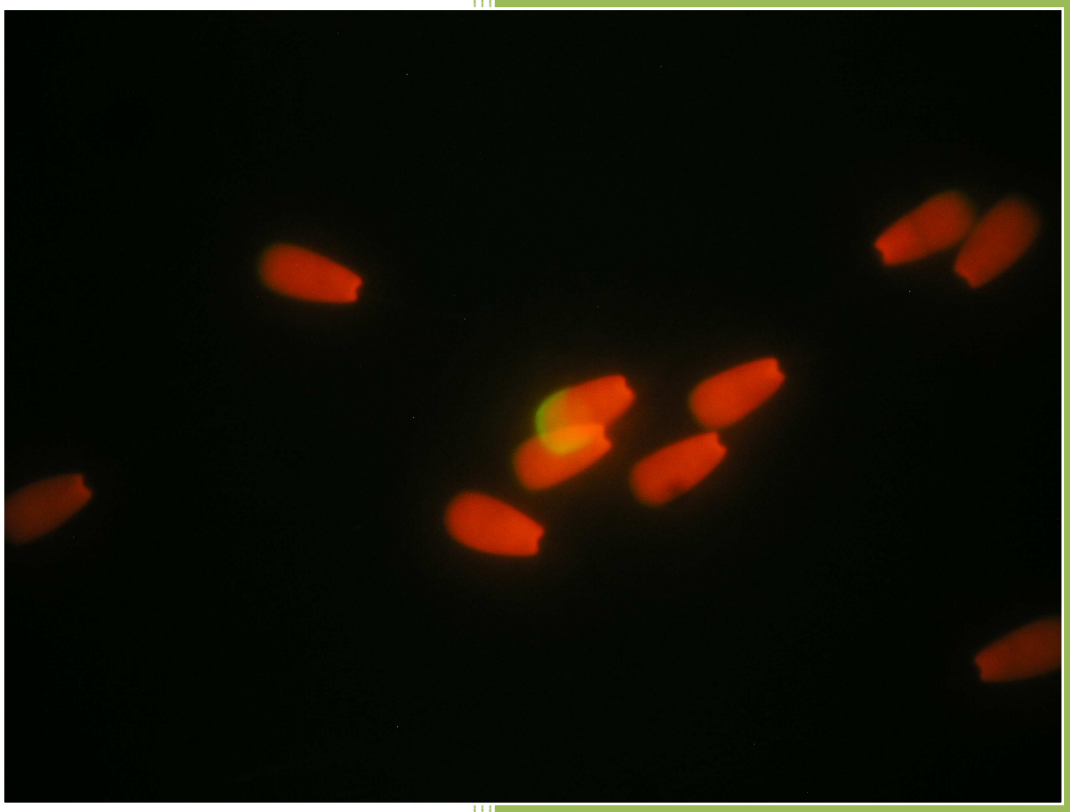
UNIVERSIDAD DE CÓRDOBA

FACULTAD DE VETERINARIA

Departamento de Medicina y Cirugía Animal

"CENTRIFUGACIÓN COLOIDAL DEL SEMEN CRIOCONSERVADO DE PERRO PARA LA SELECCIÓN DE ESPERMATOZOIDES"



Memoria para optar al grado de Doctor presentada por

Lucrecia Alcaraz Aranda

Bajo la dirección de los doctores

Jesús M. Dorado Martín
Manuel Hidalgo Prieto

Córdoba, 2013.

TITULO: *Centrifugación coloidal del semen crioconservado de perro para la selección de espermatozoides.*

AUTOR: *Lucrecia Alcaraz Aranda*

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Tesis Doctoral como compendio de publicaciones

1. Centrifugation on PureSperm® density-gradient improved quality of spermatozoa from frozen-thawed dog semen. J. Dorado, **L. Alcaraz**, N. Duarte, J.M. Portero, D. Acha, S. Demyda, A. Muñoz-Serrano, M. Hidalgo. *Theriogenology* 2011, 76: 381-385.

2. Changes in the structures of motile sperm subpopulations in dog spermatozoa after both cryopreservation and centrifugation on PureSperm® gradient. J. Dorado, **L. Alcaraz**, N. Duarte, J.M. Portero, D. Acha, M. Hidalgo. *Animal Reproduction Science* 2011, 125: 211-218.

3. Single-layer centrifugation through PureSperm® 80 selects improved quality spermatozoa from frozen-thawed dog semen. J. Dorado, **L. Alcaraz**, M.J. Gálvez, D. Acha, I. Ortiz, M. Urbano, M. Hidalgo. *Animal Reproduction Science* 2013, 140: 232-240.

TÍTULO DE LA TESIS: ***Centrifugación coloidal del semen crioconservado de perro para la selección de espermatozoides***

DOCTORANDO/A: ***Lucrecia Alcaraz Aranda***

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

D. ***Jesús M. Dorado Martín*** y **D.** ***Manuel Hidalgo Prieto***, Profesores del Departamento de Medicina y Cirugía Animal de la Facultad de Veterinaria de la Universidad de Córdoba,

INFORMAN:

Que el trabajo de tesis presentado por **Dña. Lucrecia Alcaraz Aranda**, titulado "***Centrifugación coloidal del semen crioconservado de perro para la selección de espermatozoides***" ha sido realizado bajo nuestra dirección y cumple con el artículo 24 de la norma reguladora de los estudios de Doctorado de la Universidad de Córdoba para la presentación de la tesis como compendio de publicaciones.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 16 de octubre de 2013

