 mg of medetomidine (Zalopine, 10 mg mL⁻¹; Orion Pharma Animal Health, Espoo, Finland.). After immobilisation, the bears were weighed and monitored (pulse, oxygen saturation and respiration). Prior to electroejaculation, the prepucial area was shaved and washed with physiological saline serum and the rectum was emptied of faeces. The bladder was catheterised during semen collection to prevent urine contamination. Electroejaculation was carried out with a PT Electronics electroejaculator (PT Electronics, Boring, OR, USA). The transrectal probe was 320 mm long with a diameter of 26 mm. Electric stimuli were provided until ejaculation occurred (10 V and 250 mA, on average).

To prevent urine contamination or a low cell concentration, the ejaculates were collected as isolated fractions in 15-mL graduated glass tubes. Immediately after collection, the volume of each fraction was recorded, its osmolality was measured using a cryoscopic osmometer (Osmomat-030; Gonotec, Berlin, Germany), and the pH value was determined using a CG 837 pH meter (Schott Instruments, Main, Germany). For each fraction, subjective motility was assessed with a phase-contrast microscope (100×), and urospermia was evaluated using a Merckgnost Urea Rapid Screening test (Merck, Barcelona, Spain). Fractions with low cell concentrations (<200 × 10⁶ cell mL⁻¹), low motility (<50%) or urine contamination (>80 mg urea dL⁻¹) were discarded. All valid fractions from the same electroejaculation were mixed and constituted one ejaculate. Selected samples were centrifuged at 600g for 6 min at room temperature, and the pellet was processed as described below.

Experimental design

Experiment 1: effect of soybean lecithin on sperm cryopreservation

Four extenders were prepared. The control extender (EY20) consisted of a TES-Tris-fructose (TTF)-Base (300 mOsm kg⁻¹, pH 7.1, with 6% glycerol, 2% EDTA and 1% Equex STM paste) with 20% egg yolk (de Paz *et al.* 2012). Soybean lecithin extenders were prepared with TTF-Base supplemented with 20 g L⁻¹ (2%, S2), 35 g L⁻¹ (3.5%, S3.5) or 50 g L⁻¹ (5%, S5) soybean L- α -phosphatidylcholine (Soy PC (20%); Avanti Polar Lipids, Alabaster, AL, USA) instead of egg yolk. The Soy PC (20%) contained phosphatidylcholine (PC, 24%), phosphatidylethanolamine (PE, 18.6%), phosphatidylinositol (PI, 11.5%), lysophosphatidylcholine (LPC, 4.6%) and other components (41.3%).

Fifteen brown-bear ejaculates were used. After each ejaculate had been centrifuged, the sperm pellet was divided into four aliquots, diluted with the same volume of each extender at room temperature and processed for cryopreservation (see Cryopreservation protocol, below).

Experiment 2: effect of soybean lecithin source on sperm cryopreservation

Several commercial sources of soybean lecithin are available, and each source has a unique phospholipid composition. The different lipids in the diluents may differentially affect sperm lipid packing at the membrane surface and consequently modify the chilling sensitivity of the spermatozoa (Ricker *et al.* 2006). To evaluate these differences, we tested two different sources of soybean lecithin. Type A soybean was the lecithin used in Experiment 1 and Type B was L- α -phosphatidylcholine purchased from Sigma, reported to contain from 14 to 23% phosphatidylcholine (other major phospholipid components included phosphatidylethanolamine and inositol phosphatides).

Three extenders were prepared using 50 g L⁻¹ soybean lecithin, according to the results of the previous experiment: (1) EY20 extender, (2) TTF-Base extender with 50 g L⁻¹ Type A soybean (S5-A) and (3) TTF-Base extender with 50 g L⁻¹ Type B soybean (S5-B).

Eight brown-bear ejaculates were used. The pellet was divided into three aliquots, which were diluted with the same volume of each extender at room temperature and processed for cryopreservation (see Cryopreservation protocol, below).

Experiment 3: effect of LDL on sperm cryopreservation

Four extenders were prepared using extender EY20 as a control. LDL extenders were prepared with TTF-Base supplemented (vol/vol) with 5% (LDL5), 10% (LDL10) or 15% (LDL15) LDL instead of egg yolk. For the extraction of LDL (Moussa *et al.* 2002), egg yolk was diluted 1:1 (vol/vol) in a 1.7 M NaCl solution and homogenised. The LDL was then slowly precipitated out with ammonium sulfate. The solution was dialysed for at least 6 h to remove the ammonium sulfate and then frozen at -20°C. After this step, LDL was obtained by lyophilisation of the frozen solution.

Eight brown-bear ejaculates were used. The sperm pellet was divided into four aliquots, which were diluted with the same volume of TTF-Base extender at room temperature and processed for cryopreservation (see Cryopreservation protocol, below).

Study of the total antioxidant capacity (TAC) of the extenders

The extenders used in the cryopreservation experiments were tested for their antioxidant capacities. TTF-Base was included in the analysis as a control. The other extenders tested were EY20, S2, S3.5, S5, S5-A, S5-B, LDL5, LDL10 and LDL15. Lecithin extenders were prepared in duplicate at a concentration of 5% (wt/vol) using Type A and Type B soybean lecithin. TAC assessment was carried out with the Total Antioxidant Capacity Assay Kit (BioVision, Mountain View, CA, USA) following the assay procedure recommended by the manufacturer, and the outcome was recorded in mM Trolox equivalents. The extenders were analysed after storage at 5°C for one week.

Cryopreservation protocol

The tubes with the extended samples (1:1 at 3% glycerol) were placed in beakers containing 100 mL of water at room temperature and transferred to a refrigerator, where they were slowly cooled to 5°C. Then, the samples were diluted with the same

volume of each extender containing 9% glycerol to achieve a final glycerol concentration of 6%. A final sperm concentration of 100×10^6 spermatozoa mL⁻¹ was achieved by adding the appropriate volume of each extender to 6% glycerol. After packaging into 0.25-mL plastic straws and equilibrating for 1 h at 5°C, the samples were frozen in a programmable freezer (Kryo 10 Series III; Planer PLC., Sunbury-On-Thames, UK) at -20°C min⁻¹ to -100°C and then transferred to liquid nitrogen containers. The cryopreserved samples remained in liquid nitrogen for a minimum of 1 week. Thawing was performed by plunging the straws into water at 65°C for 6 s.

Evaluation of semen motility

Sperm quality was evaluated immediately before freezing and after thawing. The motility parameters were assessed using a computer-assisted semen motility analysis system (CASA; Integrated Semen Analyser System (ISAS); Proiser, Valencia, Spain). Samples were diluted ($10\text{--}20 \times 10^6$ cells mL⁻¹) in buffered medium (HEPES 20 mmol L⁻¹, 197 mmol L⁻¹ NaCl, 2.5 mmol L⁻¹ KOH, 10 mmol L⁻¹ glucose, 1% egg yolk, pH 7, 300 mOsm kg⁻¹) and warmed on a 37°C plate for 5 min. Then, a 5- μ L sperm sample was placed in a Makler counting cell chamber (10 μ m depth; Sefi Medical Instruments, Haifa, Israel). The sample was examined at 100 $\times$ (negative phase contrast) using a microscope with a warmed stage (38°C). To classify a spermatozoon as motile, it had to meet the following standard parameters: 25 frames s⁻¹, a head area of 5 to 80 μ m² and a curvilinear velocity (VCL) > 10 μ m s⁻¹. At least five sequences or 200 spermatozoa were saved and analysed. The computer-assisted sperm analysis provided the following parameters: total motility (TM, %; defined as the percentage of spermatozoa with VCL > 10 μ m s⁻¹), progressive motility (PM, %; spermatozoa were considered progressive if VCL > 25 and straightness (STR) > 80%), average path velocity (VAP, μ m s⁻¹), curvilinear velocity (VCL, μ m s⁻¹), straight-line velocity (VSL, μ m s⁻¹), linearity (LIN, %), amplitude of lateral head displacement (ALH, μ m) and straightness (STR, %).

Flow cytometry

Sperm viability and acrosomal status were assessed using PNA-FITC-PI (PNA-FITC, Lectin from *Arachis hypogaea* (peanut) FITC conjugate; PI, Propidium iodide) double staining and flow cytometry (Martínez-Pastor *et al.* 2010). Stock solutions of the fluorochromes were prepared in phosphate-buffered saline (PBS) at 1 μ g mL⁻¹ PNA-FITC and 6 μ M PI. Sperm samples were diluted to $1\text{--}2 \times 10^6$ spermatozoa mL⁻¹ with fluorochrome solution in polypropylene tubes (300 μ L tube⁻¹). Flow cytometry analysis yielded the percentage of red events (PI-) and the percentage of green events (PNA-FITC+). These percentages were corrected according to Petrunkina and Harrison (2010) to remove the signals for non-DNA-containing particles. The number of non-sperm particles in samples was analysed with SYBR14/PI (LIVE/DEAD Sperm Viability Kit; Invitrogen, Barcelona, Spain) in parallel. Sperm samples were diluted to 5×10^6 spermatozoa mL⁻¹ with PBS, incubated with 24 μ M PI and 100 nM SYBR-14, and stored in the dark at 37°C for 20 min. We detected two populations, corresponding to

Table 1. Brown-bear semen quality parameters (mean $\pm$ s.e.m.) before freezing and after thawing in extenders containing different soybean concentrations

Control (EY20), 2% soybean (S2), 3.5% soybean (S3.5) and 5% soybean (S5). Total motility (TM, %), progressive motility (PM, %), average velocity (VAP, $\mu\text{m s}^{-1}$), curvilinear velocity (VCL, $\mu\text{m s}^{-1}$), straight-line velocity (VSL, $\mu\text{m s}^{-1}$), linearity (LIN, %), straightness (STR, %), amplitude of lateral head displacement (ALH, μm), viable spermatozoa (VIAB, PI–) and spermatozoa with damaged acrosomes (dACR, PNA–FITC+). Different letters within rows indicate significant differences between the control and the soybean-based extenders ($P < 0.05$)

Parameter	Before freezing				After thawing			
	EY20	S2	S3.5	S5	EY20	S2	S3.5	S5
TM	77.6 $\pm$ 4.4 ^a	2.1 $\pm$ 0.6 ^b	69.3 $\pm$ 4.1 ^a	70.4 $\pm$ 5.4 ^a	54.2 $\pm$ 3.5 ^a	1.0 $\pm$ 0.4 ^b	42.9 $\pm$ 4.7 ^a	52.4 $\pm$ 6.3 ^a
PM	44.0 $\pm$ 4.2 ^a	0.4 $\pm$ 0.1 ^b	24.0 $\pm$ 2.3 ^c	31.6 $\pm$ 3.1 ^c	21.5 $\pm$ 2.7 ^a	0.2 $\pm$ 0.1 ^b	4.4 $\pm$ 0.8 ^b	17.1 $\pm$ 7.1 ^b
VAP	60.9 $\pm$ 3.2 ^a	11.8 $\pm$ 3.9 ^b	55.2 $\pm$ 2.8 ^a	50.7 $\pm$ 2.9 ^a	56.6 $\pm$ 3.5 ^a	6.6 $\pm$ 1.3 ^b	42.6 $\pm$ 2.4 ^c	42.7 $\pm$ 2.8 ^c
VCL	100.3 $\pm$ 4.9 ^a	22.2 $\pm$ 7.5 ^b	102.8 $\pm$ 5.6 ^a	86.4 $\pm$ 5.2 ^a	107.0 $\pm$ 6.6 ^a	12.0 $\pm$ 2.6 ^b	86.4 $\pm$ 6.0 ^a	83.5 $\pm$ 6.4 ^a
VSL	42.7 $\pm$ 2.8 ^a	8.3 $\pm$ 3.2 ^b	30.7 $\pm$ 1.6 ^c	32.2 $\pm$ 1.7 ^c	35.1 $\pm$ 2.3 ^a	4.2 $\pm$ 1.0 ^b	25.7 $\pm$ 1.8 ^c	26.1 $\pm$ 2.1 ^c
LIN	40.9 $\pm$ 2.3 ^a	30.4 $\pm$ 4.3 ^b	30.3 $\pm$ 1.3 ^b	36.3 $\pm$ 1.6 ^{a,b}	32.3 $\pm$ 0.9	23.6 $\pm$ 4.6	28.9 $\pm$ 0.5	30.2 $\pm$ 0.7
STR	65.5 $\pm$ 1.8	55.2 $\pm$ 5.8	55.1 $\pm$ 1.7	60.9 $\pm$ 1.5	58.7 $\pm$ 0.8 ^a	40.0 $\pm$ 6.8 ^b	56.0 $\pm$ 1.1 ^a	56.2 $\pm$ 1.5 ^a
ALH	4.4 $\pm$ 0.3 ^a	1.3 $\pm$ 0.3 ^b	4.6 $\pm$ 0.2 ^a	3.8 $\pm$ 0.2 ^a	5.0 $\pm$ 0.3 ^a	0.8 $\pm$ 0.2 ^b	4.4 $\pm$ 0.3 ^a	4.2 $\pm$ 0.3 ^a
VIAB	77.7 $\pm$ 2.3 ^a	2.3 $\pm$ 1.1 ^b	23.7 $\pm$ 4.4 ^c	56.5 $\pm$ 6.0 ^d	50.1 $\pm$ 3.3 ^a	3.3 $\pm$ 2.0 ^b	16.4 $\pm$ 2.8 ^c	21.0 $\pm$ 4.1 ^c
dACR	2.8 $\pm$ 0.4 ^a	26.1 $\pm$ 5.2 ^b	21.1 $\pm$ 2.9 ^{bc}	8.6 $\pm$ 2.6 ^{ac}	11.7 $\pm$ 1.8 ^a	43.4 $\pm$ 8.3 ^b	29.7 $\pm$ 2.6 ^{bc}	23.7 $\pm$ 3.0 ^{ac}

spermatozoa (staining events in quadrants Q3 and Q4, red or green) and foreign particles (non-staining events). Finally, the percentages of viable spermatozoa (PI–, data corrected, named VIAB) and spermatozoa with damaged acrosomes (PNA–FITC+, data corrected, named dACR) were calculated.

Flow cytometric evaluations were carried out using a FACS-Calibur flow cytometer (Becton Dickinson (BD) BioSciences, San Jose, CA, USA) equipped with an argon-ion laser tuned at 488 nm and running at 200 mW with standard optics (DM 560SP and DM 640 LP). The levels of red (670 LP filter, FL3 photodetector) and green fluorescence (530/30BP filter, FL1 photodetector) of the acquired particles were recorded. In all cases, 10 000 events per sample were assessed using Cell Quest Pro Ver. 3.1 software (BD Biosciences) with a flow rate of 200 cells s^{-1} . Calibration was carried out periodically using standard beads (Calibrite; BD BioSciences). The flow cytometry data were analysed using Weasel Ver. 3.0.2 (Walter and Eliza Hall Institute, Melbourne, Australia).

Statistical analysis

The data were analysed using the SAS Ver. 9.0 package (SAS Institute Inc., Cary, NC, USA). The results are shown as the means and standard errors of the mean, unless otherwise stated. The pre-freezing and post-thawing data were analysed using linear mixed-effects models (MIXED procedure). Models were built with the extender as the fixed effect and the sample as the random effect. Significant fixed effects were further analysed using multiple comparisons of the means with Tukey contrasts. A significance level of $P < 0.05$ was used.

Results

The following quality parameters were measured in fresh brown-bear semen samples (mean $\pm$ s.e.m.): volume (2.1 ± 0.2 mL), cell concentration ($327.5 \pm 59.4 \times 10^6$ spermatozoa mL^{-1}), pH (8.2 ± 0.1) and osmolarity (293.0 ± 4.0 mOsm).

Experiment 1: effect of soybean lecithin on sperm cryopreservation

The before-freezing evaluation showed differences in all parameters analysed (except STR) between the control and S2 extender (Table 1). Soybean-based extenders caused a significant reduction in sperm quality in comparison with the control, as indicated by the PM, VSL and cell viability, whereas LIN, ALH and acrosome status did not differ between the control and S5 extender. After thawing (Table 1), the control presented better results for all the motility parameters ($P < 0.05$) except LIN (STR and ALH showed the lowest values in S2) and for sperm viability. Moreover, both the control and the S5 samples exhibited lower acrosome damage values.

Experiment 2: effect of soybean lecithin source on sperm cryopreservation

The before-freezing analysis showed that the samples diluted in the egg yolk and S5-A extenders contained significantly higher percentages of total motile spermatozoa and progressive motile spermatozoa in comparison with the spermatozoa diluted in the S5-B extender (Table 2). The kinematic parameters of the spermatozoa, the sperm viability and the proportion of damaged acrosomes were not significantly different between the three extenders. After thawing, the spermatozoa motilities in the S5-A extender group and control group were higher than that in spermatozoa frozen in S5-B, although no significant differences were observed in any variables except TM (Table 2). The percentage of viable spermatozoa was lower in diluents containing soybean lecithin than in the control, but these diluents did not affect the percentage of damaged acrosomes.

Experiment 3: effect of LDL on sperm cryopreservation

In the before-freezing study, the egg-yolk extender had the highest values for the motility parameters ($P < 0.05$) including TM, PM, VAP and VCL (Table 3). No differences were observed

Table 2. Brown-bear semen quality parameters (mean $\pm$ s.e.m.) before freezing and after thawing in extenders containing soybean lecithin from two sources

Control (EY20), 5% Type A soybean (S5-A) and 5% Type B soybean (S5-B). Total motility (TM, %), progressive motility (PM, %), average velocity (VAP, $\mu\text{m s}^{-1}$), curvilinear velocity (VCL, $\mu\text{m s}^{-1}$), straight-line velocity (VSL, $\mu\text{m s}^{-1}$), linearity (LIN, %), straightness (STR, %), amplitude of lateral head displacement (ALH, μm), viable spermatozoa (VIAB, PI-) and spermatozoa with damaged acrosomes (dACR, PNA-FITC+). Different letters within rows indicate significant differences between the control and the soybean-based extenders ($P < 0.05$)

Parameter	Before freezing			After thawing		
	EY20	S5-A	S5-B	EY20	S5-A	S5-B
TM	55.2 $\pm$ 11.6 ^{ab}	68.6 $\pm$ 3.8 ^b	33.9 $\pm$ 8.8 ^a	42.2 $\pm$ 7.2 ^a	47.2 $\pm$ 4.8 ^a	28.7 $\pm$ 4.4 ^b
PM	32.2 $\pm$ 7.0 ^{ab}	49.9 $\pm$ 3.3 ^b	26.2 $\pm$ 8.0 ^a	24.6 $\pm$ 4.9	27.4 $\pm$ 3.2	17.5 $\pm$ 4.0
VAP	55.6 $\pm$ 7.5	51.5 $\pm$ 5.4	38.6 $\pm$ 8.4	59.8 $\pm$ 7.1	50.2 $\pm$ 2.9	43.1 $\pm$ 7.3
VCL	85.1 $\pm$ 12.9	84.1 $\pm$ 11.6	56.1 $\pm$ 11.8	98.9 $\pm$ 12.9	88.9 $\pm$ 7.1	69.9 $\pm$ 12.8
VSL	37.4 $\pm$ 5.4	38.3 $\pm$ 4.3	30.4 $\pm$ 7.3	41.2 $\pm$ 5.3	34.7 $\pm$ 3.0	30.7 $\pm$ 6.2
LIN	43.1 $\pm$ 2.4	47.9 $\pm$ 2.4	45.9 $\pm$ 7.8	41.8 $\pm$ 2.8	39.3 $\pm$ 2.3	40.1 $\pm$ 8.1
STR	63.5 $\pm$ 1.5	72.2 $\pm$ 0.8	64.1 $\pm$ 9.7	64.6 $\pm$ 2.1	65.0 $\pm$ 2.2	58.2 $\pm$ 9.5
ALH	3.5 $\pm$ 0.5	3.6 $\pm$ 0.5	2.4 $\pm$ 0.4	4.2 $\pm$ 0.5	4.0 $\pm$ 0.4	3.0 $\pm$ 0.6
VIAB	77.5 $\pm$ 4.3	77.6 $\pm$ 6.0	70.8 $\pm$ 11.2	39.9 $\pm$ 2.1 ^a	26.6 $\pm$ 3.9 ^b	25.0 $\pm$ 3.5 ^b
dACR	2.9 $\pm$ 0.5	3.9 $\pm$ 0.5	7.5 $\pm$ 4.8	6.4 $\pm$ 0.9	12.6 $\pm$ 2.4	10.5 $\pm$ 3.5

Table 3. Brown-bear semen quality parameters (mean $\pm$ s.e.m.) before freezing and after thawing in extenders containing different LDL concentrations

Control (EY20), low-density lipoproteins (LDL) at three different concentrations (5, 10 and 15%; LDL5, LDL10 and LDL15, respectively). Total motility (TM, %), progressive motility (PM, %), average velocity (VAP, $\mu\text{m s}^{-1}$), curvilinear velocity (VCL, $\mu\text{m s}^{-1}$), straight-line velocity (VSL, $\mu\text{m s}^{-1}$), linearity (LIN, %), straightness (STR, %), amplitude of lateral head displacement (ALH, μm), viable spermatozoa (VIAB, PI-) and spermatozoa with damaged acrosomes (dACR, PNA-FITC+). Different letters within rows indicate significant differences between the control and the LDL-based extenders ($P < 0.05$)

Parameter	Before freezing				After thawing			
	EY20	LDL5	LDL10	LDL15	EY20	LDL5	LDL10	LDL15
TM	60.3 $\pm$ 6.4 ^a	31.7 $\pm$ 6.1 ^b	35.1 $\pm$ 6.0 ^b	28.7 $\pm$ 6.4 ^b	41.2 $\pm$ 6.4 ^a	28.9 $\pm$ 4.7 ^{ab}	29.2 $\pm$ 5.8 ^{ab}	16.9 $\pm$ 3.8 ^b
PM	34.8 $\pm$ 3.9 ^a	16.7 $\pm$ 3.8 ^b	16.1 $\pm$ 2.7 ^b	13.4 $\pm$ 2.6 ^b	22.6 $\pm$ 4.2 ^a	11.6 $\pm$ 1.6 ^b	11.9 $\pm$ 2.1 ^b	7.5 $\pm$ 1.5 ^b
VAP	56.7 $\pm$ 3.6 ^a	41.5 $\pm$ 3.5 ^b	33.5 $\pm$ 2.4 ^{bc}	26.5 $\pm$ 2 ^c	53.1 $\pm$ 5.6 ^a	38.1 $\pm$ 1.9 ^b	32.6 $\pm$ 1 ^{b,c}	26.5 $\pm$ 0.6 ^c
VCL	82.2 $\pm$ 4.7 ^a	68.4 $\pm$ 5.4 ^{ab}	56.2 $\pm$ 4.9 ^{bc}	41.5 $\pm$ 4.3 ^c	90.2 $\pm$ 7.4 ^a	66.6 $\pm$ 3 ^b	53.6 $\pm$ 2.1 ^c	40 $\pm$ 2.1 ^d
VSL	38.4 $\pm$ 2.4 ^a	26.6 $\pm$ 2.8 ^b	20.8 $\pm$ 1.5 ^{bc}	15.9 $\pm$ 1 ^c	34.8 $\pm$ 3.9 ^a	22 $\pm$ 1.6 ^b	18.5 $\pm$ 0.9 ^b	15.4 $\pm$ 0.4 ^b
LIN	46.9 $\pm$ 2.0	41.9 $\pm$ 2.3	40.4 $\pm$ 1.9	45.1 $\pm$ 3.3	38.9 $\pm$ 2.5 ^{ab}	34.7 $\pm$ 0.8 ^a	36.5 $\pm$ 0.8 ^{ab}	43.6 $\pm$ 2.8 ^b
STR	65.8 $\pm$ 1.5	63.7 $\pm$ 2.1	61.2 $\pm$ 1.6	62.1 $\pm$ 2.2	63.0 $\pm$ 1.4 ^a	56.6 $\pm$ 1 ^b	56.0 $\pm$ 0.9 ^b	57.5 $\pm$ 1.8 ^b
ALH	3.2 $\pm$ 0.2	3.1 $\pm$ 0.2	2.9 $\pm$ 0.2	2.5 $\pm$ 0.2	3.9 $\pm$ 0.2 ^a	3.3 $\pm$ 0.1 ^b	2.9 $\pm$ 0.1 ^c	2.6 $\pm$ 0.1 ^c
VIAB	72.5 $\pm$ 4.6	80.2 $\pm$ 2.8	81.9 $\pm$ 2.4	77.3 $\pm$ 5.2	40.7 $\pm$ 6.5 ^a	45.5 $\pm$ 3.3 ^a	66.2 $\pm$ 3.0 ^b	73.4 $\pm$ 3.9 ^b
dACR	3.8 $\pm$ 0.7	6.3 $\pm$ 1.9	1.8 $\pm$ 0.3	4.5 $\pm$ 2.8	6.6 $\pm$ 1.1 ^{ab}	9.6 $\pm$ 2.6 ^b	4.6 $\pm$ 0.6 ^a	4.9 $\pm$ 1.0 ^a

in TM or PM between the LDL extenders. After thawing (Table 3), sperm motility was higher in the control than in the LDL extenders. The number of damaged acrosomes was highest in the LDL5 extender ($P < 0.05$), and the percentages of viable spermatozoa were highest in the LDL10 and LDL15 extenders.

Study of the total antioxidant capacity (TAC) of the extenders

The LDL extenders showed higher Trolox equivalent antioxidant activity values after refrigerated storage than those observed for the EY20 extender (Fig. 1). Among the soybean extenders, higher values were observed for S5-A and S5-B than for the EY20, S2 and S3.5 extenders.

Discussion

The replacement of egg yolk in diluents for freezing semen to obtain chemically standardised extenders (e.g. replacement with

LDL) or to avoid the use of animal proteins (e.g. replacement with soybean) is a major priority in sperm cryopreservation. To address these objectives, we carried out three different experiments to compare sperm motility, acrosome status and viability parameters in freeze-thawed brown-bear semen preserved in TTF-based extenders containing soybean lecithin, low-density lipoproteins or egg yolk.

Soybean-based extenders

To our knowledge, this is the first study in which soybean lecithin was used in an extender for the cryopreservation of brown-bear semen. The effects of three concentrations of soybean lecithin (2%, 3.5% and 5%) on post-thaw sperm physiology were tested in comparison with conventional egg-yolk extender (Anel *et al.* 2008). We could not assess concentrations above 50 g L⁻¹ soybean lecithin (5%) due to the size of the

5

5

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10

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Fig. 1. Total antioxidant capacity (TAC, mM Trolox equivalents) of the extenders used in the present study: S5-B (5% Type B soybean), S5 (5% Type A soybean), S3.5 (3.5% soybean), S2 (2% soybean), LDL15 (15% low-density lipoprotein); LDL10 (10% LDL), LDL5 (5% LDL) and EY20 (Control).

particles and aggregates in the soybean lecithin-TTF extender emulsion (de Paz *et al.* 2010).

In the present study, the motility of post-thawed spermatozoa and the percentages of viable spermatozoa and spermatozoa with intact acrosomes increased when the concentration of soybean lecithin in the extender was increased. Papa *et al.* (2011) compared different concentrations of soybean lecithin (between 10 and 20 g L⁻¹) in freezing extenders for stallion semen and showed that spermatozoa frozen in extenders containing any concentration of soybean lecithin maintained similar percentages of motile spermatozoa. Our results showed that sperm motility, sperm viability and the percentage of intact acrosomes were significantly lower for brown-bear semen that was diluted using a soybean-lecithin extender than for semen that was diluted in an egg-yolk extender. In bovine semen, van Wagtenonk-de Leeuw *et al.* (2000) observed that semen diluted with a commercial soybean-based extender (Biociphus Plus), in comparison with semen diluted in other extenders based on egg yolk, showed significantly lower post-thawing motility. This result can be explained by the higher viscosity and presence of particulate debris in the Biociphus Plus extender. However, stallion semen frozen in Botu-Crio, which contains soybean lecithin rather than egg, exhibited similar percentages of total motile spermatozoa and spermatozoa with intact plasma membranes (Papa *et al.* 2011). These data suggest that the protective effect of soybean lecithin in semen cryopreservation, as observed for egg yolk, varies across species.

A deleterious effect on fertilisation potential has been observed in bull semen frozen with soybean lecithin compared with spermatozoa frozen in egg-yolk-containing diluent, although both types of semen showed similar sperm motility and plasma membrane integrity values (Papa *et al.* 2011). However, another fertility study determined that equine spermatozoa cryopreserved in soy phosphatidylcholine-based medium were capable of fertilisation at the same rate as spermatozoa frozen in egg-yolk-based medium, and noted that sperm viability and motility were maintained in the soy phosphatidylcholine-based medium (Ricker *et al.* 2006). However, according to our

results, extenders based on soybean lecithin cannot protect the sperm membrane during freezing as well as extenders based on EY20.

The lipid compositions of different commercial sources of soybean lecithin vary significantly, and this variation affects the preparation of media and the medium's effects on semen quality (de Paz *et al.* 2010). In the present study, two sources of soybean lecithin (50 g L⁻¹) were tested: S5-A (24% PC) and S5-B (14–23% PC). After thawing, we observed that spermatozoa showed higher total and progressive motility in the S5-A soybean-based extender than in the S5-B-based extender (only the TM was statistically significantly different between these groups), but the percentages of viable spermatozoa and spermatozoa with intact acrosomes and the kinematic parameters were similar. Our results suggest that phosphatidylcholine plays an important role in protecting sperm motility, although this effect could be due to other components present in the commercial preparation of soybean lecithin. However, sperm viability in frozen samples was not protected by the soybean extenders compared with the egg-yolk extender. Ricker *et al.* (2006) found that pure soy phosphatidylcholine-based diluents can prevent membrane damage during the cryopreservation of equine spermatozoa and that these diluents perform as well as a standard egg-yolk-based diluent in preserving sperm viability, motility and fertility.

In our study, the extender containing 5% Type A soybean lecithin protected sperm motility against cold-shock damage as well as the egg-yolk-based extender did, but no protective effect was observed for sperm viability or acrosome integrity. Different protective effects of additives on sperm motility and other sperm quality parameters have been observed (Leite *et al.* 2010). Del Valle *et al.* (2011) showed that soy lecithin is able to effectively protect certain quality characteristics of ram spermatozoa against freezing-induced damage; however, lecithin induced the loss of mitochondrial membrane potential or mitochondrial loss which, in fresh semen, was not reflected by changes in sperm motility. These authors suggest that purified soy lecithin (L- α -phosphatidylcholine) might work only through interactions with the plasma membrane, whereas egg yolk, which contains not only L- α -phosphatidylcholine but also lipoproteins, might be better able to protect mitochondria.

LDL extender

Our results showed that, before freezing, the motility parameters (TM and PM) and some kinematic parameters (VAP, VCL and VSL) were lower in spermatozoa preserved with the LDL-containing extender (5, 10 or 15%) than in spermatozoa preserved with the extender containing 20% egg yolk. However, the percentages of viable spermatozoa and spermatozoa with damaged acrosomes were similar in the LDL-based extender and in the egg-yolk extender. These differential protective effects on sperm quality after the addition of LDL instead of egg yolk to the extender have not been observed by other authors. Akhter *et al.* (2011), working with buffalo bull semen cooled to 4°C, found that sperm progressive motility, plasma membrane integrity and viability were similar in spermatozoa preserved with an extender containing 10% LDL and those preserved with an extender containing 20% egg yolk.

Yamauchi *et al.* (2009) reported that the spermatozoa cryopreserved in an extender supplemented with LDL instead of egg yolk showed better resistance to cold-shock damage during cryopreservation in comparison with semen cryopreserved in an extender supplemented with egg yolk. These authors found that the percentage of total motile spermatozoa, the extent of rapid progressive motility, the integrities of the plasmalemma and DNA, mitochondrial activity and the proteolytic activity of the acrosomal content in the post-thaw spermatozoa were higher in Agu pig spermatozoa treated with LDL (4 or 6%) than in spermatozoa frozen in an extender containing 20% egg yolk. However, our results showed that the motility of post-thaw spermatozoa was decreased in semen treated with LDL during freezing in comparison with semen in an extender containing 20% egg yolk. In contrast, the percentage of spermatozoa with normal acrosomes was not affected by replacing the egg yolk with LDL, and the percentage of viable spermatozoa was higher.

The post-thaw reduction in sperm motility, which is directly proportional to the percentage of LDL in the diluent, could indicate that the compounds present in the extender have different effects on the plasma membrane and flagellum (Vera-Munoz *et al.* 2009). The LDL extender was more effective in preserving plasma membrane integrity than the egg-yolk extender was, but sperm motility was negatively affected. Amirat *et al.* (2004) found that LDL protected sperm cells against cold shock by preventing the efflux of phospholipids and cholesterol from the sperm cell membrane. According to Ricker *et al.* (2006), exogenous lipids from the diluents are not incorporated into the sperm membrane but are strongly associated with the membrane surface; thus, these lipids may provide a physical barrier to freeze-thaw damage and prevent membrane phase separation by influencing lipid packing at the membrane surface. Additionally, the improved cryoprotection of LDL treatments may also be related to the removal of certain substances present in the egg yolk (high-density lipoproteins and aggregates) that could have deleterious effects on sperm viability (Pace and Graham 1974).

The total antioxidant capacity (TAC) of the extenders

As expected, the TTF diluent used in this experiment to obtain different extenders showed no antioxidant capacity according in the TAC assay; therefore, the antioxidant capacity of the extender was due to the addition of membrane stabilisers. The egg-yolk-based extender showed a total antioxidant capacity similar to those of the soy-lecithin-based extenders (S2 and S3.5). Consistent with this observation, the greatest sperm viability and the lowest percentage of damaged acrosomes were observed in samples preserved with egg-yolk-based extender. In this regard, the LDL-based extenders, which were associated with higher TAC values than egg-yolk-based extenders, are less able to protect the sperm motility in post-thawed samples but have a greater protective effect on sperm viability. These results suggest that there is no direct relationship between the antioxidant capacity of the extender and its protective effect on post-thawed sperm quality.

The presence of polyunsaturated fatty acids in plasma membranes is accepted as the main cause of sperm membrane

oxidation during cryopreservation. Thus, it is possible that the loss of cell function observed in post-thawed spermatozoa involves the aforementioned oxidative stress. Furthermore, although Kasimanickam *et al.* (2007) observed that lipid peroxidation and plasma membrane integrity had a cause and effect relationship, sperm motility was an independent factor. One way to prevent oxidative stress in spermatozoa after thawing is to add antioxidants to the freezing extender (Fernández-Santos *et al.* 2007), but variable results have been obtained with the addition of antioxidants to freezing diluents for different species (bull, Stradaoli *et al.* 2007; red deer, Fernández-Santos *et al.* 2007; ram, da Silva Maia *et al.* 2010; pig, Buranaamnuay *et al.* 2011). Hu *et al.* (2011) found that an extender containing LDL reduced the negative impact of ROS on bull spermatozoa and that 8% LDL was associated with better semen quality parameters and anti-oxidant enzyme activity than was egg yolk.

In summary, our results confirm that LDL extenders have greater anti-oxidant activity than egg-yolk extenders. High concentrations of LDL (10 or 15%) could be useful as a substitute for egg yolk in extenders used for cryopreservation because LDL at high concentrations is better able to protect the viability of brown-bear spermatozoa and does not affect the acrosomal integrity, although sperm motility is negatively affected. Soybean lecithin did not protect the viability of freeze-thawed brown-bear spermatozoa, although the sperm motility was similar in the extender containing 5% Type A soybean and the extender containing egg yolk. Further studies are needed to more precisely determine the type and concentration of soybean lecithin suitable for use in freezing diluents for brown-bear semen.