Firma de los directores

Fdo: Jesús M. Dorado Martín. Manuel Hidalgo Prieto

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Resumen

Resumen

La crioconservación del esperma es un pilar básico en los programas de reproducción asistida en la especie canina, al eliminar ésta los inconvenientes del transporte de animales, permitir la reproducción de perros genéticamente superiores en aquellos casos en los que el apareamiento natural resulta imposible y al ofertar la posibilidad de almacenar el esperma indefinidamente para su posterior uso. Pese a todas estas ventajas, la fertilidad potencial del esperma congelado se ve comprometida debido a las alteraciones en la estructura y fisiología del espermatozoide que se producen tras el proceso de congelación y descongelación. Por ello, nace la necesidad de desarrollar técnicas de congelación más eficaces y procedimientos que contrarresten estas limitaciones. En la actualidad, las técnicas de separación de espermatozoides mediante la centrifugación coloidal son capaces de mejorar la calidad del esperma criopreservado, seleccionando los espermatozoides más viables (potencialmente fértiles) de una población donde la mayoría están dañados o muertos. En este sentido, la selección de espermatozoides podría ser un prerrequisito para obtener tasas de gestación óptimas tras la inseminación artificial con esperma congelado-descongelado de perro.

El gradiente de densidad PureSperm® fue diseñado para la selección de espermatozoides viables y morfológicamente intactos en humanos. No obstante, ha sido empleado con éxito en varias especies animales (p. ej. primates, toro y carnero). Sin embargo, según la bibliografía consultada, no ha sido evaluado en la especie canina. Por lo expuesto, el objetivo principal de esta Tesis Doctoral fue estudiar la eficacia del coloide comercial PureSperm® para la selección de espermatozoides caninos crioconservados, evaluando diferentes parámetros de calidad seminal (motilidad, morfoanomalías, vitalidad espermática e integridad de acrosoma) en muestras seminales frescas, no seleccionadas (crioconservadas o control) y seleccionadas (crioconservadas y centrifugadas en PureSperm®).

En la primera y segunda publicación se evaluó la influencia del proceso de crioconservación y de la centrifugación a través del gradiente de densidad discontinuo PureSperm® 40/80 en la calidad seminal y la estructura de las subpoblaciones cinéticas del esperma canino crioconservado. En la tercera publicación buscamos simplificar la

técnica de separación, realizando en esta ocasión una centrifugación en una sola capa de PureSperm® 80. Durante el periodo experimental se emplearon un total de 9 perros adultos y sanos de diferentes razas (3 Galgos españoles, 1 Braco Alemán, 1 Mestizo y 4 Beagles). Un total de 20 eyaculados fueron obtenidos mediante estimulación manual (10 para las publicaciones uno y dos y 10 para la tercera publicación), 1 ó 2 veces por semana, y la fracción espermática del eyaculado fue separada. Las muestras seminales fueron crioconservadas empleando un protocolo estándar (método Uppsala modificado), para después de su descongelación ser centrifugadas a través de los coloides PureSperm® 40/80 o PureSperm® 80. Las muestras seminales (inmediatamente después de la recogida, tras la descongelación y después de la selección mediante centrifugación coloidal PureSperm® 40/80 y PureSperm® 80, según experimentos) fueron evaluadas para el movimiento (mediante el sistema de análisis de esperma asistido por ordenador - CASA, Sperm Class Analyzer), la morfología espermática (en muestras teñidas con Diff-Quick®), y la integridad de las membranas plasmática y acrosómica (tinción triple de Yoduro de Propidio, Isocianato de fluoresceína conjugado con la lectinas *Arachis Hipogea* y Rodamina 123).

La criopreservación afectó significativamente ($P < 0,001$) a todos los parámetros seminales analizados. Asimismo, influyó significativamente ($P < 0,001$) en la frecuencia de distribución de los espermatozoides móviles dentro de cada subpoblación y los parámetros cinéticos de las mismas. La centrifugación a través del gradiente discontinuo de densidad PureSperm® 40/80 mejoró la calidad de las muestras crioconservadas en cuanto a movimiento y vitalidad ($P < 0,001$). Por otra parte, las muestras seleccionadas fueron enriquecidas en la Subpoblación 4 (espermatozoides muy veloces y progresivos), alcanzando proporciones del 31,9% en comparación con las muestras no seleccionadas (24,9%). En conclusión, el gradiente de densidad PureSperm® 40/80 es un método relativamente simple que permite mejorar la calidad del esperma canino crioconservado.

En cuanto a la centrifugación del esperma canino crioconservado a través de una sola capa del coloide PureSperm® 80, las muestras seleccionadas presentaron mejores porcentajes de movimiento, morfología, vitalidad e integridad de acrosoma ($P < 0,001$), en comparación con las muestras no seleccionadas (control). Asimismo, la centrifugación en una sola capa de PureSperm® 80 incrementó el porcentaje de espermatozoides muy veloces y progresivos (Subpoblación 2: 44,1%). De los resultados

obtenidos podemos concluir que la centrifugación a través del coloide PureSperm® 80 puede ser una alternativa al gradiente discontinuo PureSperm® 40/80 y un método satisfactorio para mejorar la calidad del esperma canino crioconservado.

Abstract

Abstract

Sperm cryopreservation is an integral part of assisted reproductive programs and eliminates disadvantages of animal transportation, and allows genetically superior dogs to reproduce even if natural mating is impossible, and allows breeders to store semen for future use. However, cryopreservation induces both a loss of sperm viability and an impairment of functionally of the spermatozoa surviving the thawing, thus reducing fertilizing ability. Therefore, it is important to develop a suitable technique to cryopreserve mammalian spermatozoa. Currently, centrifugation techniques through layers of colloid has been satisfactorily applied to improve the quality of sperm following cryopreservation, thus selecting the most viable spermatozoa (potentially fertile) from a population where the majority has been damage or is dead. In this sense, sperm selection might be one of the prerequisites for achieving optimal conception rates after artificial insemination with frozen-thawed dog semen.