Acknowledgements

This work was supported in part by CICYT (CGL 2010–19213/BOS) and CANTUR SA. Felipe Martínez-Pastor was supported by the Ramón y Cajal program (Spanish Ministry of Science and Innovation). The authors thank Miguel Ángel Maraón and Miguel Angel Prieto, the gamekeepers of the Cabárceno Nature Park, as well as Susana Gomes-Alves, María Mata-Campuzano, Elena López-Urueña and Patricia Manrique.

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CAPÍTULO 9

PUBLICACIÓN 4. THE ADDITION OF HEAT SHOCK PROTEIN HSPA8 TO CRYOPROTECTIVE MEDIA IMPROVES THE SURVIVAL OF CANTABRIAN BROWN BEAR SPERMATOZOA DURING CHILLING AND AFTER CRYOPRESERVATION

The addition of heat shock protein HSPA8 to cryoprotective media improves the survival of brown bear (*Ursus arctos*) spermatozoa during chilling and after cryopreservation

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ARTICLE INFO

Article history:

Received 21 September 2012

Accepted 1 November 2012

Keywords:

Brown bear

Sperm cryopreservation

Heat shock proteins

Artificial mucus

ABSTRACT

The Cantabrian brown bear survives as a small remnant population in northern Spain and semen cryopreservation for future artificial insemination is one of the measures being implemented for conservation of this species. As part of this program we investigated the value of adding heat shock protein A8 (HSPA8) to media (N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid-TRIS-fructose with 20% egg yolk) used for chilling and cryopreserving the spermatozoa. Semen samples from eight brown bears were obtained by electroejaculation during the breeding season. In experiment 1, we tested three concentrations of HSPA8 (0.5, 1, and 5 µg/mL) to determine whether sperm motility (computer assisted sperm analysis system) and sperm survival could be improved during refrigeration (5 °C) up to 48 hours. Results showed that sperm viability (test with propidium iodide) was improved by the addition of 0.5 and 5 µg/mL HSPA8. In experiment 2, HSPA8 was added to the cryopreservation media (6% final glycerol concentration) before the freezing process. Though there were no differences in sperm viability immediately after thawing (analyses to 0 hours), plasma membrane permeability (test with YO-PRO-1) was significantly lower by the presence of HSPA8 (1 µg/mL) and acrosomal damage (test with peanut agglutinin-fluorescein isothiocyanate conjugate) was reduced by higher concentrations of HSPA8 (1 and 5 µg/mL) (analyses after thermal stress test incubating over 2 hours to 37 °C). In experiment 3, results of a simple progression test carried out through artificial mucus (hyaluronic acid 4 mg/mL) showed a significant decrease in the total number of sperm able to swim a distance of 0.5 to 2 cm through a capillary tube for all HSPA8-based extenders. Nevertheless, the distance traveled by the vanguard spermatozoa, which represent a highly motile subpopulation, was restored by the inclusion of 1 and 5 µg/mL HSPA8 in the cryopreservation media. Thus, the HSPA8 addition to extender improves the quality of brown bear (*Ursus arctos*) sperm during chilling (viability) and after cryopreservation (number of sperm with damaged acrosomes and “apoptotic-like” changes).

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1. Introduction

The Cantabrian brown bear (*Ursus arctos*) is the last indigenous brown bear population in the Iberian Peninsula and is considered to be at risk of extinction (Real Decreto

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439/1990, regulation of the National Catalogue of Endangered Species) because of habitat loss and population fragmentation (approximately 150 individuals restricted to two isolated populations in the North of Spain). The development of a genome resource bank for this species, mainly consisting of frozen semen for future use with artificial insemination, has been proposed as a useful adjunct to other conservation methods because it would maximize the reproductive potential of the male by overcoming the constraints of time and space [1].

The development of a species-specific conservation protocol that includes artificial insemination with frozen semen has to include the validation of an effective method for freezing semen [2]. The application of these technologies must be adapted to individual species because spermatozoa clearly respond differently depending on the origin of the sample, creating a need to conduct research into sperm cryopreservation [3].

In brown bears, recent studies have focused on the optimization of extender composition, including egg yolk [4–6], glycerol [7,8], and several additives [9], and glycerol concentration and freezing rate used [10]. Together, these studies have resulted in improved methods for semen storage *in vitro*; however, recent investigations have indicated that there might be some additional benefits to be gained by exploiting the physiological ability of the female reproductive tract to prolong sperm viability *in vivo*. Several studies of sperm storage in the mammalian female reproductive tract have reported that the ascent of spermatozoa is regulated by the female, and that a successful spermatozoon must be equipped with a particular suite of proteins for entry into the oviduct, and then remain viable until ovulation has occurred [11].

Moreover, it is increasingly believed that the oviduct is not a passive conduit for the circulation of spermatozoa and that a sperm–oviduct dialogue is an important feature of sperm transport [12]. Contact between the spermatozoa and the surface of oviductal epithelial cells has developed a new paradigm in this regard, by showing that the arrival of spermatozoa stimulates the *de novo* expression of genes and the synthesis of novel proteins [13–15]. Heat shock proteins are among the novel proteins studied and experimentally it has been shown that one of these (heat shock protein A8 (HSPA8); formerly known as heat shock cognate protein 73 kD [HSC70]) is able to prolong the viability of boar, bull, and ram fresh spermatozoa *in vitro* [16,17]. Because this specific protein is highly conserved across species (for review, see [18]) and its bioactivity for mammalian spermatozoa is not species-specific, the objective of this study was to determine whether the protein could also be beneficial for the long- (cryopreservation) and short-term (chilling at 5 °C) storage of brown bear spermatozoa. Apart from evaluating spermatozoa by the use of routine parameters such as viability, motility, and acrosomal integrity, we also tested the frozen/thawed spermatozoa for their ability to migrate through capillary tubes containing hyaluronic acid. This method is regarded as providing physiologically-relevant information about the ability of spermatozoa to colonize the oviduct and penetrate oocytes during *in vitro* fertilization [19].

2. Materials and methods

2.1. Reagents and animal regulation

All the products were obtained from Sigma (Madrid, Spain), except Equex STM Paste (Minitüb, Tiefenbach, Germany); bovine recombinant HSPA8 was provided by Dr. Fazeli (The Academic Unit of Reproductive and Developmental Medicine, University of Sheffield, UK).

2.2. Animals and sample collection

Semen samples from eight sexually mature male Brown bears were obtained by electroejaculation during the breeding season (late April to early July). Animals were housed in a half-freedom regime in Cabárceno Park (Cantabria, Spain; 43° 21' N, 3° 50' W, altitude: 142 m), and fed with a diet based on chicken meat, bread, and fruit. Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65.

The males were immobilized by teleanesthesia, using 750 mg of zolazepam HCl with tiletamine HCl (Zoletil100; Virbac, Carros, France) and 6 mg of medetomidine (Zalopin, Orion Pharma Animal Health, Finland; 10 mg/mL). After immobilization, the males were weighed and monitored (pulse, oxygen saturation, and respiration). Before electroejaculation, the prepuce was shaved and washed with physiological saline serum, and the rectum was emptied of feces. The bladder was catheterized during semen collection to prevent urine contamination. Electroejaculation was carried out with a PT Electronics electroejaculator (Boring, OR, USA). The transrectal probe was 320 mm long with a diameter of 26 mm. Electric stimuli were given until ejaculation (10 V and 250 mA, on average).

To prevent urine contamination or low cellular concentration, the ejaculates were collected as isolated fractions in 15 mL graduated glass tubes. Immediately after collection, the volume of each fraction was recorded, osmolality was measured using a cryoscopic osmometer Osmomat-030 (Gonotec; Berlin, Germany) and the pH value was determined by a CG 837 pH meter (Schott Instruments, Mainz, Germany). For each fraction, subjective motility was assessed with a phase contrast microscope (magnification $\times 100$) and urospermia checked by DiaSys Ecoline test (Diagnostics Systems GmbH, Holzheim, Germany). Fractions with a reduced concentration ($<150 \times 10^6$ cells per mL), low motility ($<50\%$), or contaminated urine (>80 mg urea per dL) were rejected. All valid fractions of the same electroejaculation were mixed and constituted one ejaculate. The selected samples were centrifuged at $600 \times g$ for 6 minutes and the pellet was processed according to the experimental design.

2.3. Experimental design

Two extenders were prepared. The control extender (EY) for refrigeration storage was N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid-TRIS-fructose 300 mOsm/kg, pH 7.1, with 2% EDTA and 1% Equex STM paste with 20% egg yolk (based on Anel et al. [4], without glycerol).

The EY for cryopreservation was the same as for refrigeration process but 6% (vol/vol) glycerol was added (Anel et al. [4], modified by de Paz et al. [10]).

2.3.1. Experiment 1. Use of HSPA8 for the refrigeration storage (5 °C) of semen samples

The pellets were divided into four aliquots and extended 1:1 with four different extenders: EY (0 µg/mL HSPA8), EY with 0.5 µg/mL HSPA8 (HA), EY with 1 µg/mL HSPA8 (HB), and EY with 5 µg/mL HSPA8 (HC). The tubes with the extended samples were put into beakers containing 100 mL of water at room temperature (22 °C) and transferred to a refrigerator, where samples were slowly cooled (−0.25 °C per minute) to 5 °C. Then, the samples were diluted with the appropriate volume of each extender to achieve the final sperm concentration (100×10^6 sperm per mL). After that, the samples were refrigerated (5 °C) to 48 hours and analyzed for motility and membrane status parameters at 0, 24, and 48 hours (see section 2.4.).

2.3.2. Experiment 2. Use of HSPA8 in the cryopreservation of semen samples

The samples were divided in four aliquots and extended 1:1 with four different extenders: EY, HA, HB, and HC. The tubes with the extended samples (1:1 with 3% glycerol extender [N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid-TRIS-fructose 300 mOsm/kg, pH 7.1, with 2% EDTA and 1% Equex STM paste] with 20% egg yolk [4]) were put into beakers containing 100 mL of water at room temperature (approximately 22 °C) and transferred to a refrigerator, where they were slowly cooled (−0.25 °C per minute) to 5 °C. Then, the samples were diluted with the same volume of each extender containing 9% glycerol, to achieve the final glycerol concentration of 6% [10]. Final sperm concentration (100×10^6 spermatozoa per mL) was achieved by adding the appropriate volume of each extender to the 6% glycerol. After packaging into 0.25-mL plastic straws and equilibrating for 1 hour at 5 °C, the samples were frozen in a programmable biofreezer (Kryo 10 Series III; Planer Plc., Sunbury-On-Thames, UK) at −20 °C per minute to −100 °C, and then transferred to liquid nitrogen containers. The cryopreserved samples remained in liquid nitrogen for a minimum of 1 week. Thawing was performed by dropping the straws into water at 65 °C for 6 seconds. After that, an aliquot of sperm sample was analyzed (See section 2.4.) and another aliquot was subjected to a thermal stress test, incubating to 37 °C over 2 hours, to check for latent damage to the sperm (adapted from Alvarez-Rodríguez et al. [7]).

2.3.3. Experiment 3. Assessment of frozen-thawed spermatozoa using a hyaluronic acid migration test

The sperm migration test was carried out immediately after thawing the samples. The sperm samples (200 µL [100×10^6 sperm per mL]) were placed in small glass tubes. Then, flat capillary tubes (VITROTUBES capillary [Mod. 3520-050 (0.20 × 2.00 × 50 mm); CM Scientific Ltd., Silsden, UK]) were filled with a solution of hyaluronic acid: 4 mg/mL hyaluronic acid (Sigma-Aldrich; 53747; 1.5 to 1.8×10^6 d) in a PBS buffer containing 0.05% BSA (to avoid sperm agglutination). The capillaries were placed inside the glass tube containing the sperm suspension and incubated at 39 °C for 10 minutes. Thereafter, sperm progression was assessed under

a phase-contrast microscope (magnification $\times 200$) (Olympus BH2; negative-high phase contrast). The distance in mm traveled by the vanguard spermatozoon (spermatozoa capable of achieving the greatest distance in the capillary) and the number of spermatozoa per capillary tube accumulated between 0.5 and 2 cm from the base of the capillary were measured. The first segment (0 to 0.5 cm) was discarded because of sperm concentration because of direct sample contact, which was not significant to real sperm progression [20]. Sperm counts that progressed beyond 2 cm were irrelevant.

2.4. Semen evaluation

Sperm quality was evaluated at different points: refrigeration storage times (0, 24, and 48 hours) for experiment 1 and before freezing and after thawing (postthawing and after 2-hour incubation [37 °C]) for experiment 2.

2.4.1. Sperm motility

The assessment of motility parameters was performed using a computer assisted semen motility analysis system (Integrated Semen Analyser System; Proiser, Valencia, Spain). The standard settings of the computer assisted sperm analysis used correspond to the values of dog spermatozoa defined by the Integrated Semen Analyser System (Proiser). The settings used to define progressive motility are specific to bears and are defined by our previous experience [4]. Samples were diluted (10 to 20×10^6 cells per mL) in buffered medium (HEPES 20 mm/L, 197 mmol/L NaCl, 2.5 mmol/L KOH, 10 mmol/L glucose, 1% egg yolk; pH 7; 300 mOsm/kg), and warmed on a 37 °C plate for 5 minutes. Then, a 5 µL sperm sample was placed into a Makler cell counting chamber (10 µm depth; Sefi Medical Instruments, Haifa, Israel). The sample was examined at magnification $\times 100$ (negative phase contrast) in a microscope with a warmed stage (38 °C). The standard parameter settings were set at 25 frames per second, 5 to 50 µm² for head area and curvilinear velocity (VCL) >10 µm/s to classify a spermatozoon as motile. At least five sequences or 200 spermatozoa were saved and analyzed afterward. Reported parameters were average velocity (µm/s) and VCL (µm/s). Total motility was defined as the percentage of spermatozoa with VCL >10 µm/s, and progressive motility was defined as the percentage of spermatozoa with VCL >25 µm/s and straightness $>80\%$ (also provided by the system).

2.4.2. Flow cytometric evaluation

Viability and acrosomal status were assessed using the double stain peanut agglutinin-fluorescein isothiocyanate conjugate (PNA-FITC)/propidium iodide (PI) and flow cytometry. Stock solutions of the fluorochromes were prepared in PBS at 1 µg/mL PNA-FITC and 1.5 µM PI. Sperm samples were diluted with fluorochrome solution down to 5×10^6 spermatozoa per mL in polypropylene tubes (300 µL per tube). The flow cytometry analysis yielded the percentage of spermatozoa viable and with intact acrosomes (PI−) and the percentage of spermatozoa with damaged acrosomes (PNA-FITC+).

Early changes in plasma membrane permeability was assessed with 0.1 μM YO-PRO-1 and 1.5 μM PI. This probe allows us to evaluate the increment in membrane permeability that usually occurs after refrigeration or cryopreservation processes.

Flow cytometry evaluations were carried out using a FACScalibur flow cytometer (Becton Dickinson BioSciences,

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; PI) and green fluorescence (FL1 photodetector; YO-PRO-1 and FITC) of acquired particles were recorded. In all the cases we assessed

Fig. 1. Motility parameters for the use of heat shock protein A8 (HSPA8) protein in the refrigeration storage (5 °C) of semen samples (mean $\pm$ SEM). Extenders: N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid-TRIS-fructose-20% egg yolk with 0 $\mu\text{g/mL}$ HSPA8 (EY), EY with 0.5 $\mu\text{g/mL}$ HSPA8 (HA), EY with 1 $\mu\text{g/mL}$ HSPA8 (HB), and EY with 5 $\mu\text{g/mL}$ HSPA8 (HC). Time represents the refrigeration time (hours) of the samples at 5 °C (0, 24, and 48 hours). Motility parameters: total motility (TM; %), progressive motility (PM; %), average velocity (VAP; $\mu\text{m/s}$), and curvilinear velocity (VCL; $\mu\text{m/s}$). * Differences ($P < 0.05$) respect to the control (EY).

10,000 events per sample, with a flow rate of 200 cells per second.

2.5. Statistical analysis

Data were analyzed using the SAS V.9.1. package (SAS Institute Inc., Cary, NC, USA). Results are shown as mean and standard error of the mean, unless otherwise stated. Residuals were tested for normality (Shapiro–Wilk test). The data of progression tests were normally distributed. Percentage data were arcsin square-root transformed when necessary. Analyses of the prefreezing, postthawing, and migration test data were carried out using linear mixed-effects models (MIXED procedure, ML method). Models were built with (1) refrigeration storage variables (concentrations of HSPA8 and storage time) or (2) freezing–thawing variables (concentrations of HSPA8 and test time) or (3) progression test variables (concentrations of

HSPA8) as fixed effect and males and samples in the random part of the model (random effect). Significant fixed effects were further analyzed using multiple comparisons of means with Tukey contrasts. A significance level of $P < 0.05$ was used.

The relationships between the result of the vanguard spermatozoon test and the HSPA8 concentration in extender was analyzed using a univariate test of significance for planned comparison with some specific contrasts within the ANOVA procedure.

3. Results

The fresh Brown bear semen samples showed the following values for volume, pH, osmotic pressure, and cellular concentration (mean $\pm$ SEM): 4.5 ± 1.0 mL, 8.00 ± 0.20 , 294.3 ± 4.9 mOsm/kg, and $229.3 \pm 88.5 \times 10^6$ spermatozoa per mL.

Fig. 2. Flow cytometry parameters for the use of heat shock protein A8 (HSPA8) protein in the refrigeration storage (5 °C) of semen samples (mean $\pm$ SEM). Extenders: N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid-TRIS-fructose-20% egg yolk with 0 μ g/mL HSPA8 (EY), EY with 0.5 μ g/mL HSPA8 (HA), EY with 1 μ g/mL HSPA8 (HB), and EY with 5 μ g/mL HSPA8 (HC). Times represent the refrigeration time (hours) of the samples at 5 °C (0, 24, and 48 hours). Flow cytometry analysis: viable and with intact acrosomes (VIAB) (propidium iodide [PI]–); viable sperm and acrosomes (ACR) (Peanut agglutinin-Fluorescein isothiocyanate conjugate); number of damaged acrosomes and changes to membrane permeability (APOPT) (YO-PRO-1+); sperm with early changes in their membrane (apoptotic-like). * Differences ($P < 0.05$) with respect to the control (EY).

3.1. Experiment 1. Use of HSPA8 in the refrigeration storage (5 °C) of semen samples

Motility parameters (Fig. 1) showed higher values (HC with respect to the control; $P < 0.05$) at 0 hours for total motility (Fig. 1A) and 24 hours in HB. Progressive motility at 48 hours (Fig. 1B) showed lower values for HB and HC.

In addition, viability (Fig. 2A) showed higher values in HA at 0, 24, and 48 hours ($P < 0.05$), and also HC at 48 hours, with respect to the control. Number of sperm with damaged acrosomes (Fig. 2B) showed lower values ($P < 0.05$) for all concentrations of protein at 48 hours and for HC at 24 hours. Apoptotic index measured with YO-PRO-1 (Fig. 2C) showed lower values at 0 hours for all concentrations of HSPA8 ($P < 0.05$).

3.2. Experiment 2. Use of HSPA8 in the cryopreservation of semen samples

Prefreezing motility parameters (Fig. 3) and membrane status (Fig. 4) showed no differences.

Also, no differences in postthaw motility parameters were found (Fig. 3), but early changes to membrane permeability showed lower values for HB after a 37 °C incubation test over 2 hours (Fig. 4C). In contrast, immediately after thawing HC showed higher value of changes to membrane permeability. Moreover, HB and HC showed lower values after the incubation test.

3.3. Experiment 3. Assessment of frozen-thawed spermatozoa using a hyaluronic acid migration test

Results for the maximum distance that one single spermatozoon was able to travel showed that the lower HSPA8 concentration significantly reduced the distance traveled by the vanguard sperm with respect to control (Fig. 5A) but that the higher HSPA8 concentrations restored the progressive ability of this sperm with respect to the control. In contrast, univariate tests of the vanguard spermatozoon results (Fig. 6) showed a significant linear relationship (dose-dependent) between HA, HB, and HC ($P < 0.05$).

The total number of sperm measured in a specific distance (from 0.5 cm to 2 cm) (Fig. 5B) showed

Fig. 3. Motility prefreezing, postthawing, and 2-hour incubation (37 °C) parameters for the use of heat shock protein A8 (HSPA8) in the cryopreservation of semen samples (mean $\pm$ SEM). Extenders: N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid-TRIS-fructose-20% egg yolk with 0 μ g/mL HSPA8 (EY), EY with 0.5 μ g/mL HSPA8 (HA), EY with 1 μ g/mL HSPA8 (HB), and EY with 5 μ g/mL HSPA8 (HC). Motility parameters: total motility (TM; %), progressive motility (PM; %), average velocity (VAP; μ m/s), and curvilinear velocity (VCL; μ m/s). * Differences ($P < 0.05$) with respect to the control (EY).

Fig. 4. Flow cytometry prefreezing, postthawing, and 2-hour incubation (37 °C) parameters for the use of heat shock protein A8 (HSPA8) in the cryopreservation of semen samples (mean $\pm$ SEM). Extenders: N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid-TRIS-fructose-20% egg yolk with 0 μ g/mL HSPA8 (EY), EY with 0.5 μ g/mL HSPA8 (HA), EY with 1 μ g/mL HSPA8 (HB), and EY with 5 μ g/mL HSPA8 (HC). Flow cytometry analysis: viable and with intact acrosomes (VIAB) (propidium iodide [PI]–) viable sperm, acrosomes (ACR) (Peanut agglutinin-Fluorescein isothiocyanate conjugate); number of damaged acrosomes and changes to membrane permeability (APOPT) (YO-PRO-1+); sperm with early changes in their membrane (apoptotic-like). * Differences ($P < 0.05$) respect to the control (EY).

a significant decrease of the number of spermatozoa for all the concentrations of HSPA8, with respect to the control.

4. Discussion

This study evaluated the semen quality after of the use of HSPA8 protein (0.5, 1, and 5 μ g/mL) as an additive in the refrigeration and cryopreservation of Brown bear sperm samples in order to improve the sperm preservation in this species. Moreover, we carried out a sperm progression test to evaluate the effect of protein addition on sperm motility.

In the first experiment, progressive motility showed a significant decrease at 48 hours of refrigeration after the use of HSPA8 protein, but HSPA8 showed also a significant enhancement of viability at the lower concentration 0.5 μ g/mL for 0, 24, and 48 hours of refrigeration storage, and by 48 hours the number of acrosomes damaged was significantly reduced for all concentrations. The effect on Brown bear sperm refrigeration was consistent with the results obtained for other species: boar and bull (studies carried out at 38 °C) [16] and ram (studies carried out at 5 °C and 38 °C) [17]. We interpret this result as being a consequence of the conserved nature of this protein across species [18]. Heat shock protein A8 is a member of the HSP70 family of proteins (70 kDa), and is regarded as among the most conserved protein families in evolution. It is found in all

organisms from archabacteria and plants to humans, and the prokaryotes.

One of the objectives of the present study was to assay the protective activities of the protein by exogenous addition and to optimize the amount of protein needed for sperm quality preservation. Results from the refrigeration study were encouraging and showed a beneficial effect on viability that confirmed the potential role of HSPA8 on maintaining the quality of Brown bear samples. There were few improvements of the flow cytometry parameters when these were assessed immediately after thawing, but the 2-hour incubation stress test revealed that the HSPA8 significantly protected the sperm plasma membrane against permeabilization (HB extender) and reduced the proportion of sperm with damaged acrosomes (HB and HC extender). These results suggest improved survival of frozen-thawed spermatozoa in the female reproductive tract, like the study on cryopreserved ram sperm which concluded that the addition of seminal plasma proteins increased the sperm resistance to damage from cold shock [21]. In this sense, the severely shortened lifespan of cryopreserved spermatozoa within the female tract is a major cause of poor fertility and the ability of HSPA8 to protect spermatozoa against cold injury could help improve this result.

Spermatozoa are not capable of synthesizing heat shock proteins (HSPs) in response to stress, unlike somatic cells,

Fig. 5. Data (mean $\pm$ SEM) of migration test of frozen-thawed sperm through artificial mucus (hyaluronic acid). Extenders: N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid-TRIS-fructose-20% egg yolk with 0 μ g/mL HSPA8 (EY), EY with 0.5 μ g/mL HSPA8 (HA), EY with 1 μ g/mL HSPA8 (HB), and EY with 5 μ g/mL HSPA8 (HC). (A) Vanguard spermatozoon (the linear distance covered by the foremost sperm cell) and (B) total number of sperm counted between 0.5 cm and 2 cm of capillary tube. * Differences ($P < 0.05$) with respect to the control (EY).

because ejaculated spermatozoa are transcriptionally inactive. Therefore, they depend on preformed HSPs for protection against various stresses [22]. But the HSP might be outside the sperm, being produced by the oviduct. Thus, it has been reported that when sperm reach the oviduct they stimulate the upregulation of HSPA8 and that it is translocated into the extracellular environment (the lumen of the oviduct) [23]. This extracellular HSP activity has been increasingly recognized and studied over the past few years [15,24]. In addition, it seems that HSPA8 interacts with lipid rafts and cholesterol, and this might be important in terms of protecting the sperm [25].

On the other hand, another possible role of the HSP70 family is inhibition of the accumulation of protein aggregates, thus removing the stimulus that might constitute an apoptosis signaling pathway for the cells [26]. The early changes in plasma membrane permeability (apoptotic-like changes) obtained in refrigeration storage (experiment 1),

Fig. 6. Linear distance covered by the vanguard spermatozoon (foremost sperm cell) (mean $\pm$ SEM) obtained in the migration test of frozen-thawed sperm through artificial mucus (hyaluronic acid) analyzed with the extenders, N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid-TRIS-fructose-20% egg yolk with 0.5 μ g/mL heat shock protein A8 [HSPA8] (HA), HA with 1 μ g/mL HSPA8 (HB), and HA with 5 μ g/mL HSPA8 (HC). Line represents a significant linear relationship between HA, HB, and HC (univariate test with specific contrast, $P = 0.0077$).

showed lower value for all concentrations at 0 hours and a slight tendency to decrease the apoptotic index at the other refrigeration times (24 and 48 hours). In contrast, the cryopreservation process showed no clear tendency to decrease the apoptotic index, except after the thermal stress test in which we obtained a protective effect of 1 μ g/mL, whereas the higher concentration produced a detrimental effect on sperm immediately after thawing. In terms of number of acrosomes damaged, results showed a significant decrease in values for both HB and HC after the incubation test. One explanation of these differences could be that the constant changes in sperm membrane physiology during this process, together with the egg yolk and glycerol present in the extender could interfere in the protein activity. In this regard, a freezing experiment without egg yolk and glycerol might be a good assay to test the isolated effect that the protein could have on sperm protection during cryopreservation.

We carried out a simple test using hyaluronic acid in order to see whether the presence of HSPA8 might improve the ability of spermatozoa to migrate through a viscous medium. Hyaluronic acid was chosen for this experiment because some authors have suggested that normal spermatozoa in other species have hyaluronic acid-binding receptors (CD44) [27]. Moreover, hyaluronic acid has been shown to stimulate human sperm motility by increasing intracellular Ca^{2+} concentration [28]. In practical applications, studies carried out with human spermatozoa showed that effective selection of spermatozoa without DNA fragmentation could be achieved using hyaluronic acid binding [29] and that there are some beneficial effects on intracytoplasmic sperm injection outcomes if hyaluronic acid was used in a sperm selection step [30].

The effects of hyaluronic acid sperm selection in this experiment were especially interesting because there were two different and distinct outcomes. As a whole sperm

population the functional motility was systematically suppressed by the presence of HSPA8. This effect was significantly dose-dependent and was detectable at the lowest dose tested. Paradoxically, however, the maximum distances traveled within the capillary tubes by the vanguard sperm revealed the opposite tendency. A very clear positive relationship between maximum distance and the HSPA8 concentration was observed; this implies that a distinctive but small sperm subpopulation present within the bulk samples could retain its functional swimming ability when cryopreserved in the presence of 1 and 5 $\mu\text{g}/\text{mL}$ of HSPA8. This is not unlike the situation inside the oviduct, where most sperm are not highly active, and a small number are sufficiently active to progress toward the oocyte for fertilization [11]. These effects could also be because of selective blocking by HSPA8 of the passage of the poorer motility sperm, and allowing some high motility sperm to enhance their motility. This interpretation is consistent with the reduced polyspermy rate that is found in porcine IVF when spermatozoa are exposed to HSPA8 before, or during, IVF [16].

The effect of the HSPA8 protein might therefore be related to a poorly defined mechanism that allows spermatozoa to remain within the sperm reservoir in the female tract. The protein reduces the motility of the overall sperm population, but the vanguard sperm must represent a very special subpopulation, which can be “rescued” by the protein. In this sense, the nonmotile sperm could be differentially sensitive to inhibitory signaling activity from the HSPA8 [31]. But further studies should be carried out to determine whether the effect of HSPA8 might be linked to the establishment and maintenance of the oviductal sperm reservoir.

In vitro tests involving sperm migration through capillary tubes have been used previously to examine sperm quality in several species: in goats, Cox et al. [19] observed a correlation between migration distance and the ability to colonize the oviduct and penetrate eggs during *in vitro* fertilization. These authors also found that sperm migration efficiency in homologous mucus could be related to velocity parameters, linearity and lateral head displacement of the vanguard sperm population. Other authors (bull [32]; bovine [33]) have suggested that there is no relationship between mucus penetration and field fertility, but these results could be influenced by the use of methyl cellulose instead of hyaluronic acid [32] and the elaboration of pools of samples that varied between 10 and 210 straws per sample for each insemination [33].

4.1. Conclusions

We have presented some preliminary evidence to show that low concentrations of HSPA8 (0.5 $\mu\text{g}/\text{mL}$) could be useful for improving existing diluents for Brown bear semen refrigeration storage. Postthawing results showed a beneficial effect of the protein in improving the acrosomal status, and also a protective effect of HSPA8 (1 $\mu\text{g}/\text{mL}$) on sperm submitted to a thermal stress test (37 °C during 2 hours). The thermal stress response was improved and the migration efficiency of a small population was also improved by the addition of HSPA8 to Brown bear sperm extender.

Acknowledgments

This work was supported in part by MICINN (CGL 2010-19213/BOS) and CANTUR S.A. Felipe Martínez-Pastor was supported by the Ramón y Cajal program (RYC-2008-02560, MICINN, Spain). The authors thank Miguel Ángel Marañón and the game keepers of the Cabárceno Nature Park, María Mata Campuzano, Julio Tamayo Canul, Leticia Ordás Bandera, Carmen Martínez Rodríguez, Susana Gomes Alves, María Nicolás, Elena López Urueña, and Patricia Manrique Revuelta and Alireza Fazeli's lab members of the Department of Human Metabolism in Sheffield, for their help in the artificial mucus test.

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CAPÍTULO 10

**PUBLICACIÓN 5. BROWN BEAR SPERM DOUBLE FREEZING:
EFFECT OF ELAPSED TIME AND USE OF PURESPERM®
GRADIENT BETWEEN FREEZE-THAW CYCLES**

Brown bear sperm double freezing: effect of elapsed time and use of PureSperm® gradient between freeze-thaw cycles

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Article
Full Title:	Brown bear sperm double freezing: effect of elapsed time and use of PureSperm® gradient between freeze-thaw cycles
Short Title:	Brown bear sperm double freezing
Corresponding Author:	Paulino de Paz Universidad de León León, SPAIN
Keywords:	brown bear, consecutive freezing-thawing cycles, sperm cryopreservation, PureSperm
Abstract:	Sex pre-selection of offspring through the use of sexed spermatozoa has great potential as a captive population management strategy for endangered wildlife. The practical problem of this is that the sex-sorting facility is a long distance from the semen collection position and to resolve this problem two repeated freeze-thaw cycles may be necessary. In the present study, we analyze different effects of refreezing on quality parameters of brown bear electroejaculated samples. We carried out two different experiments: 1) to assess the effects of the two freezing-thawing cycles on sperm quality and to analyze three different elapsed times between freezing-thawing cycles (30, 90 and 180 min), and 2) to analyze the efficacy of the use of PureSperm between freezing-thawing cycles to select sperm subpopulations that could improve the sperm freezability. The motility parameters, viability and acrosomal status of spermatozoa were evaluated immediately before freezing and after thawing on both cycles. The motility, viability and undamaged acrosomes were significantly reduced after the second thawing respect to first thawing into each elapsed time group, but the elapsed times did not significantly affect the viability and acrosome status although motility was damaged. Our results showed higher values of viability in pellet sample respect to the rest of the groups and it also showed a significant decrease in the number of acrosome damaged. This effect was highlighted just after the gradient centrifugation, where the motility and viability was superior in comparison to the non-selected sample. In summary, the double freezing of bear semen selected by gradient centrifugation adversely affects the quality of sperm but could be useful to carry out a sex-sorting of frozen-thawed bear spermatozoa before to send to biobank the cryopreserved sample.
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**Brown bear sperm double freezing: effect of elapsed time and use of
PureSperm® gradient between freeze-thaw cycles**

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Short title: Brown bear sperm double freezing

Abstract

Sex pre-selection of offspring through the use of sexed spermatozoa has great potential as a captive population management strategy for endangered wildlife. The practical problem of this is that the sex-sorting facility is a long distance from the semen collection position and to resolve this problem two repeated freeze-thaw cycles may be necessary. In the present study, we analyze different effects of refreezing on quality parameters of brown bear electroejaculated samples. We carried out two different experiments: 1) to assess the effects of the two freezing-thawing cycles on sperm quality and to analyze three different elapsed times between freezing-thawing cycles (30, 90 and 180 min), and 2) to analyze the efficacy of the use of PureSperm between freezing-thawing cycles to select sperm subpopulations that could improve the sperm freezability. The motility parameters, viability and acrosomal status of spermatozoa were evaluated immediately before freezing and after thawing on both cycles. The motility, viability and undamaged acrosomes were significantly reduced after the second thawing respect to first thawing into each elapsed time group, but the elapsed times did not significantly affect the viability and acrosome status although motility was damaged. Our results showed higher values of viability in pellet sample respect to the rest of the groups and it also showed a significant decrease in the number of acrosome damaged. This effect was highlighted just after the gradient centrifugation, where the motility and viability was superior in comparison to the non-selected sample. In summary, the double freezing of bear semen selected by gradient centrifugation adversely affects the quality of sperm but could be useful to carry out a sex-sorting of frozen-thawed bear spermatozoa before to send to biobank the cryopreserved sample.

Keywords: brown bear, consecutive freezing-thawing cycles, sperm cryopreservation, PureSperm.

1. Introduction

The establishment of brown bear Genome Resource Banks (GRBs) required the development of a specific methodology for freezing brown bear semen [1] allowing the reproductive potential of each male to be maximised. The collection and handling of samples in wildlife is complex, and thus the different technologies must be adapted to these species.

The Cantabrian brown bear (*Ursus arctos*) is the last indigenous brown bear population in the Iberian Peninsula. Due to habitat loss and population fragmentation (approximately 150 individuals restricted to two isolated populations in the North of Spain) it is considered to be at risk of extinction (Real Decreto 439/1990, Regulation of the National Catalogue of Endangered Species).

Unbalanced sex ratios can have negative effects on small population of wildlife, and sex pre-selection of offspring through the use of sexed spermatozoa has great potential as a captive population management strategy for endangered wildlife [2].

The practical problem of this is that the sex-sorting facility is a long distance from the semen collection position and to resolve this problem two repeated freeze-thaw cycles may be necessary. The benefits of the double freezing technique for endangered species conservation are enormous [3], [4].