The PureSperm® density-gradient was designed to select viable and morphologically intact human spermatozoa. However, it has been used inter-species (i.e. primates, bull, and ram) with acceptable results. However, to our knowledge, studies on the effect of Puresperm® centrifugation on frozen-thawed dog semen have not been reported. The main aim of this Doctoral Thesis was to investigate if centrifugation through PureSperm® can improved the post-thaw quality of dog semen, based on the evaluation of sperm motility, morphology, viability and acrosome integrity in fresh semen, unselected semen samples (post-thawing or control) and selected preparations (after centrifugation of thawed samples through PureSperm®).

In the first and second papers of this series we evaluated the effects of both cryopreservation and discontinuous density-gradient centrifugation through PureSperm® 40/80 on semen quality and structures of motile sperm subpopulations of frozen-thawed dog semen. The third paper tried to simplify the sperm selection technique used in the other ones, using single-layer centrifugation through PureSperm® 80 after cryopreservation. During the experimental period, nine healthy, mature dogs of different breeds (3 Spanish Greyhounds, 1 German Pointer, 1 Crossbreed and 4 Beagles) were used. In total, twenty ejaculates were collected by digital manipulation (10 ejaculates for

the first and second papers and 10 ejaculates for the third paper), once or twice per week, and the sperm-rich fraction of the ejaculate was collected. Semen samples were cryopreserved following a standard method (modified Uppsala method) and selected by either density-gradient centrifugation on PureSperm® 40/80 or single-layer centrifugation through PureSperm® 80. Fresh semen, unselected sperm samples and selected preparations were evaluated for sperm motility (assessed by computerized-assisted semen analysis - CASA), morphology (Diff-Quick® staining) and viability (triple fluorescent stain of Propidium iodine / Isothiocyanate-labeled peanut (*Arachis hypogaea*) / Rhodamine 123).

Cryopreservation had a significant ($P < 0.001$) effect on all studied semen parameters. Likewise, cryopreservation had a significant ($P < 0.001$) effect on both the frequency distribution of spermatozoa within subpopulations and the motion characteristics of each subpopulation. PureSperm® 40/80 centrifugation yielded sperm suspensions with improved motility and viability ($P < 0.001$). On the other hand, the selected sperm samples was enrich in Subpopulation 4 (high speed and progressive spermatozoa), reaching a proportion of 31.9% of the present spermatozoa, in contrast with the unselected sperm samples, where this sperm subpopulation accounted for 24.9% of the total. In conclusion, PureSperm® 40/80 centrifugation is a relatively simple method for successfully improving the quality of frozen-thawed dog spermatozoa.

With regard to single-layer centrifugation results, PureSperm® 80 yielded sperm suspensions with improved motility, morphology, viability and acrosome integrity ($P < 0.001$). The selected sperm samples were enriched in Subpopulation 2 (high speed and progressive spermatozoa), reaching a proportion of 44.1% of the present spermatozoa. From these results, we conclude that single-layer centrifugation with PureSperm® 80 may be an alternative and successful method for improving the quality of frozen-thawed dog spermatozoa.

Introducción

Introducción

La crioconservación de esperma constituye una parte integral en los programas de reproducción asistida y tiene aplicación en distintas circunstancias, tales como aquellas que implican problemas de fertilidad en el macho, en Técnicas de Reproducción Asistida (TRA) y donantes en bancos de esperma (Kim et al., 2010). Sin embargo, este procedimiento induce una serie de cambios osmóticos, químicos y mecánicos al espermatozoide, causando la muerte de algunas células y graves daños en aquellas que sobreviven tras el proceso de congelación y descongelación (Watson, 1995; 2000). Estos daños son acumulativos y pequeñas lesiones pueden resultar en importantes cambios perjudiciales al final del proceso (Nicolas et al., 2012), lo que se traduce en una disminución de la viabilidad del espermatozoide y la consecuente disminución de su capacidad fertilizante (Watson, 2000). Así, la tasa de gestación y los porcentajes de nacidos vivos mediante las TRA actuales no son completamente satisfactorios cuando usamos esperma canino criopreservado (Kim et al., 2010). Por lo expuesto, la selección de los espermatozoides más viables (potencialmente fértiles) en muestras seminales procesadas (congeladas-descongeladas) donde la mayoría están dañados o muertos podría ser uno de los requisitos previos para lograr tasas de concepción óptimas tras la inseminación artificial (IA) u otras TRA con semen canino congelado-descongelado.