Studies of sperm re-freezing have been carried out in several species (bull [3], [4]; stallion [5], [6]; ram [7]; mouse [8] evaluating the motility and viability of sperm following two freeze-thaw cycles. During the time elapsed between the two freezing-thawing cycles, the thawed sample undergoes changes that can affect sperm viability. When motility, viability and acrosome integrity of bull spermatozoa were compared, conventional single freezing was significantly superior to the double freezing [4]. Nevertheless, these studies showed that due to improvements in assisted reproductive techniques, the amount of sperm needed for the insemination has decreased and they could freeze straws with less viable cells concentration in the second cycle without detrimental effect in final fertility.

Sperm selection techniques could be a good alternative to minimize the percentage of damaged spermatozoa from the first cycle but these techniques involve a significant decrease in terms of sperm recovery after the gradient selection [9], [10]. The effects of different extenders, dilution rates and factors of centrifugation on the recovery of brown bear spermatozoa and semen quality parameters after thawing have been evaluated [11], [12].

Density gradient centrifugation has been widely used to select motile sperm subpopulations and to remove bacterial contamination [13]. PureSperm® (Nidacom, Gothenburg, Sweden) is a product designed for the density gradient centrifugation that selects viable and morphologically intact human spermatozoa in Assisted Reproduction Technology [14]. PureSperm has been previously tested for brown bear sperm and it is suitable for improving spermatozoa qualitative characteristics in fresh and post-thawed semen [15]. In summary, the double freezing of selected spermatozoa by density gradient can increase possibilities in the management of cryopreserved spermatozoa from a Genome Resource Banks.

Thus, the two main objectives of this study were: (a) to assess the effects of the two freezing-thawing cycles on sperm quality and to analyze three different elapsed times between freezing-thawing cycles (30, 90 and 180 min), and (b) to assess the efficacy of using of PureSperm between freezing-thawing cycles to select sperm subpopulations that could improve the sperm freezability.

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2. Materials and methods

2.1 Reagents and animal regulation

All the products were obtained from Sigma (Madrid, Spain), except the Equex STM Paste (Minitüb, Tiefenbach, Germany).

2.2. Animals and sample collection

Semen samples from 18 sexually mature male brown bears were obtained by electroejaculation during the breeding season (late April to early July). The animals were housed in a half freedom regime in Cabárceno Park (Cantabria, Spain; 43° 21' N, 3° 50' W, altitude: 142 meters), and fed with a diet based on chicken meat, bread and fruits. Animal manipulations were performed in accordance with Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65.

The males were immobilized by teleanaesthesia, using 750 mg of zolazepam HCl+tiletamine HCl (Zoletil100®; Virbac, Carros, France) and 6 mg of medetomidine (Zalopin®, Orion Pharma Animal Health, Finland, 10 mg/mL). After immobilization, the males were weighed and monitored (pulse, oxygen saturation and respiration). Prior to electroejaculation, the prepucial area was shaved and washed with physiological saline serum, and the rectum was emptied of feces. The bladder was catheterized during semen collection to prevent urine contamination. Electroejaculation was carried out with a PT Electronics® electroejaculator (PT Electronics, Boring, OR, USA). The transrectal probe was 320 mm long with a diameter of 26 mm. Electric stimuli were given until ejaculation (10 V and 250 mA, on average).

To prevent urine contamination or low cellular concentration, the ejaculates were collected as isolated fractions in 15 mL graduated glass tubes. Immediately after collection, the volume of each fraction was recorded, osmolality was measured using a cryoscopic osmometer Osmomat-030 (Gonotec™, Berlin, Germany) and the

pH value was determined by a CG 837 pH meter (Schott Instruments, Mainz, Germany). For each fraction, subjective motility was assessed with a phase contrast microscope (100x) and urospermia evaluated using Urea test strips, (DiaSys Ecoline® GmbH, Holzheim, Germany). Fractions with a reduced concentration ($<200 \times 10^6$ cell/mL), low motility ($< 50\%$) or contaminated urine (>80 mg urea/dL) were rejected. All valid fractions of the same electroejaculation were mixed and constituted one ejaculate. The selected samples were centrifuged at $600 \times g$ for 6 minutes and the pellet was processed according to the experimental design.

2.3. Cryopreservation protocol

The cryopreservation process was carried out following our standard protocol ([16] modified by [17]). The tubes with the 1:1 extended samples (3% glycerol) were put in glasses containing 100 mL of water at room temperature and transferred to a refrigerator, where they were slowly cooled down to 5 °C ($-0,25$ °C/min). Then, the samples were diluted with the same volume of each extender containing 9% glycerol, to achieve the final glycerol concentration of 6%. Final sperm concentration (100×10^6 spermatozoa/mL) was achieved by adding the appropriate volume of each extender to the 6% glycerol. After packaging into 0.25-mL plastic straws and equilibrating for 1 h at 5°C, the samples were frozen in a programmable biofreezer (Kryo 10 Series III; Planer plc., Sunbury-On-Thames, UK) at $-20^\circ\text{C} / \text{min}$ down to -100°C , and then transferred to liquid nitrogen containers. The cryopreserved samples remained in liquid nitrogen for a minimum of two weeks. The straws were thawed by dropping them in water at 65°C for 6 s.

2.4. Experimental design

2.4.1. Experiment 1. Re-freezing effects (30 min). Effects of elapsed time between freezing/thawing cycles on thawed sample (30, 90 and 180 min).

For this experiment, we used 20 ejaculates from 18 males. After thawing, the sperm sample (~ 1.2 mL of final volume) was divided into three aliquots each of

which was maintained at room temperature ($\sim 22^{\circ}\text{C}$) for a different time (30, 90 and 180 min). After of the different elapsed times, each sample was processed for a second cryopreservation applying the same procedure as in the first freezing-thawing cycle (see 2.3. Cryopreservation protocol). The samples were evaluated at four points during treatment: 1) Before the first freezing cycle (P1), 2) After first thawing (T1), 3) After the time elapsed at room temperature before the second freezing cycle (P2) and 4) After second thawing (T2).

2.4.2. Experiment 2. Use of PureSperm® between freezing/thawing cycles.

PureSperm® gradient was used for selecting 8 brown bear semen samples following the protocol used by our group [15]. After thawing eight straws, the sperm sample ($\sim 1,6$ mL of final volume) was divided into four aliquots corresponding to Control (CTL; room temperature storage without any experimental process), Centrifuged Control (CTL_W; subjected to the centrifugation process ($600\times g/20$ min) to evaluate its possible detrimental effect on sperm samples), Pellet (PS_P; pellet obtained after the sample was treated using PureSperm® density gradient centrifugation) and Interphase (PS_I; obtained after PureSperm® density gradient centrifugation in a phase between two gradient concentrations of PureSperm®). Each sample was then processed for a second cryopreservation (see 2.3. Cryopreservation protocol). The samples were evaluated as in experiment 1.

2.5. Semen evaluation

Sperm quality was evaluated immediately before freezing and after thawing in both processes (first and second freezing-thawing processes). The motility parameters were assessed by a computer assisted semen motility analysis system (ISAS, Integrated Semen Analyser System; Proiser, Valencia, Spain). The standard settings of the CASA used correspond to the values of dog spermatozoa defined by ISAS. The settings used to define progressive motility are specific to bears and are defined by our previous experience [16]. Samples were warmed on a 37°C plate

for 5 min. Then, a 5- μ L sperm sample was placed into a Makler cell counting chamber (10 μ m depth; Sefi Medical Instruments, Haifa, Israel). The sample was examined at $\times 100$ (negative phase contrast) in a microscope with a warmed stage (38 $^{\circ}$ C). The standard parameter settings were set at 25 frames/s, 5 to 50 μ m² for head area and VCL > 10 μ m/s to classify a spermatozoon as motile. At least five sequences or 200 spermatozoa were saved and analyzed afterwards. Computer-assisted sperm analysis provides the following parameters: total motility (TM, %; was defined as the percentage of spermatozoa with VCL > 10 μ m/s), progressive motility (PM, %; spermatozoa were considered progressive if VCL>25 and STR>80%), average path velocity (VAP, μ m/s), curvilinear velocity (VCL, μ m/s), linearity (LIN, %) and amplitude of lateral head displacement (ALH, μ m).

Sperm viability and acrosomal status were assessed using the double stain PNA-FITC/PI and flow cytometry [18]. Stock solutions of the fluorochromes were prepared in PBS at 1 μ g/mL PNA-FITC and 6 μ M PI. Sperm samples were diluted with fluorochrome solution down to $1\text{--}2\times 10^6$ spermatozoa/mL in polypropylene tubes (300 μ L/tube). The flow cytometry analysis yielded the percentage of viable spermatozoa (PI–, named VIAB) and the percentage of spermatozoa with damaged acrosomes (PNA-FITC+, named dACR).

Flow cytometry evaluations were carried out using a FACScalibur flow cytometer (Becton Dickinson BioSciences, 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/s.

Recovery rates were used in two different ways in the present study: a) recovery rate of spermatozoa quality, defined for each parameter as post-thawing 2 data /post-thawing 1 data ($\times 100$); and b) recovery rate of spermatozoa quantity defined

as total number of spermatozoa after PureSperm® /total number of spermatozoa before PureSperm® (x100).

2.6. Statistical analysis

Data were analyzed using the SASTM V.9.1. package (SAS Institute Inc., Cary, NC, USA). Results are shown as means and standard errors of the mean. Residuals were tested for normality (Shapiro-Wilk test). Percentage data were arcsin square-root transformed when necessary. Analyses of the data were carried out using linear mixed-effects models (MIXED procedure, ML method). Models were built with (1) elapsed time between freezing/thawing cycles or (2) PureSperm gradient as fixed effect, and males and samples in the random part of the model (random effect). Significant fixed effects were further analysed using multiple comparisons of means with Tukey contrasts. A significance level of $P < 0.05$ was used.

3. Results

The fresh brown bear semen samples showed the following parameters of quality (mean $\pm$ SEM). Experiment 1: volume (2.56 ± 0.21 mL), cell concentration ($273.27 \times 10^6 \pm 31.17$ spermatozoa/mL), pH (7.86 ± 0.07) and osmolality (262.46 ± 8.10 mOsm/kg). Experiment 2: volume (2.22 ± 0.27 mL), cell concentration ($268.27 \times 10^6 \pm 21.09$ spermatozoa/mL), pH (8.05 ± 0.12) and osmolality (282.00 ± 8.26 mOsm/kg).

3.1. Experiment 1. Re-freezing effects. Elapsed time between freezing/thawing cycles effects.

The results obtained on 30 min elapsed time (as a reference of the effect of the re-freezing on brown bear sperm) showed a detrimental effect between the two freezing/thawing cycles (T1 vs T2, $p < 0.05$) for motility (**Figure 1**), viability and acrosome status post-thawing (**Figure 2**).

The quality parameters of spermatozoa after each freeze-thaw cycle (T1 vs T2, Figure 1 and Figure 2) showed numerous significant differences into each elapsed time group. The motility, viability and undamaged acrosomes were significantly reduced after the second thawing.

Analyzing the effect of elapsed time in double freezing spermatozoa quality (P2 and T2, **Table 1**), the viability and acrosome status showed no significant differences among the three elapsed times either P2 and T2. However, TM, PM and LIN in P2 sample showed the lower value for 180 min (P2_180) but these differences were not observed in T2.

3.2. Experiment 2. Use of PureSperm® gradient between cycles.

When the samples were pre-selected in PureSperm after the first thawing (P2, **Table 2**), we observed generally higher motility and viability for the spermatozoa in the PS_Pellet sample. After the second thawing cycle (T2, **Table 2**), spermatozoa motility showed no differences between PS_Pellet and both controls (CTL and CTL-W), but we observed differences with PS Interphase spermatozoa. Viability and number of intact acrosomes showed higher values for the PS Pellet in comparison with the other groups.

Recovery rates of motility parameters (**Figure 3**) between freezing-thawing cycles (T2/T1 x100) showed no differences but recovery rates of VIAB (**Figure 4**) showed higher values for the PS-Pellet in comparison with control ($p < 0.05$).

Recovery of spermatozoa number after the gradient selection experiment, showed a significant decrease ($p < 0.01$) on both fractions (pellet and interphase) with regard to the control (Control: 71.4 ± 3.4 % vs. PS-P: 11.6 ± 2.9 % vs. PS-I: 26.8 ± 5.9 %).

4. Discussion

The main objective of the present study was to evaluate the use of two freezing-thawing cycles in brown bear sperm samples, in order to establish the most suitable cryopreservation protocol for this species and to increase the availability of bio-resources [8]. Moreover, studies of elapsed time between freezing-thawing cycles could allow us to establish a period of time in order to carry out different techniques such as sperm-sorting.

We tested the motility, viability and acrosome integrity of brown bear spermatozoa after double freezing and thawing and selected the 30 min elapsed time as a control of the re-freezing process. Results obtained in the present study showed a significant reduction in several functional parameters of spermatozoa between the first and the second freezing-thawing cycle. These data are in agreement with the results obtained in re-freezing experiments in several species. Thus, **Saragusty et al [4]** after double freezing bovine sperm, observed that motility, viability and acrosome integrity decreased significantly when compared with the single freezing. Working with stallion semen, **McCue et al [6]** obtained a significant decrease in total motility ($P < 0.05$) from $91.8 \pm 3.1\%$ prior to freezing to $45.7 \pm 10.4\%$ after the second freezing. However, sperm viability only showed a tendency ($P = 0.09$) toward a decrease after refreezing.

Similar results were obtained in human sperm [19], showing that the second freezing cycle decreased ($p < 0.01$) both motility and viability for slow freezing and rapid freezing. **Verza and Esteves [20]** also obtained a clear detrimental effect ($p < 0.01$) on the second thawing both for motility (first thawing 42 % vs. second thawing 22.5 %) and viability (first thawing 45 % vs. second thawing 20 %), confirming again the results obtained in the present study.

Results obtained in the previous study allow highlight the importance of using double freezing-thawing to improve the availability of sperm. A useful tool to confirm the quality of the double freezing samples was to assess the fertility data obtained from other studies. Thus, results in bull sperm **Arav et al [3]** showed a

pregnancy rate of 44% after insemination with double freezing semen in comparison with 45.5% for the control group (conventional freezing). In stallion, the ability of refrozen spermatozoa to initiate embryo development after ICSI was confirmed by **Choi et al [5]**. Studies in sheep also confirmed the efficacy of sperm submitted to double freezing in terms of the pregnancy rate obtained **[21], [22]**. Some studies were carried out on human sperm showing that 1) the numbers of both normal motility and viability sperm parameters after 3 serial freeze-thaw cycles were adequate for bringing about fertilization via ICSI **[19]**, and 2) refreezing of human semen allows the retrieval of viable spermatozoa after thawing **[20]**.

These studies evaluated the use of the cycles as a way to improve the availability of the samples to apply different ART techniques. ICSI techniques could be applied without good motility parameters, but the improvement of these characteristics must be mandatory in order to possibility the real application of the samples in wildlife management. Thus, it would be necessary to improve as much as possible the post-thawing motility parameters to be able to use the sperm on insemination programs on which the management of the female could be less complex.

On the other hand, results obtained for the different elapsed times could be useful for sex-sorting of frozen-thawed bear spermatozoa with the possibility to re-freezing the sample obtained after this selection and sending the cryopreserved sample with acceptable motility and viability after the second thawing to the biobank **[23]**. Results obtained in this experiment allow us to establish a period of time (up to 180 min) to carry out different manipulations obtaining no differences after the second freezing/thawing.

The use of density gradient centrifugation after the first freezing-thawing cycle was carried out to improve the quality of treated seminal samples, as demonstrated in other species (ram **[24]**; bull **[25]**; stallion **[26]**; bear **[15]**; human **[27]**). This improvement aims to increase the freezability of brown bear sperm subjected to a second freeze-thawing cycle. Our results showed higher values of viability the pellet

sample with respect to the rest of the groups and it also showed a significant decrease in the number of acrosomes damaged. This effect was highlighted just after the gradient centrifugation, when the samples increased both motility and viability with respect to the non-selected sample. To our knowledge, the present study is the first to use a gradient centrifugation technique between freezing/thawing cycles of brown bear spermatozoa, improving the efficacy of using bio-resources after double freezing.

Mousset-Simeón et al [28] suggest that the PureSperm gradient could be considered for the selection of human frozen-thawed sperm in assisted reproduction techniques. Our findings regarding the quality of spermatozoa selected with the PureSperm gradient allow us to conclude that the PureSperm gradient could improve the effectiveness of the second freezing-thawing cycle, obtaining similar values of total motility, viability and acrosomal status in the PS-Pellet after the second cycle in comparison with control values in the first cycle. These results agree with **Morrell et al [29]**, who showed that the PureSperm density gradient could improve human sperm survival and DNA integrity, probably by removing reactive oxygen species from the media of the sperm. Thus, sperm centrifugation could minimize the risk of sperm abnormalities in order to optimize the results of assisted reproduction techniques **[28]**. Testing the maintenance of motility and acrosome integrity, results obtained in the present study agree with **[30]**, who reported good result for bull spermatozoa after double freezing and thawing and the use of PureSperm between cycles.

However, the efficiency of the selection procedure is compromised by low recoveries of the total number of spermatozoa in the PS pellet. We obtained higher values of spermatozoa collected from the interphase between the gradient density compared to that obtained in PS pellet, but these spermatozoa showed a significant reduction in quality parameters which could be due to the sub-lethal damaged of the sperm that was highlighted by the second freezing-thawed process. These values were similar to those observed in previous studies by our group. **Nicolas et**

al. [15] showed that a high proportion of the spermatozoa were lost after PureSperm density gradient centrifugation and, on average, only 12.5% of post-thawed sperm were recovered in the pellet and 26.4% in PS-Interface.

In summary, the results of brown bear spermatozoa double freezing, applying three elapsed times and/or PureSperm density gradient centrifugation, lead us to conclude: 1) further studies should be carried out in order to minimize detrimental effects on sperm quality in the re-freezing process 2) the storage of samples for up to 180 min at room temperature between freezing/thawing cycles could be used since the damage on sperm are not modified by time and 3) The PureSperm gradient centrifugation technique could be applied for the improvement of sperm quality after two freezing-thawing cycles, as selected spermatozoa showed a higher capacity to survive the freeze-thaw process, but with a significant lower sperm recovery.

Aknowledgements

The authors thank Miguel Ángel Marañón and the game keepers of the Cabárceno Nature Park, Susana Gomes Alves, María Nicolás, Patricia Manrique, María Mata Campuzano, Julio Tamayo Canul, Felipe Martinez-Pastor and Leticia Ordás Bandera.

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469 **TABLES**

470

471 **Table 1.** Parameters for the two consecutive freezing/thawing cycles in brown bear spermatozoa: elapsed time (30, 90 and 180 min)
 472 between cycles. Mean±SEM. Different letters indicate differences between elapsed times for each individual sampling time in pre-freezing
 473 2 (P2) or post-thawing 2 (T2) (p<0.05).

474

	Pre-freezing 2			Post-thawing 2		
	P2_30	P2_90	P2_180	T2_30	T2_90	T2_180
TM	53.9±3.1 a	54.1±4.1 a	34.2±8.6 b	37.5±3.1	37±4.1	26.3±6.3
PM	22.2±1.6 a	19.9±1.6 a,b	13.1±2.9 b	13.9±1.2	13±1.5	12.5±2.9
VAP	44.9±2	40.7±2.6	40.9±6.3	41.1±1.9	37.7±2.7	46.9±4.5
VCL	83.3±3.7	75.5±4.6	82±13.5	76.7±3.6	73.5±5.4	92.2±9.9
LIN	33.6±0.6 a	32.1±0.7 a,b	27±4.2 b	32.2±0.3	30.7±0.5	34.3±2
VIAB	58.3±1.9	53.2±2.7	51.3±3.2	40±1.9	40.7±2.7	37.5±1.9
dACR	7.9±0.9	9.8±1.1	11.6±1.5	21±1.4	21.3±2.2	26.5±2.6

475

476 Motility parameters: total motility (TM; %), progressive motility (PM; %), average velocity (VAP; µm/s), curvilinear velocity (VCL; µm/s)
 477 and percentage of linearity (LIN; %). Membrane status measured by flow cytometry: viable spermatozoa (VIAB: PI-) and spermatozoa
 478 with damaged acrosomes (dACR: PNA+).

479

480 **Table 2.** Parameters for the two consecutive freezing/thawing cycles in brown bear spermatozoa: use of PureSperm between cycles.
 481 Mean±SEM. Different letters indicate differences between experimental groups for each individual sampling time (pre-freezing 2 -P2- or
 482 post-thawing 2 -T2-) (p<0.05).
 483

	Pre-freezing 2				Post-thawing 2			
	P2_CTL	P2_CTL-W	P2_PS-P	P2_PS-I	T2_CTL	T2_CTL-W	T2_PS-P	T2_PS-I
TM	34.2±8.6 a	33.6±7.4 a	64.9±6.1 b	15±3.7 c	26.3±6.3 a,b	25.1±6.4 a,b	34.6±8.3 a	21.2±5.3 b
PM	13.1±2.9 a	14±4.3 a	28.5±4.6 b	6.5±1.6 a	12.5±2.9	12±3.3	13±4.2	7.6±2.3
VAP	40.9±6.3 a	55.1±4.9 b	59±4.9 b	40.3±5.1 a	46.9±4.5	52.2±4.1	53.3±3.7	42±4.6
VCL	82±13.5 a,c	105.5±7.9 a,b	114.1±6.3 b	78.1±7.6 c	92.2±9.9 a,b	107.1±7.7 a,b	112.8±6.6 a	86.2±8.9 b
LIN	27±4.2	29.8±1.5	32±1.9	33.6±1.7	34.3±2 a	31±1.4 a,b	28.6±1.5 b	30.7±2.1 a,b
VIAB	51.3±3.2 a	49±3.2 a,b	67.9±2.3 c	40.8±3.2 b	37.5±1.9 a	34.9±3.2 a	51.3±5.1 b	34.9±3.4 a
dACR	11.6±1.5	13.8±1.4	10.9±2.1	16.4±2.3	26.5±2.6 a	28.3±2.8 a	20.2±2.4 b	31.4±2.2 a

484
 485 Control (CTL; without treatment), Control washed (CTL-W; centrifugation without PureSperm®), pellet (PS-P; obtained after PureSperm®
 486 gradient treatment) and interphase (PS-I; obtained in a phase between gradient concentrations of PureSperm®). Motility parameters:
 487 total motility (TM; %), progressive motility (PM; %), average velocity (VAP; µm/s), curvilinear velocity (VCL; µm/s) and percentage of
 488 linearity (LIN; %). Membrane status measured by flow cytometry: viable spermatozoa (VIAB: PI-) and spermatozoa with damaged
 489 acrosomes (dACR: PNA+).
 490

FIGURES LEGENDS

Figure 1. Brown bear sperm refreezing. Motility parameters for the three groups of elapsed times between cycles (30, 90 and 180min) represented individually in relation to analyzed sampling points (P1 (pre-freezing 1), T1 (thawing 1), P2 (pre-freezing 2) and T2 (thawing 2)). Motility parameters: total motility (TM; %), progressive motility (PM; %), average path velocity (VAP; $\mu\text{m/s}$), curvilinear velocity (VCL; $\mu\text{m/s}$), linearity (LIN; %) and amplitude of the lateral movement of the head (ALH; μm).. Mean $\pm$ SEM. Different letters indicate that means differ between sampling points for each time of elapsed times between cycles ($p<0.05$).

Figure 2. Brown bear sperm refreezing. Membrane integrity parameters for the three groups of elapsed times between cycles (30, 90 and 180min) represented individually in relation to analyzed sampling points (P1 (pre-freezing 1), T1 (thawing 1), P2 (pre-freezing 2) and T2 (thawing 2)). Viable spermatozoa (VIAB: PI-) and spermatozoa with damaged acrosomes (dACR: PNA-FITC +) Mean $\pm$ SEM. Different letters indicate that means differ between sampling points for each time of elapsed times between cycles ($p<0.05$).

Figure 3. Brown bear sperm refreezing. Recovery rates of motility parameters between the two freezing/thawing cycles ($(T2/T1) \times 100$) of sperm parameters for the four groups of PureSperm experiment: CTL (Control), CTL-W (Control washed with PBS 1X and centrifugated at 600xg for 6 min), PS-P (Pellet; spermatozoa that penetrate both phases of the PureSperm gradient) and PS-I (Interphase; sperm recovery between phases of the gradient). Motility parameters: total motility (TM; %), progressive motility (PM; %), average velocity (VAP; $\mu\text{m/s}$), curvilinear velocity (VCL; $\mu\text{m/s}$), percentage of linearity (LIN; %) and amplitude of the lateral movement of the head (ALH; μm). Mean $\pm$ SEM. Different letters indicate that means differ between groups ($p<0.05$).

Figure 4. Brown bear sperm refreezing. Recovery rates of membrane integrity parameters between the two freezing/thawing cycles ($(T2/T1) \times 100$) of sperm parameters for the four groups of PureSperm experiment: CTL (Control), CTL-W (Control washed with PBS 1X and centrifugated at 600xg for 6 min), PS-P (Pellet; spermatozoa that penetrate both phases of the PureSperm gradient) and PS-I (Interphase; sperm recovery between phases of the gradient). (VIAB: PI-) and spermatozoa with damaged acrosomes (dACR: PNA-FITC +) Mean $\pm$ SEM. Different letters indicate that means differ between groups ($p<0.05$).

Figure1
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CAPÍTULO 11

DISCUSIÓN GENERAL

DISCUSIÓN GENERAL

La conservación de las características fisiológicas originales de las células es un requisito básico en los procedimientos de crioconservación de muestras biológicas, y es la base fundamental para el establecimiento de los bancos de recursos genéticos (BRG). Estos Bancos han permitido la puesta en marcha de los programas de conservación de diversas especies con el estatus de especie amenazada, como es el caso del oso pardo de las montañas cantábricas.

En este sentido, la optimización y estandarización de los protocolos de conservación espermática hacen necesario el estudio de los diferentes parámetros que condicionan la calidad final de las muestras espermáticas congeladas. En el presente trabajo analizamos el efecto de diferentes factores básicos del protocolo de congelación: 1) el agente crioprotector en cuanto a el momento de adición del glicerol a la muestra; 2) la concentración óptima del glicerol en los diluyentes de congelación y su interacción con tres rampas de congelación; 3) el empleo de sustitutos de la yema de huevo (la lecitina de soja o las lipoproteínas de baja densidad –LDL– de la yema de huevo) como protectores de membrana; 4) el efecto de las proteínas de choque térmico (HSPA8) sobre la calidad espermática después de la refrigeración o la congelación.. Por último, hemos evaluado el proceso de recongelación espermática, como una alternativa metodológica que permitiría la aplicación de biotecnologías específicas en estos biomateriales obtenidos en el medio natural.

Los resultados de esta tesis doctoral persiguen incrementar la eficacia del BRG en esta especie, contribuyendo al desarrollo de herramientas útiles, cuyo fin es maximizar el rendimiento de los escasos recursos disponibles de oso pardo.

11.1 MODO DE ADICIÓN DEL GLICEROL

El momento exacto de adición del glicerol en las muestras previamente a la crioconservación es importante en cuanto a minimizar el efecto tóxico que puede producir el glicerol en los espermatozoides en muestras de alto valor biológico.

En el *Capítulo 6* se describen los resultados obtenidos en nuestro estudio que indican que la movilidad y la viabilidad de los espermatozoides de oso pardo no presentan un cambio significativo en los diferentes modelos de adición del glicerol ensayados. Sólo existen diferencias, tras la realización de un test de termorresistencia de 4h a 37 °C, en el parámetro de movilidad VAP que es significativamente más alto en la adicción

después de la refrigeración, y en la adicción fraccionada en dos pasos con respecto a la adición total del glicerol a temperatura ambiente.

Estos resultados difieren notablemente de los que se han encontrado en otras especies. Así, algunos autores han demostrado que el glicerol debe ser añadido a las muestras espermáticas después de la refrigeración (en perro: **Silva et al. 2003, Rota et al. (2005)**; en ovino: **Gil et al., 2003**; en cerdo: **Almlid y Johnson, 1988**) y en otros estudios la recomendación es incorporarlo antes de iniciar la refrigeración (en caballo: **Vidament 2005**; en ciervo: **Fernandez-Santos et al. 2005**; en humano: **Deppe et al. 2004**). No obstante, **Silva et al. (2006)** concluyeron que la adición del glicerol al semen de perro puede ser realizada, indistintamente, a temperatura ambiente o tras la refrigeración. En los estudios de congelación de espermatozoides de osos, la adición del glicerol se ha realizado después de la congelación y no es posible comparar resultados.

Esta variabilidad de resultados entre especies la interpretamos en base al coeficiente de permeabilidad del glicerol a las membranas que varía entre especies dada la especificidad de especie en la composición y estructura de las membranas celulares. De manera general, nuestros resultados pueden estar relacionados con una alta tolerancia de los espermatozoides de oso pardo a los cambios en las muestras producidos por algún tipo de estrés osmótico inducido por el diluyente, y que ha sido documentado por **Songsasen et al. (2002)** en la congelación de espermatozoides de perro.

En resumen, en la congelación de espermatozoides de oso pardo no existe un efecto diferencial en cuanto al modo de adición de glicerol, lo que establece un amplio margen de flexibilidad posibilitando la adaptación del protocolo de congelación en función de las diversas condiciones de campo que nos podemos encontrar (limitación de infraestructuras, condiciones de transporte...).

11.2 CONCENTRACIÓN DE GLICEROL Y RAMPAS DE CONGELACIÓN

A la hora de elaborar el protocolo de conservación seminal, cobran especial relevancia dos factores: la concentración del glicerol y la rampa de congelación utilizada, parámetros que son estudiados en el *Capítulo 7*.

Tasas del glicerol comprendidas entre el 4 y 8 % han sido estudiadas en oso pardo (**Anel et al., 2010; Okano et al., 2006**), con el fin de testar unas concentraciones del crioprotector que permitan alcanzar un equilibrio entre el efecto crioprotector del glicerol y su propia citotoxicidad (**Pegg, 2002**). De acuerdo a estos estudios, y considerando la falta de conclusiones entre el rango 4-8% de un estudio previo de

nuestro grupo (**Anel et al., 2010**) decidimos analizar el rango 2–10 % con el fin de definir la concentración óptima del glicerol en la congelación de espermatozoides eyaculados de oso pardo.

Las muestras criopreservadas con el 10 % de glicerol presentaron daños acrosomales así como una reducción de los parámetros cinéticos. A pesar de utilizar un protocolo fraccionado de adición del glicerol, parece que durante el tiempo de equilibrado (1 h) periodo en que la muestra espermática tiene la concentración final del glicerol, se magnifican los efectos deletéreos de este compuesto.

En contraste a lo acontecido con la concentración más elevada de glicerol (10%), la utilización de la menor concentración (2%) presenta buenos resultados pre-congelación, pero la acción crioprotectora se ve disminuida lo que se refleja tanto en los datos cinéticos como en la viabilidad, parámetros que presentan unos valores sub-óptimos tras la descongelación.

En cuanto a las rampas de congelación, el estudio muestra solamente diferencias en la movilidad progresiva, siendo menor en la rampa lenta (-10 °C/min) con respecto a la media (-20°C/min).

El estudio combinado de ambos parámetros, concentración de glicerol y rampa de congelación, ofrece resultados interesantes ya que, aunque cabría esperar que el uso de una baja concentración de glicerol (2%) requeriría una rampa lenta (-10 °C/min) para permitir una amplia deshidratación celular antes de que se inicie la formación de cristales de hielo, el resultado es el inverso y se produce una mejora significativa al utilizar una rampa rápida de congelación (-40 °C/min). Nosotros interpretamos que el daño observado en los espermatozoides con un 2% de glicerol es inducido por el estrés osmótico o la precipitación de sales más que por la formación de hielo intracelular durante la criopreservación (**Muldrew, 2008**).

Del mismo modo, las altas concentraciones de glicerol (10%) presentan mejores resultados de viabilidad e integridad acrosomal cuando se combinan con la rampa lenta, efecto posiblemente inducido por la aparición de daños sub-letales producidos por una mayor probabilidad de formación de hielo intracelular (**Curry and Watson 1994; Woods et al. 2004**).

En resumen, la concentración ideal a utilizar en la criopreservación de oso pardo se encuentra dentro del rango del 4-8% de glicerol, para el cual no existen diferencias en la rampa de congelación utilizada, aunque el análisis de la motilidad y la viabilidad mediante un ajuste de curvas con un modelo cuadrático nos indica que el mejor valor

para estos parámetros se alcanza en torno al 6.4% de glicerol. Teniendo en cuenta todos los parámetros utilizados en este estudio, proponemos una concentración del 6% de glicerol en combinación con una rampa de congelación media (-20 °C/min).

11.3 SUSTITUTOS DE LA YEMA DE HUEVO: SOJAS Y LDL

Los protectores de membrana constituyen un elemento esencial en los diluyentes de criopreservación espermática, ya que son los responsables de minimizar los efectos deletéreos del frío sobre las membranas celulares, preservando la movilidad, la viabilidad y la integridad acrosomal. La yema de huevo es uno de los protectores de membrana más utilizados (**Salamon and Maxwell, 2000**).

El *Capítulo 8* de la presente memoria recoge la evaluación de dos posibles sustitutos de la yema de huevo: la lecitina de soja y las lipoproteínas de baja densidad de la yema de huevo (LDL).

La lecitina de soja se evaluó en dos experimentos diferenciados. En el primero de ellos, se comparan las concentraciones de soja del 2, 3,5 y 5% con respecto al control (20% yema de huevo) y se demuestra que las muestras criopreservadas con yema de huevo presentan, tras la descongelación, una mayor viabilidad en todos los casos, aunque la movilidad total no se ve significativamente afectada en las dos concentraciones superiores de soja con respecto al control. Estos resultados concuerdan con un estudio en semen de caballo (**Papa et al. (2011)**), en el que se observaron porcentajes similares de movilidad y viabilidad espermática en las muestras congeladas con soja respecto al control con yema. En cambio, **van Wagtendonk-Leeuw et al. (2000)** trabajando con semen de toro, observaron un efecto perjudicial de la soja sobre la movilidad espermática. Estos resultados contradictorios podrían ser explicados por la respuesta diferencial que podrían presentar diferentes especies ante el efecto protector de la lecitina de soja.

En el segundo experimento se emplean dos fuentes diferentes de lecitina de soja (tipo A con 24% fosfatidilcolina y tipo B con un rango del 14 al 23% de fosfatidilcolina) en una concentración del 5% (según resultados del experimento 1). Las muestras congeladas con diluyentes de ambos tipos de soja presentaron un descenso significativo de la viabilidad espermática respecto al grupo control (yema 20%), aunque solo los espermatozoides congelados con soja tipo A presentaron tras la descongelación una movilidad equivalente a la del grupo control. Este efecto diferencial de la soja A puede ser interpretado en base a su distinta composición al igual que lo hacen **del Valle et al.**

(2012) para explicar las diferencias entre diluyentes con soja o con yema en la protección de las mitocondrias durante la congelación. Estos autores observan que la soja purificada compuesta por fosfatidilcolina protege solo la membrana plasmática, mientras que la yema de huevo, que presenta otras lipoproteínas en su composición, interactúa no solo con la membrana plasmática sino también con la membrana mitocondrial ejerciendo una más amplia acción protectora.