Actualmente, existen diversos procedimientos (p. ej. migración, filtración y centrifugación a través de coloides) disponibles para la selección de espermatozoides funcionales en base a distintas cualidades espermáticas (Morrell et al., 2011). En concreto, la centrifugación a través de gradientes de densidad ha sido indicada para las selección de espermatozoides en reproducción asistida (Morrell et al., 2009). Esta técnica ha sido utilizada satisfactoriamente para separar espermatozoides móviles, con la cromatina intacta y morfológicamente normales del resto del eyaculado (Morrell et al., 2009). Recientemente, se han desarrollado diferentes formulaciones especie-específica para su uso en reproducción asistida humana y animal (Morrell and Rodriguez-Martinez, 2009), incluida la especie canina (Morrell et al., 2008a). PureSperm® (Nidacon International AB, Gotemburgo, Suecia), uno de estos productos comerciales, fue originalmente diseñado para seleccionar espermatozoides humanos

viables y morfológicamente intactos para su posterior uso en reproducción asistida (Söderlund and Lundin, 2000; Morrell et al., 2004; Mousset-Siméon et al., 2004). PureSperm® ha sido también empleado con éxito en eyaculados y muestras procesadas de numerosas especies animales, como primates (O'Brien et al., 2003), titis (Hernández-López et al., 2005), toros (Hollinshead et al., 2004a; Maxwell et al., 2007; Underwood et al., 2009), carneros (O'Brien et al., 2003; Hollinshead et al., 2004a), osos (Nicolas et al., 2012) y perros (Dorado et al, 2011a, 2011b; Phillips et al., 2012), con el fin de separar los espermatozoides del plasma seminal o diluyentes y enriquecer también la cantidad de células espermáticas con morfología normal y/o motiles para ser usado posteriormente en TRA. Sin embargo, según la bibliografía consultada, no existen estudios sobre el efecto de la centrifugación de muestras de semen canino congelado-descongelado a través de gradientes de PureSperm®.

Un nuevo método de selección de espermatozoides mediante centrifugación coloidal ha sido recientemente desarrollado por Morrell et al. (2008a), el cual emplea una sola capa de coloide. Esta técnica es más simple que la centrifugación a través de gradientes, pero aparentemente igual de eficaz (Morrell et al., 2009). Así, usando diferentes coloides, la centrifugación en una sola capa de coloide ha sido empleada con éxito para mejorar la calidad de muestras de esperma en humanos (Zhou et al., 2010), sementales (Morrell et al., 2010; Gutiérrez-Cepeda et al., 2011) verracos (Martinez-Alborcia et al., 2012), toros (Thys et al., 2009), perros (Morrell et al., 2009) y gatos (Chatdarong et al., 2010). Una modificación de la técnica de centrifugación en el gradiente de densidad PureSperm®, centrifugación a través de una sola capa de coloide, ha sido realizada en la especie humana con resultados aceptables (Zhang et al., 2004). En cambio, según nuestro conocimiento, no existen estudios sobre el efecto que la selección de espermatozoides mediante la centrifugación en una sola capa de PureSperm® pudiera tener sobre la calidad del esperma de perro a la descongelación.

El movimiento espermático juega un papel importante en el transporte del esperma dentro del tracto reproductor femenino, principalmente para la penetración de la zona pelúcida. Clásicamente se ha considerado el eyaculado como una población homogénea con una distribución estadística normal, utilizándose los valores medios de movimiento para clasificar los eyaculados o para evaluar el efecto de un tratamiento o un procedimiento biotecnológico, lo que a día de hoy es considerado erróneo

(Mortimer, 1997). Actualmente, la identificación de subpoblaciones de espermatozoides móviles dentro de un mismo eyaculado en mamíferos se ha convertido en un tema de máximo interés para la correcta evaluación de los eyaculados. Sin embargo, no existe un consenso sobre el papel fisiológico que representan estas subpoblaciones en el eyaculado. Además es ampliamente conocido que existen distintas subpoblaciones de espermatozoides en los eyaculados de diferentes especies (Holt, 1996; Abaigar et al., 1999; Rigau et al., 2001; Quintero-Moreno et al., 2007; Dorado et al., 2010), las cuales se caracterizan por parámetros cinéticos con valores precisos, obtenidos por los sistemas de análisis de esperma asistido por ordenador (CASA). De esta manera, la presencia de estas subpoblaciones cinéticas se ha relacionado con la resistencia a la criopreservación (Martinez-Pastor et al., 2005; Núñez-Martínez et al., 2006; Núñez Martínez et al., 2006; Flores et al., 2009), la presencia de estimulantes (Abaigar et al., 1999), la respuesta a la refrigeración y la fertilidad (Quintero-Moreno et al., 2003; Quintero-Moreno et al., 2004; 2007). No obstante, según nuestro conocimiento, no se han realizado estudios en referencia a la influencia de la centrifugación del semen canino crioconservado bien a través del gradiente de densidad PureSperm® o a través de una sola capa de este coloide comercial sobre la estructura de las subpoblaciones cinéticas del espermatozoide de perro.

Objetivos

Objetivos

El objetivo principal de la presente Tesis Doctoral fue valorar la eficacia del coloide comercial PureSperm® en la selección del espermatozoide canino crioconservado, en base a la calidad seminal de las muestras procesadas.

Para alcanzar tal objetivo se plantearon diversos objetivos específicos, desarrollados en cada una de las tres publicaciones que incluye esta Tesis Doctoral:

Objetivo 1: Evaluar los efectos de la crioconservación del esperma canino en un diluyente comercial (CaniPRO™ Freeze A & B, Minitüb, Tiefenbach, Alemania) sobre la calidad seminal (Dorado et al., 2011a) y la distribución de los espermatozoides dentro de las diferentes subpoblaciones cinéticas identificadas (Dorado et al., 2011b).

Objetivo 2: Investigar si la centrifugación a través del gradiente de densidad discontinuo PureSperm® 40/80 mejora la calidad espermática de dosis seminales crioconservadas (Dorado et al., 2011a), incluyendo su efecto sobre las subpoblaciones espermáticas (Dorado et al., 2011b)

Objetivo 3: Valorar cómo la centrifugación del esperma canino crioconservado a través de una sola capa del coloide PureSperm® 80 mejora la calidad seminal a la descongelación, en base a su capacidad de separar subpoblaciones de espermatozoides con buen movimiento, morfología, vitalidad e integridad de acrosoma (Dorado et al., 2013).