Las lipoproteínas de baja densidad (LDL) también ofrecen unos resultados contradictorios tras la descongelación ya que observamos que la viabilidad es mayor en las muestras congeladas en un diluyente con altas concentraciones de LDL (10-15%) respecto al control con yema de huevo, el estatus del acrosoma no ve afectado pero la movilidad se ve significativamente reducida. Este efecto diferencial de las LDL sobre los distintos compartimentos del espermatozoide podría interpretarse en base a la observación de **Vera-Munoz et al. (2009)** que nos indican que los componentes presentes en los diferentes diluyentes pueden ejercer distintos efectos protectores sobre la membrana plasmática o sobre el flagelo del espermatozoide. Así se ha observado (**Amirat et al. 2004**) que las LDL protegen a los espermatozoides durante la congelación impidiendo la pérdida de fosfolípidos y de colesterol desde la membrana plasmática, asociándose para ello con la superficie celular y conformando una barrera física que podría prevenir los cambios de fase (Ritker et al. 2006). La mejor viabilidad de las muestras congeladas con LDL podría deberse a la ausencia de algunos factores negativos presentes en la yema de huevo.

En relación con esta hipótesis, el estudio de la capacidad antioxidante (TAC) de los distintos diluyentes nos muestra una clara relación dicha capacidad y la concentración de soja o LDL en el diluyente. Obviamente, la solución base utilizada en nuestros diluyentes no presenta capacidad antioxidante alguna y el efecto observado es debido a los protectores de membrana empleados. La yema de huevo ofrece valores TAC similares a los mostrados por las sojas 2 y 3,5%, mientras que los diluyentes con LDL 10-15% presentaron unos valores notablemente más altos. **Hu et al. (2011)** encontraron que los diluyentes con LDL reducían el impacto negativo de las especies reactivas del oxígeno (ROS), dando mejores resultados que los diluyentes con yema de huevo en cuanto a parámetros de calidad espermática y en relación con la actividad de enzimas antioxidantes.

En resumen, las LDL en concentraciones del 10-15% pueden constituir un buen sustituto de la yema de huevo en los diluyentes de congelación del semen de oso pardo,

pero la lecitina de soja aunque mantiene la motilidad no protege adecuadamente la viabilidad espermática. Otros estudios serán necesarios para determinar el tipo y la concentración de lecitina de soja adecuada para la criopreservación de espermatozoides de oso pardo.

11.4 ADICIÓN DE PROTEÍNAS DE CHOQUE TÉRMICO (HSPA8) EN LOS DILUYENTES DE REFRIGERACIÓN Y CONGELACIÓN ESPERMÁTICA

La proteínas de choque térmico HSPA8 (0,5, 1 y 5 µg/mL) es analizada en el *Capítulo 9* para evaluar su posible efector protector durante la refrigeración o congelación del semen de oso. El trabajo se divide en dos experimentos: 1) refrigeración hasta 48 h (5 °C) y 2) congelación espermática.

En el primer caso, los resultados indican que la proteína ejerce un mayor efecto protector sobre la viabilidad espermática y el estatus del acrosoma durante la refrigeración, principalmente a las 48 h, respecto a lo observado con el control. Un efecto similar ha sido observado en otras especies utilizando rangos de concentración de proteína similares a los de nuestro estudio: en toro (muestras incubadas a 37 °C durante 48h; **Elliott et al., 2009**), en cerdo (incubación a 39 °C durante 24h; **Elliott et al., 2009**) y en ovino (conservación a 5 °C y 38 °C hasta 48h; **Lloyd et al., 2009**). Estos resultados permiten corroborar la naturaleza conservada de esta proteína durante la evolución en diferentes especies (**Daugaard et al., 2007**).

En el segundo experimento no se observan grandes diferencias en los parámetros de calidad estudiados inmediatamente después de la congelación. No obstante, la incubación de las muestras descongeladas a 37 °C durante 2 horas produce un descenso significativo del número de acrosomas dañados (con 1 y 5 µg/mL HSPA8) y una reducción en el índice apoptótico (con 1 µg/mL HSPA8). En un estudio llevado a cabo en ovino, **Colás et al. (2009)** observan el efecto beneficioso de la adición de proteínas del plasma seminal al diluyente de congelación con una mejora de la calidad seminal. En este sentido, la formación de HSPA8 en el oviducto es estimulada por los espermatozoides (**Georgiou et al., 2005**) y esta proteína parece prolongar la viabilidad de los espermatozoides en el tracto de la hembra (cerdo y toro: **Elliott et al. 2009**; carnero: **Lloyd et al. 2009**). Este efecto parece estar relacionado con una interacción de la proteína HSPA8 con ciertos dominios lipídicos y el colesterol de la membrana espermática (**Chen et al., 2005**).

En el estudio de movilidad llevado a cabo con el test de migración en ácido hialurónico, se pueden destacar dos resultados. Por una parte, el número de espermatozoides que avanza entre 0,5-2 cm en el test decrece significativamente con respecto al control, de una forma dosis-dependiente. En cambio, la distancia máxima recorrida por el espermatozoide de vanguardia aumenta de manera lineal a medida que aumenta la concentración de proteína, llegando a equipararse la de la muestra con mayor concentración (5 µg/mL) al control. Este resultado parece indicarnos la existencia de una pequeña sub-población espermática que conserva su movilidad cuando se da la más alta concentración de proteína (5 µg/mL). Este hecho puede relacionarse con lo acontecido en el oviducto, donde la mayoría de los espermatozoides no son altamente activos, pero una pequeña proporción constituye la población capaz de progresar hacia el oocito para su fecundación (Suarez, 2008). En este sentido, se ha señalado la existencia de un mecanismo mediante el cual los espermatozoides no móviles serían inhibidos específicamente por HSPA8 y de este modo se mantendría un reservorio espermático en el oviducto (Hernandez et al., 2009).

En conclusión, nuestros resultados muestran que HSPA8, a bajas concentraciones, podría ser útil para mejorar la calidad de las muestras refrigeradas de semen de oso pardo. Además, en las muestras descongeladas se observa una disminución de los acrosomas dañados (1 y 5 µg/mL) y del índice apoptótico (1 µg/mL) después de una incubación de las muestras a 37 °C durante 2h.

11.5 RECONGELACIÓN ESPERMÁTICA

Los biomateriales obtenidos en todas las especies de especial relevancia económica, social o biológica, tanto las domésticas como las silvestres, deben ser aprovechados al máximo y por ello se hace necesario el desarrollo de nuevos procedimientos encaminados a optimizar el uso eficaz del material genético conservado.

En el *Capítulo 10* se estudia el efecto de la recongelación espermática sobre la calidad de las muestras de oso pardo, así como el uso de un gradiente de selección con PureSperm entre ambos ciclos de congelación consecutivos. Esta doble congelación debe permitir una intervención tecnológica diferida, como por ejemplo el sex-sorting, que no es posible aplicar en condiciones de campo.

Los resultados de la recongelación espermática muestran una reducción significativa en los parámetros de movilidad y viabilidad espermática y un aumento en el daño acrosomal. En general, es el segundo ciclo de congelación el que tiene un efecto más

deletéreo sobre la calidad espermática y por ello se hace necesario estudiar con mayor profundidad las variables de este procedimiento para intentar corregir dichos efectos.

Estudios llevados a cabo en ovino (**Hollinnshead et al, 2004; de Graaf et al, 2007; Morton et al, 2006**), confirman la pérdida de calidad espermática tras la recongelación, aunque con la realización de pruebas de fertilidad demuestran la eficiencia de los espermatozoides recongelados en la producción de descendencia. **Arav et al. (2002)**, aplicando una doble congelación a semen de toro, observaron un porcentaje de preñez similar al del grupo control (no recongelado). También se han realizado ensayos de recongelación con semen humano en los que se observaron buenos resultados de fertilización heteróloga aplicando inyección espermática intra-citoplasmática de espermatozoides recongelados (**Bandularatne and Bongso, 2002**).

Cuando se estudiaron diferentes tiempos de espera entre ciclos de congelación (30, 90 y 180 min), tiempos en los cuales se podrían aplicar distintas técnicas a la muestra, como por ejemplo el sexado espermático, podemos observar que no se aprecian diferencias significativas en los parámetros de movilidad (excepto LIN) y viabilidad entre los tres tiempos.

En el segundo experimento del estudio de la recongelación aplicamos un gradiente de PureSperm para seleccionar la muestra entre los dos ciclos de congelación. Nuestros resultados permiten afirmar que el uso de este gradiente mejora la movilidad total, la viabilidad y el estatus acrosomal de los espermatozoides recongelados, con respecto a las muestras no seleccionadas. Los gradientes de selección para mejorar la calidad de las muestras seminales han sido aplicados en diferentes especies (ovino: **García-Álvarez et al, 2010**; toro: **Rodríguez-Martínez et al, 1997**; caballo: **Morrel et al, 2009**; y humano: **Classens et al, 1998**). En un estudio previo de nuestro grupo, **Nicolás et al. (2012)** describen el uso de un gradiente de PureSperm en la precongelación de semen de oso pardo, obteniendo una población espermática seleccionada con mejor calidad espermática (movilidad, viabilidad, estatus acrosomal) aunque el rendimiento de la técnica era negativo por la pérdida de espermatozoides. Esta misma pérdida ha sido observada en nuestro actual estudio.

La calidad de la muestra seleccionada después del segundo ciclo de congelación podemos relacionarla con la existencia de una población espermática característica que presenta una mejor supervivencia al ser sometida al segundo ciclo de congelación y que, además, sería seleccionable de manera eficaz con el uso del PureSperm. No obstante, el principal problema del uso de gradientes es la baja tasa de recuperación de

espermatozoides, por lo cual tenemos que establecer un compromiso entre la capacidad selectiva del gradiente y la pérdida de espermatozoides que se produce. Esta estrategia implica la modificación de las condiciones de la técnica aplicando, entre otros posibles factores, gradientes con menor densidad.

En conclusión, la recongelación espermática en el oso pardo, especie en que las muestras tienen un alto valor biológico por su escasez y la dificultad de su obtención, ofrece una opción técnica viable que permite la aplicación de nuevas biotecnologías. Nosotros hemos comprobado la posibilidad de emplear un tiempo de espera entre ciclos de hasta 180 min, así como el uso del PureSperm en ese periodo, como un procedimiento eficaz en cuanto a resultados de calidad espermática postdescongelación.

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CAPÍTULO 12

CONCLUSIONES

CONCLUSIONES

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

1. El momento de adición del glicerol durante el proceso de congelación de espermatozoides de oso pardo (antes y/o después de la refrigeración a 5 °C) no tuvo efecto sobre la calidad seminal post-descongelación, lo que facilitará la adaptación del protocolo de congelación a las condiciones de campo.

2. La concentración de glicerol óptima para la congelación de espermatozoides de oso pardo, definida mediante una curva de ajuste con un modelo cuadrático para los parámetros de calidad espermática post-descongelación, se situó en el 6%. Para las concentraciones del 4 y 6% de glicerol no se observaron diferencias respecto a las rampas de congelación utilizadas (-10, -20 y -40 °C/min).

3. Las lipoproteínas de baja densidad obtenidas de yema de huevo (LDL) pueden ser un buen sustituto de la yema de huevo en los diluyentes de congelación para oso pardo, atendiendo a los resultados obtenidos. En cambio, la lecitina de soja de las fuentes ensayadas en el presente trabajo, no representa una alternativa válida para sustituir a la yema de huevo en dichos diluyentes.

4. La proteína de choque térmico A8 (HSPA8) mejoró de forma significativa los parámetros de calidad espermática en oso pardo, tanto en refrigeración (0,5 µg/mL) como en congelación (1 µg/mL). No obstante, la movilidad de los espermatozoides, medida mediante un test de progresión espermática, se redujo significativamente en el diluyente con HSPA8 frente al control. Solo una pequeña población, representada por el espermatozoide de vanguardia presentó valores de progresión similares a los del control cuando se empleó una concentración de 5 µg/mL de HSPA8).

5. En el proceso de doble congelación, los espermatozoides de oso pardo sufre una pérdida significativa de calidad. En este sentido, no se observan diferencias en esta pérdida de calidad si el tiempo de espera entre ambos ciclos es de 30, 90 o 180 minutos. La utilización de un gradiente de selección espermática PureSperm® entre ciclos de congelación mejora la calidad de los espermatozoides de oso pardo después de la segunda descongelación.

CAPÍTULO 12

CONCLUSIONS

CONCLUSIONS

Here follow the conclusions derived from the results obtained in the studies presented in this thesis:

1. The moment of glycerol addition during the freezing process of brown bear semen (before, before and after (split) or after cooling to 5 °C) had no effect on post-thaw sperm quality, facilitating the adaptation of the freezing protocol to specific field conditions.

2. The optimum concentration of glycerol for freezing brown bear spermatozoa, defined by a curve fitting using a quadratic model for post-thawing quality parameters, was 6%. For concentrations of 4% and 6%, no differences were found between freezing rates (-10, -20 and -40 °C/min).

3. Low density lipoproteins obtained from egg yolk (LDL) could be a good substitute for egg yolk in freezing extenders for brown bear semen, according to the results obtained. Contrarily, soy lecithin, at least the kinds tested in this study, is not a valid alternative to replace egg yolk in such extenders.

4. The heat shock protein A8 (HSPA8) significantly improved sperm quality parameters in brown bear sperm, both for cooling (at 0.5 mg/mL) and freezing (at 1 mg/mL) storage. However, the sperm motility, measured by a test of sperm progression, was significantly reduced in the diluent with HSPA8 versus control. Only a small population, represented by the vanguard sperm progression, showed similar values to those of the control when HSPA8 was used at a concentration of 5 mg/mL.

5. In the process of double freezing, brown bear sperm samples suffered a significant loss of quality. In this sense, no differences were found in this loss of quality when the elapsed time between freezing cycles was 30, 90 or 180 min. The use of the PureSperm® sperm selection gradient between freezing cycles improved the quality of brown bear sperm after the second thawing.

AGRADECIMIENTOS

AGRADECIMIENTOS

A Luis Anel por permitirme disfrutar de una beca FPI del Ministerio. A Paulino de Paz por sugerirme la idea de la investigación como salida profesional y por su inmensa labor de co-dirección en este trabajo.

A Santiago Borragán por demostrarme repetidamente su cariño y apoyo. Estoy seguro de que personas como tú existen pocas. A Maraón por ser una persona con un corazón enorme.

A Felipe, por ser mi asesor y estar siempre dispuesto a ayudarme en todo aquello que necesitaba, incluido facilitarme mis estancias en diversos centros de investigación del Reino Unido y contactos con la profesora Jane Morrel. Sin olvidarme de sus consejos en citometría y también por su amistad y apoyo personal. Gracias.

To Bill Holt, for their support in conducting this work and for his ability to teach me many things in a simple conversation. Thank you very much!.

A Merce por su ayuda en el trabajo de laboratorio. A los compañeros del laboratorio sin los cuales, sin duda, no hubiera sido posible la realización de este trabajo: Susana, María Nicolás, Leti, Elena, Carmen y Patri. A Julio y a María Mata, por hacerme sentir en el laboratorio como mi segunda casa. No hubiera podido superar muchas cosas sin vosotros. Muchas gracias por los congresos que hemos pasado, por las experiencias que hemos vivido y por haberme demostrado vuestro apoyo en múltiples ocasiones. Julio, nos vemos pronto. María, mis mejores deseos tanto personales como para la carrera investigadora que apenas comienza. Al resto de compañeros y amigos que me he ido encontrando en la Universidad: Cristian, Pancho, David, Jenny, Adrián, Adelino...

La universidad me permitió encontrarme con dos personas muy especiales para mí. En primer lugar: Raquel. Siempre dispuesta a un café, o una cerveza y hablando con la confianza de unos amigos que se conocen de toda la vida. En segundo lugar: Luis. Mi hermano. Porque a veces pienso que el destino, sólo pone zancadillas a mi paso pero, sin embargo, en ocasiones te brinda el mejor de los regalos: la amistad verdadera. Con él se completó mi familia ya que, con el calor del abrazo de unos padres, me reciben cada vez que les veo Ernesto y María. Muchas gracias por apoyarme en el peor momento de mi vida y gracias por formar parte de muchos de los mejores momentos de ella.

A Javi y a Merce de Vitatene, y a Maribel, por su más que agradable compañía y por hacer menos cuesta arriba cada día de trabajo.

Al resto de gente maravillosa que me encontré en el periodo universitario: Laura, Gemma, Carla, Raquel, Christopher, Aida; Sara, Ana Belén,... Gracias a todos y siento si me olvido de alguien.

A Fidel y Garmilla. Gracias por las escapadas juntos, por los vinos disfrutados y por los que nos quedan por disfrutar. Gracias.

A José Luis Acebes Arranz, del Área de Fisiología Vegetal, por demostrarme que otro tipo de investigación existe y por su preocupación por mi bienestar personal. Gracias por los cafés de máquina y las charlas cargadas de ánimo. Y sin olvidar al resto de compañeros del Área, que amablemente me ofrecen una sonrisa al llegar al laboratorio (Asier, África, Toño, Penélope, Ana, M^a Luz...). A José Miguel Domingo, compañero de aventuras extraescolares, ex-profesor y, siempre amigo y maestro.

A mi familia en general. A mi tío Jose, por demostrarme en el día a día que los lazos de sangre no significan nada para considerar a alguien familia. A mi prima Patri, una futura médica brillante y una prima excelente. Te quiero peque. A Flory, porque no hace falta hablar de ciertos temas para saber que cada día nos acordamos de ELLA. Gracias por tu sonrisa al otro lado del teléfono y la calidez de tus palabras. Te quiero!. A mi abuelo desde mis travesuras en el campo de Colominas hasta su preocupación por todos y cada uno de los nuevos proyectos que emprendo. Abuelo, el destino nos la ha robado pero estoy seguro de que guía nuestros pasos en nuestros corazones y espero que, por fin, podrá fundirse en un abrazo con la abuela.

A mi familia de Orallo, por mostrarme cariño y afecto en cada una de las ocasiones que nos vemos . En especial a Maruja y a Aurelia por mantener en mi vivo el espíritu de Cecilio y Francisca y poder imaginar, hasta donde llega mi memoria, al abuelo esperando en la valla de entrada del pueblo.

A mi otra familia: Celia, Leandro, Raquel y Berni, porque, a pesar de los delicados momentos que he atravesado, han sabido comprenderme y ofrecerme su cariño. Del mismo modo, a los padres de Berni: Erundina y Vicente, por los buenos momentos compartidos en las fiestas de Trobajo y en los cumpleaños. A Yoigo, Micky y al peque que está en camino... Gracias.