Material y Métodos

Material y Métodos

1. Animales

A lo largo del periodo experimental se emplearon un total de 9 perros adultos y sanos de diferentes razas (3 Galgos españoles, 1 Braco alemán, 1 mestizo y 4 Beagles), con edades comprendidas entre los 3 y 8 años y de fertilidad desconocida. Los animales fueron alojados en las instalaciones del Hospital Clínico Veterinario de la Universidad de Córdoba SL y alimentados con pienso comercial y agua *ad libitum.*

2. Obtención y valoración de las muestras seminales

Para la primera y segunda publicación se recogieron un total de 8 eyaculados (2 eyaculados por individuo) de 4 de los perros, mientras que en la tercera publicación recolectamos un total de 10 eyaculados (2 eyaculados por individuo) de 5 de los perros. Los eyaculados fueron recolectados en días alternos mediante estimulación manual (Linde-Forsberg, 1991). La segunda fracción del eyaculado (fracción espermática) se recolectó en tubos graduados (BD Falcon™ Tubes, BD Biosciences, Erembodegem, Bélgica) atemperados a 38ºC, despreciándose las otras dos fracciones (preespermática y prostática).

Inmediatamente tras la recogida, las muestras seminales fueron evaluadas para el volumen, la concentración, el movimiento y la morfología espermática. El volumen de la fracción espermática fue registrado en tubos calibrados. La concentración espermática se evaluó mediante fotómetro (SpermaCue®, Minitüb, Tiefenbach, Alemania), como describió Peña et al. (2003). La morfología espermática se valoró subjetivamente en extensiones teñidas con la tinción Diff-Quick® (Baxter DADE Diagnostics AG, Düdingen, Suiza). Un mínimo de 200 espermatozoides por muestra fueron evaluados en microscopio de campo claro (Olympus BH-2, Olympus Optical Co., LTD, Tokio, Japón), obteniendo el porcentaje de espermatozoides anormales (ASM, %). Solo fueron incluidos en los estudios aquellos eyaculados cuya concentración espermática fuera superior a 200 x 10^6 espermatozoides/ml, volumen

superior o igual a 0,8 ml, con una motilidad total superior al 65% y un porcentaje de morfoanomalías inferior o igual al 30%.

3. Protocolo de congelación y descongelación del esperma canino

Las muestras seminales fueron procesadas según el método Uppsala (Peña and Linde-Forsberg, 2000), modificado por Núñez-Martínez et al. (2006). Tras la recogida, las muestras seminales fueron diluidas al 1:1 (v:v) en diluyente Tris-ácido cítrico (Biladyl® A, Minitüb, Tiefenbach, Alemania) y centrifugadas a 700 x g durante 10 minutos a temperatura de laboratorio (22ºC). Tras la centrifugación, se eliminó el sobrenadante y el sedimento resultante se resuspendió, a temperatura de laboratorio, con el volumen necesario de CaniPRO™ Freeze A suplementado con un 20% de yema de huevo para alcanzar una concentración aproximada de 300-400 x 10^6 espermatozoides/ml. La muestra diluida se refrigeró lentamente hasta 5ºC durante 1 hora en Equitainer™ I (Hamilton Research, Inc., Danvers, MA, USA). A continuación, realizamos una segunda dilución con CaniPRO™ Freeze B (5ºC) con un 20% de yema de huevo hasta conseguir una concentración final aproximada de 150-200 x 10^6 espermatozoides/ml. Finalmente, las muestras fueron envasadas manualmente en pajuelas de plástico de 0,5ml, congeladas horizontalmente en vapores de nitrógeno liquido, 4 cm por encima de la superficie del nitrógeno líquido, durante 10 minutos y almacenadas directamente en nitrógeno liquido a – 196ºC. Tras 24-48 horas de almacenamiento, las pajuelas seleccionadas al azar fueron descongeladas mediante inmersión en baño maría a 37ºC durante 30 segundos para su posterior análisis.

4. Selección de espermatozoides

Para la separación y selección de espermatozoides en dosis de semen criopreservado de perro, empleamos dos técnicas de selección diferentes: 1) el gradiente de densidad discontinuo para las dos primeras publicaciones y 2) la centrifugación coloidal en una sola capa para la tercera publicación.

4.1. Centrifugación en gradiente discontinuo de densidad (PureSperm® 40/80)

La selección de espermatozoides a través del gradiente de densidad discontinuo PureSperm® fue realizada según las indicaciones del fabricante (Nidacon International AB), a temperatura de laboratorio. En resumen, 2 ml del coloide PureSperm® 80 es pipeteado en un tubo de centrifugación cónico de 15 ml (BDFalcon™ Tubes) y, seguidamente, aplicamos cuidadosamente encima de ésta una segunda capa de 2 ml del coloide PureSperm® 40. Finalmente, depositamos una alícuota (1,5 ml) de esperma congelado-descongelado (tres pajuelas de 0,5 ml que contenían entre 150-200 espermatozoides/ml) sobre el gradiente de densidad. A continuación realizamos una primera centrifugación a 300 x g durante 20 minutos (Eppendorf Centrifuge 5702RH, Eppendorf Ibérica SLU, Madrid, España), tras la que aspiramos y eliminamos las capas superiores (diluyente y espermatozoides no viables). El pellet (espermatozoides seleccionados) se resuspendió en 5 ml de tampón fosfato salino atemperado (PBS, Sigma-Aldrich Chemie GMBH, Steinheim, Alemania) y centrifugado de nuevo a 500 x g durante 10 minutos. Por último, el sedimento es resuspendido en PBS hasta obtener una concentración de 30 x 10^6 espermatozoides/ml para su posterior análisis. Además, determinamos la concentración espermática de las muestras seleccionadas para calcular el rendimiento del procedimiento.

4.2. Centrifugación coloidal en una sola capa de PureSperm® 80

La técnica empleada para la centrifugación en una sola capa fue una modificación del protocolo por Morrell et al. (2012). En resumen, 2 ml del coloide PureSperm® 80, equilibrado a 22ºC, fue vertido en un tubo Falcon™. Seguidamente, dos pajuelas de un eyaculado individual, conteniendo 80-100 x 10^6 espermatozoides/ml, son descongeladas al mismo tiempo y su contenido mezclado. Después, pipeteamos el esperma descongelado (1 ml) sobre el coloide y centrifugamos a 300 x g durante 20 minutos. Finalmente, resuspendimos el pellet de espermatozoides en un nuevo tubo cónico con PBS hasta alcanzar una concentración final de 30 x 10^6 espermatozoides/ml para su posterior análisis. Al igual que en el protocolo anterior, determinamos la concentración espermática de la muestra seleccionada para calcular el rendimiento del procedimiento.

5. Evaluación de los parámetros de calidad seminal

La calidad seminal fue valorada 1) antes de la congelación (muestras frescas), 2) inmediatamente después de la descongelación (muestras no seleccionadas o control), 3) tras la centrifugación coloidal, bien en el gradiente de densidad o en una sola capa de coloide (muestras seleccionadas), y 4) tras el lavado de las muestras seleccionadas mediante centrifugación en gradiente discontinuo de densidad (primera publicación).

5.1. Morfología espermática

La morfología espermática fue evaluada en muestras teñidas con Diff-Quick® (Kim et al., 2010), bajo microscopía de campo claro (Olympus BH-2, Olympus Optical Co., LTD, Tokio, Japón). Al menos 200 espermatozoides por preparación fueron contados para determinar el porcentaje de morfoanomalías.

5.2. Movimiento espermático

El movimiento espermático se valoró objetivamente mediante el sistema de análisis de imagen asistido por ordenador Sperm Class Analyzer® (Microptic SL, Barcelona, España), como describieron Núñez-Martínez et al. (2006) para el perro. El análisis cinético se basó en la captura y posterior análisis de 25 imágenes digitalizadas consecutivas, obtenidas de un único campo microscópico. Las imágenes fueron capturadas en un lapso de tiempo de 1 segundo, correspondiéndose con una velocidad de captura de 1 fotograma cada 40 milisegundos. Las muestras analizadas fueron previamente diluidas hasta aproximadamente 25 x 10^6 espermatozoides/ml en el diluyente correspondiente y se mantuvieron en estufa a 38ºC durante 5 minutos. En cada análisis, dos alícuotas de 5 µl fueron evaluadas empleando un microscopio de contraste de fases (Eclipse 50i, Nikon, Tokio, Japón) equipado con una placa térmica a 38ºC. Tres campos microscópicos fueron capturados aleatoriamente en cada alícuota. Los objetos identificados erróneamente como espermatozoides fueron eliminados sobre el monitor

mediante la función playback. Los parámetros cinéticos evaluados fueron: velocidad curvilínea (VCL, µm/s: media de la velocidad del recorrido de los espermatozoides desde el punto actual al punto real siguiente en la trayectoria descrita por la célula espermática), velocidad rectilínea (VSL, µm/s: velocidad del recorrido medio en línea recta desde el punto inicial al punto final de la trayectoria), velocidad lineal o promedio (VAP, µm/s: velocidad del recorrido promedio realizado por el espermatozoide), índice de linealidad (LIN, %: resultado del cociente VSL/VCL, estima la proximidad de la trayectoria real del espermatozoide a una línea recta), índice de rectitud (STR, %: resultado del cociente VSL/VAP, estima la proximidad del recorrido del espermatozoide a una línea recta), oscilación (WOB, %: resultado del cociente VAP/VCL, estima la oscilación de la trayectoria curvilínea respecto de la trayectoria media), amplitud de desplazamiento lateral de la cabeza (ALH, µm: magnitud del desplazamiento lateral de la cabeza del espermatozoide con respecto a su trayectoria promedio) y frecuencia de cruces (BCF, Hz: promedio de la frecuencia con que la cabeza del espermatozoide cruza la trayectoria promedio en ambas direcciones). El movimiento total (MOT, %) fue definido como el porcentaje de espermatozoides con una VAP > 15 µm/s, mientras que los objetos con una VAP < 10 µm/s fueron considerados inmóviles. Además, el movimiento progresivo (PMOT, %) fue definido como el porcentaje de espermatozoides con una VAP > 65 µm/s y un STR >75%.