El final de estos agradecimientos es para mis tres soportes vitales: Laura, Manuel y Emilia.

A Laura por entender cada una de mis adversidades, por vivir junto a mi muy buenos momentos y por saber entenderme en cada momento. Espero y deseo una larga vida juntos en que por fin pueda entender que sirvo para algo y hacerte sentir como te mereces: como una princesa. “Porque nada valgo...”. Te quiero.

A papá, porque todo lo que soy depende de ti. Dar lo mejor en cada momento es una tarea imposible, pero tú lo consigues cada día. Gracias por estar ahí y a seguir luchando por tu sitio en esta vida: aún nos quedan mil experiencias que vivir juntos.

A mamá. Como hablar de la persona más dulce del mundo. Aún me arroja en mi cama como si fuese un niño pequeño y, cada noche cuando preparo meticulosamente mi ropa para ir al trabajo, la imagino a mi lado ayudándome. Siento que hayas tenido que partir tan pronto, pero no te preocupes por mí. Estoy bien. Sólo me hace falta asimilar tu falta.

APÉNDICE I: ANEXO GRÁFICO

FOTO 1.

Foto 1. Ejemplar adulto de oso pardo (*Ursus arctos*) en el parque de la Naturaleza de Cabárceno, Cantabria, España.

Foto 2. Oso pardo (*Ursus arctos*) en el parque de la Naturaleza de Cabárceno, Cantabria, España. A) Panorámica de parte del recinto del Parque destinado a los osos. B) Osa con dos crías. C) Monta en la época reproductiva. D) Foto desde la parte superior del recinto de osos. E) Osa amamantando a sus dos crías.

FOTO 2.

Foto 3. Captura de los animales en el recinto del Parque de la Naturaleza de Cabárceno. A) Coche de alimentación y coche de vigilancia. B) Disparo del dardo anestésico. C) Animal con el dardo anestésico. D) y E) Animales con efecto del anestésico.

FOTO 3.

Foto 4. Manejo de los animales y proceso de electroeyaculación. A) Pesaje del animal. B) Rasurado del área prepucial y limpieza con suero salino. C) y D) Sondaje de la vejiga urinaria. E) Toma de muestras sanguíneas y colocación de la vía. F) Animal monitorizado para las constantes vitales y saturación de O₂, inmovilizado en la mesa de trabajo mediante cadenas y arneses. G) Proceso de electroeyaculación. H) e I) Embudo de recolección de las muestras espermáticas conectado a un tobo de vidrio de centrifuga de 15 mL. J) Ejemplo de muestra seminal obtenida del proceso, para su posterior análisis y tratamiento.

FOTO 4.

Foto 5. Evaluación inicial, captura y análisis de la calidad de las muestras espermáticas. B) Microscopio de contraste de fases y cámara de transmisión de imágenes al PC. C) Sistema de análisis computarizado de la movilidad espermática (CASA: Proiser Microptic. Barcelona). D) Ejemplo de campos capturados por el software de captura y análisis de la movilidad. E) Citómetro de flujo FACScalibur (Becton Dickinson Immunochemistry Systems, San Jose, CA, USA). F) Análisis PNA-FITC/PI. Diferenciamos cuatro poblaciones espermáticas. PNA-FITC-/PI-: viables con el acrosoma intacto; PNA-FITC+/PI-: viables con el acrosoma dañado; PNA-FITC-/PI+: no viables con el acrosoma intacto; PNA-FITC+/PI+: no viables con el acrosoma dañado. G) Análisis SYBR-14/PI. Diferenciamos tres poblaciones espermáticas. SYBR-14+/PI-: vivos; SYBR-14+/PI+: moribundos; SYBR-14-/PI+: muertos). H) Análisis YO-PRO-1/PI. Diferenciamos tres poblaciones espermáticas. YO-PRO-1-/PI-: vivos; YO-PRO-1+/PI-: apoptóticos; YO-PRO-1-/PI+: muertos. Los dotplot F,G y H proceden de *Reprod Dom Ani* 45 (2), 67-78 (2010).

FOTO 5.

Foto 6. Equipo de laboratorio para la congelación de muestras espermáticas. A) Biocongelador (Planner Krio II). B) Impresora de pajuelas. C) Selladora de pajuelas. D) Peine y soporte para la creación de cámara de aire previa al sellado y rack de congelación con las pajuelas. E) Empajuelado de las muestras para su congelación. F) Racks con muestras sumergidos en N₂ tras su extracción del biocongelador. G) Tanque de N₂ para la conservación de las pajuelas. H) Baño de descongelación de pajuelas.

FOTO 6.

Foto 7. Instalaciones y material necesario para el test de progresión a través de *mucus* artificial de ácido hialurónico. A) Microscopio de contraste de fase para la visualización y conteo de los espermatozoides dentro del capilar. B) Incubador programable. C) Jeringuilla para el llenado por absorción de los capilares. D) Capilar sellado por un extremo tras el llenado con el *mucus* e introducido en el tubo con la muestra seminal. E) Los capilares utilizados en este experimento son capilares VITROTUBES™ (Mod. 3520-050 (0.20 x 2.00 x 50), CM Scientific Ltd., Silsden, UK). F) Fórmula empírica del ácido hialurónico. G) Porta con un canal central y milimetrado para el contaje espermático y para la correcta identificación del espermatozoide de vanguardia.

FOTO 7.

APÉNDICE II: FACTOR DE IMPACTO DE LAS PUBLICACIONES

Factor de impacto (FI) de las publicaciones según el Journal Citation Reports del año 2011

REVISTA	FI	CATEGORÍA	PUESTO EN LA CATEGORÍA	REVISTAS EN LA CATEGORÍA
Theriogenology	1,963	Veterinary Sciences	16	143
Reproduction in Domestic Animals	1,356	Veterinary Sciences	37	143
Reproduction, Fertility and Development	2,109	Zoology	22	146
PLOS One	4,092	Biology	12	85

APÉNDICE III: JUSTIFICANTES ACEPTACIÓN/ENVÍO ARTÍCULOS

PAPER 3.

The antioxidant effects of soybean lecithin- or low-density lipoprotein-based extenders for the cryopreservation of brown bear (*Ursus arctos*) sperm.

From: editorial.rd@csiro.au

To: ppazc@unileon.es

Subject: Reproduction, Fertility and Development – Decision on Manuscript ID RD12181.R2

Dear Dr Paz

Thank you for sending the revised version of this paper (The antioxidant effects of soybean lecithin- or low-density lipoprotein-based extenders for the cryopreservation of brown bear (*Ursus arctos*) sperm) and for dealing so thoroughly with the referees' comments. I have now had the opportunity to examine your revised manuscript and *I pleased to accept it for publication in Reproduction, Fertility and Development.*

My only comment is that the pages 30-33 of the manuscript (corrections for non-cell particles and colour figures illustrating the approach) should be published as supplementary information online – in this way the information will be accessible for the readers, but would not dilute the major message of the study: and also colour figure published online would not incur charges. Please consider that publication of colour images in print if you decide to go for this option will be associated with charges.

You will hear in due course from the Production Editor regarding the copyedited manuscript, page proofs, etc. From what I can see, your files are acceptable in the format you have supplied, but you may be asked for different file formats if needed.

Thank you for your excellent contribution. On behalf of the Editors of Reproduction, Fertility and Development, we look forward to your continued contributions to the Journal.

Sincerely,

Professor Anna Petrunkina

Associate Editor, Reproduction, Fertility and Development

Editorial.rd@csiro.au

PAPER 4.

The addition of heat shock protein HSPA8 to cryoprotective media improves the survival of brown bear (*Ursus arctos*) spermatozoa during chilling and after cryopreservation.

----- Mensaje original -----

Asunto: Your Submission THERIO-D-12-00610

Fecha: 1 Nov 2012 16:52:07 +0000

De: Fulvio Gandolfi <therio.europe@unimi.it>

Para: ppazc@unileon.es

Ms. Ref. No.: THERIO-D-12-00610

Title: The addition of heat shock protein HSPA8 to cryoprotective media improves the survival of brown bear (*Ursus arctos*) spermatozoa during chilling and after cryopreservation

Theriogenology

Dear Sr PAZ CABELLO,

I am pleased to confirm that your manuscript, "The addition of heat shock protein HSPA8 to cryoprotective media improves the survival of brown bear (*Ursus arctos*) spermatozoa during chilling and after cryopreservation, *has been accepted for publication in Theriogenology*.

I would also like to congratulate for the quality of your work you since it is very rare that a manuscript is recommended for acceptance by both reviewers without a single change.

The files have been forwarded to the publisher and you will be contacted in due course regarding galley proofs.

Thank you for submitting your work to this journal.

Sincerely,

Fulvio Gandolfi, DVM, PhD

Co-Editor-in-Chief

Theriogenology

PAPER 5.

Brown bear sperm double freezing: effect of elapsed time and use of PureSperm® gradient between freeze-thaw cycles.

Asunto: Submission Confirmation for Brown bear sperm double freezing: effect of elapsed time and use of PureSperm® gradient between freeze-thaw cycles

De: "PLOS ONE" <plosone@plos.org>

Fecha: 12/12/2012 12:06

Para: "Paulino de Paz" <ppazc@unileon.es>

Dear Dr. de Paz,

Your submission entitled "Brown bear sperm double freezing: effect of elapsed time and use of PureSperm® gradient between freeze-thaw cycles" has been received by PLOS ONE. You will be able to check on the progress of your paper by logging on to Editorial Manager as an author. The URL is <http://pone.edmgr.com/>.

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal.

Kind regards,

PLOS ONE

APÉNDICE IV: INFORMES INTERNACIONALES TESIS

INFORME 1

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

2012-11-19

1(2)

Jane M. Morrell, Professor in Veterinary Reproductive Biotechnologies

To Whom It May Concern

*Report on the thesis entitled: Improvement and development of the freezing protocols of brown bear ejaculated semen samples (*Ursus arctos*).*

By the PhD student: Manuel Álvarez-Rodríguez.

The thesis describes a conservation-based project, featuring experiments to improve the quality of cryopreserved spermatozoa from the brown bear, *Ursus arctos*, which is an endangered species in Spain. The results of the experiments enabled practical conclusions to be drawn concerning methodology that can be applied directly to enhance sperm cryopreservation for conservation purposes. The project resulted in five scientific articles, four of which have been accepted in good journals dealing with applied reproductive techniques. The remaining article is currently under review. The experiments were designed to investigate the effects on sperm quality of various components of cryopreservation media, namely the timing and proportion of glycerol addition for effective cryopreservation, the effect of replacing egg yolk with soy bean proteins or low density lipoproteins, the effect of adding heat shock proteins, and lastly the conditions under which a double freezing protocol might be used, to permit additional biotechniques such as sex sorting to take place at a different location to the site of semen collection. The series of experiments increase in complexity from article 1 (timing of glycerol addition) to article 4 (effect of heat shock proteins).

The glycerol studies, together with different rates of cooling, indicate that brown bear spermatozoa may tolerate a range of conditions during cryopreservation. The use of a quadratic model elegantly enabled the best combination of parameters to be chosen to devise a protocol for sperm cryopreservation in this species. The third study involved investigations of replacements for egg yolk in cryopreservation media, for example with low density lipoproteins (LDL) or soybean lecithin (phosphatidyl choline type A and B). The results were conflicting for LDL in that sperm viability and antioxidant activity, but not motility, were higher in the media containing LDL. However, LDL could be used as a substitute for egg yolk whereas egg yolk was significantly better as a cryoprotectant than the lecithin-containing media. The fourth article identified two concentrations of heat shock protein 8 that could be used for enhancing sperm survival for fresh and cryopreserved brown bear spermatozoa, respectively. The results of the hyaluronic acid migration test were particularly interesting, in that although mean sperm progression was decreased in the presence of heat shock protein 8, the vanguard sperm subpopulation showed increased progression. The authors interpret this observation as reflecting the mechanism of selection of fertilizing spermatozoa in the female reproductive tract. The final article describes an investigation of the use of a double freezing protocol to enable other manipulations to be carried out between the two cryopreservation cycles, as has been attempted in other species.

According to his cv, the candidate has participated in several reproductive projects in different species, gaining a useful set of skills in wildlife Assisted Reproduction. Apart from the five first-author publications comprising the thesis, he has been a co-author on seven scientific papers and has made a number of conference presentations, showing his ability both to conduct his own scientific research and to collaborate with others. The publications describe a range of techniques, incorporating both practical applied methodology and advanced analytical tools in studies on ejaculated and epididymal spermatozoa from ram, brown bear and red deer. Based on the evidence provided, I can absolutely recommend him for the PhD degree.

Jane M. Morrell, BVetMed, BSc(Hons), PhD, MBA, FRCVS, leg vet, docent,

INFORME 2.

FACULTY OF VETERINARY MEDICINE
approved by the EAEVE

Department of **Reproduction, Obstetrics
and Herd Health**

3 December 2012

Dear Professor,

Concerning the PhD thesis of D. Manuel Alvarez Rodriguez (Faculty of Veterinary Medicine, University of León, Spain)

Title: Improvement and development of freezing protocols of brown bear ejaculated sperm samples (*Ursus arctos*)

Directors: Dr. Luis Anel Rodríguez and Dr. Paulino de Paz Cabello

Currently the brown bear (*Ursus arctos*) is seriously endangered in Spain due to their isolation and high vulnerability and therefore this species is considered to be at risk of extinction. Optimization of freezing protocols in combination with the application of several assisted reproductive techniques would support efforts to conserve this endangered species.

In the present PhD thesis, several experiments were performed concerning various aspects for the optimization of cryopreservation procedures of the brown bear (*Ursus arctos*) ejaculated sperm. This research resulted in 3 accepted peer-reviewed papers (2 published and 1 in press), 1 paper accepted with minor revisions and 1 paper currently under review. All papers were accepted or are submitted to internationally recognized scientific journals with high impact factors and well-known in the field of reproduction. The studies which are performed are interesting and original especially regarding the limited number of studies on this specific topic. The manuscripts and thesis are well-prepared and written understandably. The conclusions follow logically from the presented data and the findings are thoroughly discussed.

In my opinion, this thesis is scientifically sound and valid. Additionally, the candidate demonstrated that he can initiate and perform scientifically sound experiments, report the results and critically evaluate and discuss the main findings. Moreover, looking at the candidates *curriculum vitae* it becomes obvious he is a co-author on several other papers and has been active on international congresses. In my opinion, I consider the applicant suitable to obtain the PhD degree. The candidate may in my opinion therefore proceed to the oral defense of the thesis.

Sincerely yours and with kind regards

Tom Rijsselaere

Tom Rijsselaere, DVM, MSc LAS, PhD, Dipl ECAR
Head of Clinic for Small Animal Reproduction
Vice President of EVSSAR
Department of Reproduction, Obstetrics and Herd Health
Faculty of Veterinary Medicine - Ghent University
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APÉNDICE V: ESTANCIAS INTERNACIONALES TESIS

MINISTERIO
DE CIENCIA
E INNOVACIÓN

SUBDIRECCION GENERAL DE
FORMACION E INCORPORACION
DEL PERSONAL INVESTIGADOR

CERTIFICADO DE REALIZACIÓN DE ESTANCIA BREVE

Apellidos, nombre:	ÁLVAREZ RODRÍGUEZ, MANUEL	NIF/NIE:	09814159J
Referencia de la ayuda:	BES-2008-003151	Referencia del proyecto:	CGL2007-63748

ORGANISMO: ZOOLOGICAL SOCIETY OF LONDON (ZSL)

CENTRO: INSTITUTE OF ZOOLOGY

DEPARTAMENTO: REPRODUCTIVE BIOLOGY

PAÍS: REINO UNIDO

El abajo firmante certifica que el/la investigador/a en formación a quien se refiere el presente documento ha permanecido en el centro de trabajo desde el día 3 de SEPTIEMBRE de 2011 hasta el día 23 de DICIEMBRE de 2011(*)

Nombre y apellidos del firmante; WILLIAN VINCENT HOLT

Cargo: HEAD OF REPRODUCTIVE BIOLOGY

Fecha: 23 de DICIEMBRE de 2011

ZSL
LIVING CONSERVATION

INSTITUTE OF ZOOLOGY
THE ZOOLOGICAL SOCIETY OF LONDON
REGENT'S PARK, LONDON NW1 4RY

Firma y sello

W. V. Holt

(*) TO BE COMPLETED BY THE HOST RESEARCH DIRECTOR

The undersigned certifies that the scholar has remained in this centre from (day) of (month) of 200- until (day) of (month) of 200-

CORREO ELECTRONICO

becaspi@mec.es

MINISTERIO
DE CIENCIA
E INNOVACIÓN

SUBDIRECCION GENERAL DE
FORMACION E INCORPORACION
DEL PERSONAL INVESTIGADOR

CERTIFICADO DE REALIZACIÓN DE ESTANCIA BREVE

Apellidos, nombre:	Álvarez Rodríguez, Manuel	NIF/NIE:	09814159 J
Referencia de la ayuda:	BES-2008-003151	Referencia del proyecto:	CGL2007-63748

ORGANISMO: University of Sheffield
CENTRO: Unit of Reproductive and Developmental Medicine
DEPARTAMENTO: Department of Human Metabolism
PAÍS: Reino Unido

El abajo firmante certifica que el/la investigador/a en formación a quien se refiere el presente documento ha permanecido en el centro de trabajo desde el día 29 de Enero de 2012 hasta el día 1 de Abril de 2012 (*)

Nombre y apellidos del firmante: Alireza Fazeli
Cargo: Head of Department of Human Metabolism
Fecha: 1 de Abril de 2012

Firma y sello
DEPARTMENT OF
HUMAN METABOLISM
UNIVERSITY OF SHEFFIELD
U.K.

(*) TO BE COMPLETED BY THE HOST RESEARCH DIRECTOR
The undersigned certifies that the scholar has remained in this centre from (day) of (month) of 2012 until (day) of (month) of 2012

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becaspi@mec.es