5.3. Vitalidad espermática

La vitalidad espermática fue evaluada en términos de integridad de las membranas plasmática y acrosómica y de la función mitocondrial. Estas características fueron valoradas simultáneamente usando una modificación de la técnica de triple tinción para fluorescencia descrita por Graham et al. (1990), la cual incluye los fluorocromos yoduro de propidio (PI), rodamina 123 (R123) e isocianato de fluoresceína conjugado con la lectina *Arachis Hipogaea* (FITC-PNA). Todos los reactivos fueron adquiridos de Sigma-Aldrich Chemie GMBH (Steinheim, Alemania). En resumen, 200 µl de esperma diluido (aproximadamente 25-50 x 10^6 espermatozoides/ml) fueron incubados a 38ºC en la oscuridad con 5 µl de R123 (0,1 mg/ml). El exceso de fluorocromo fue eliminado mediante centrifugación (400 x g durante 6 minutos) y el pellet de espermatozoides es a continuación resuspendido en

200 µl de PBS. Seguidamente, una mezcla de 10 µl de PI (0,1mg/ml) y 5 µl de FITC-PNA (0,1 mg/ml) fue añadida a la muestra, y las células fueron después incubadas durante 10 minutos a 38ºC. Un total de 300 espermatozoides por muestra fueron evaluados bajo microscopia de epifluorescencia (Olympus BX40, Tokio, Japón), empleando un filtro U-ND25-2 (filtro de excitación de 460 a 490 nm) y objetivo de 100x. Cuatro patrones de tinción pudieron identificarse: 1) espermatozoides vivos con un acrosoma intacto (VS), 2) espermatozoides vivos con reacción acrosómica (VS-AR), 3) espermatozoides muertos con un acrosoma intacto (Olds-Clarke) y 4) espermatozoides muertos con reacción acrosómica (DS-AR). Los valores fueros expresados como porcentajes.

6. Identificación de las subpoblaciones cinéticas

Las observaciones obtenidas por el sistema CASA en los eyaculados frescos, en las muestras seminales no seleccionadas (congeladas y descongeladas) y en las preparaciones seleccionadas tras la descongelación fueron incluidas en una única base de datos Excel®. A continuación, empleando el análisis clúster multivariante, los espermatozoides del conjunto de datos fueron clasificados y agrupados en un número reducido de subpoblaciones, de acuerdo a sus patrones de movimiento, siguiendo la metodología descrita por Martínez-Pastor et al. (2005). En resumen, empleamos en primer lugar el procedimiento PRINCOMP para realizar un análisis de componentes principales (PCA) sobre los datos cinéticos. A continuación, se aplicó un análisis clúster no jerárquico (procedimiento FASTCLUS) sobre los componentes principales seleccionados (variables). Como tercer paso, aplicamos un procedimiento clúster jerárquico (método AVERAGE) para agrupar los clúster obtenidos en el paso anterior. Para determinar finalmente el número de clúster o subpoblaciones (cuarto paso), se estudió la evolución lo largo del proceso de agrupación de tres estadísticos proporcionados por el análisis estadístico: *pseudo-T^2*, *pseudo-F*, y *criterio de agrupamiento cúbico*.

7. Análisis estadístico

Los resultados fueron expresados en media ± error estándar de la media (SEM). Los datos fueron estudiados empleando un modelo lineal general (PROC GLM) en el que los efectos de los machos y de la réplica dentro de cada macho fueron considerados como efectos aleatorios, así como la interacción entre ellos. Las variables dependientes expresadas como porcentajes fueron transformadas a arco seno antes el análisis. Las diferencias entre los valores medios fueron analizadas mediante el test de Duncan. Además, se calcularon las frecuencias relativas de cada subpoblación cinética, las cuales fueron comparadas mediante el análisis de varianza (ANOVA) y el test Chi-cuadrado (procedimiento FREQ). Todos los análisis fueron realizados mediante el paquete estadístico SAS v9.0 (SAS Institute Inc., Cary, NC, USA). Los valores fueron considerados estadísticamente significativos cuando $P<0,05$.

Resultados y Discusión

Resultados y Discusión

- Primera Publicación: ***Centrifugation on PureSperm® density-gradient improved quality of spermatozoa from frozen-thawed dog semen.*** *J. Dorado, L. Alcaraz, N. Duarte, J.M. Portero, D. Acha, S. Demyda, A. Muñoz-Serrano, M. Hidalgo*. Theriogenology 2011, 76: 381-385.

Nuestros resultados mostraron que la criopreservación indujo un aumento significativo ($P < 0,001$) en los valores medios de ASM, DS y DS-AR, mientras que se observó lo contrario para los valores de VS y VS-AR. Los parámetros derivados del CASA fueron significativamente ($P < 0,001$) superiores en el semen fresco que en las muestras congeladas-descongeladas. Esta disminución en la calidad de la cinética del espermatozoide canino probablemente se deba a que las mitocondrias son las estructuras del espermatozoide más sensibles al daño criogénico (Peña et al., 2003). No obstante, los resultados de calidad seminal obtenidos tras la descongelación tanto en las muestras seleccionadas como en las no seleccionadas son comparables a los obtenidos en otros estudios (Peña et al., 2003; Batista et al., 2006).

Hubo diferencias significativas ($P < 0,05$) entre los perros estudiados para los parámetros de calidad espermática, tanto en muestras frescas como congeladas-descongeladas, como también observamos un efecto significativo ($P < 0,001$) de la réplica. Además, observamos interacción ($P < 0,001$) entre los perros y la réplica para los parámetros evaluados en estas muestras. Por el contrario, no hubo diferencias significativas ($P > 0,05$) entre los perros para los parámetros de calidad espermática evaluados en las muestras seleccionas, como tampoco observamos interacción ($P > 0,05$) entre los perros y la réplica. Sin embargo, si observamos un efecto significativo ($P < 0,001$) de la réplica. La variabilidad individual observada en la calidad seminal a la descongelación sugiere que cada individuo puede tener diferente susceptibilidad a la crioconservación del esperma, como ha sido señalado anteriormente (Batista et al., 2006; Silva et al., 2006).

La centrifugación de espermatozoides congelados-descongelados a través de PureSperm® produjo un aumento significativo ($P < 0,001$) de los valores medios de

PMOT, VSL, STR, ALH y BCF. No se observaron diferencias significativas ($P > 0,05$) entre las muestras no seleccionadas y las seleccionadas en gradiente de densidad para VCL, VAP y LIN. Los valores medios de MOT y WOB fueron significativamente ($P < 0,001$) mayores en las muestras originales que en las seleccionadas a través de PureSperm®. Los valores medios de VS y VS-AR mejoraron significativamente ($P < 0,001$) tras la centrifugación en el gradiente de densidad, mientras que observamos lo contrario para DS. No se encontraron diferencias significativas ($P < 0,05$) entre las muestras no seleccionadas y las seleccionadas para los valores medios de ASM y DS-AR. Este es el primer intento de utilizar la centrifugación a través de PureSperm® para separar el esperma canino criopreservado. De acuerdo con los resultados de estudios previos (Hernández-López et al., 2005; Maxwell et al., 2007; Underwood et al., 2009), el protocolo PureSperm® seleccionó una población de espermatozoides con una calidad mejorada en lo que se refiere a una mayor motilidad, vitalidad e integridad del acrosoma. Aunque existió una tendencia a mejorar los espermatozoides morfológicamente normales, estas diferencias no resultaron significativas. Debido a diferentes factores tales como la fuerza de centrifugación, el volumen de los gradientes y el volumen de esperma utilizado, los resultados obtenidos con éste protocolo deben ser interpretados con cautela.

Además, los espermatozoides seleccionados a través de PureSperm® mostraron mayor velocidad que los no seleccionados. La relevancia biológica de este hallazgo en el perro ha sido recientemente establecida por Silva et al. (2006), demostrando que la presencia de altas velocidades en el espermatozoide son puntos de referencia para la fertilidad tanto *in vivo* como *in vitro*. Así, es de suponer que los espermatozoides seleccionados tras la centrifugación en gradiente discontinuo son las alícuotas de aquellos espermatozoides con mayor capacidad fecundante potencial. Futuras investigaciones son necesarias para determinar si modificaciones en este protocolo (formulación específica para el esperma canino, centrifugación en una sola capa, densidad del coloide, fuerza de centrifugación, etc.) son requeridas para mejorar la capacidad fecundante del semen de perro criopreservado y seleccionado mediante gradientes de densidad, incluyendo su uso para la inseminación artificial de perras.

Por otra parte, el lavado del sedimento tras la centrifugación en gradientes de densidad discontinuo redujo significativamente ($P < 0,001$) todos los parámetros CASA

evaluados. Valores superiores de ASM, VS-AR y DS ($P < 0,001$) y menores de VS y DS-AR ($P < 0,05$) fueron observados también tras el lavado del pellet resultante de la centrifugación coloidal. Es interesante destacar que este lavado tras la centrifugación coloidal en PureSperm® fue perjudicial para los espermatozoides crioconservados del perro, resultando en una disminución del porcentaje de espermatozoides motiles progresivos, viables y con acrosoma intacto. Estos resultados indican que no hubo necesidad de incluir una etapa de lavado en el protocolo, ahorrando así tiempo y esfuerzo en la preparación de las muestras.

Con respecto a la tasa de recuperación espermática, obtuvimos un rendimiento del 40,83% tras la centrifugación en PureSperm® y del 17,31% tras el lavado del pellet resultante de éste procedimiento. El volumen de la suspensión de esperma recuperado tras la centrifugación en PureSperm® osciló entre 0,5 y 1 ml y fue menor a 0,5 ml detrás el lavado.

- Segunda Publicación: ***Changes in the structures of motile sperm subpopulations in dog spermatozoa after both cryopreservation and centrifugation on PureSperm® gradient.*** *J. Dorado, L. Alcaraz, N. Duarte, J.M. Portero, D. Acha, M. Hidalgo***.** Animal Reproduction Science 2011, 125: 211-218.

Como esperábamos, la crioconservación indujo grandes cambios en los valores medios de los parámetros cinéticos del espermatozoide de perro. Las muestras congeladas-descongeladas (no seleccionadas y seleccionadas) mostraron una disminución significativa ($P < 0,001$) en los porcentajes de los parámetros CASA, con la excepción de STR en las muestras seleccionadas (75,36 ± 0,22% frente a 74,11 ± 0,76%; $P > 0,05$). La crioconservación implica estrés osmótico y la formación o reorganización del hielo intracelular durante la congelación y de nuevo durante la descongelación, lo que resulta en alteraciones de la fisiología de la célula. En el presente estudio, en lugar de un alto porcentaje de espermatozoides inmóviles tras la descongelación observamos principalmente una disminución en la calidad de la cinemática del espermatozoide, probablemente porque, como se ha propuesto anteriormente en perros, las mitocondrias del espermatozoide son las estructuras más sensibles al daño criogénico (Peña et al., 2003). No obstante, los valores obtenidos en el semen congelado son comparables a los de otros estudios (Silva et al., 1996; Ström et al., 1997; Peña et al., 1998; Rota et al., 1999; Yildiz et al., 2000; Peña et al., 2003; Silva et al., 2003; Batista et al., 2006).

Hubo diferencias significativas ($P < 0,05$) entre los perros estudiados para los parámetros de calidad espermática, tanto en muestras frescas, congeladas-descongeladas como seleccionadas, como también observamos un efecto significativo ($P < 0,001$) de la réplica. Además, observamos interacción ($P < 0,001$) entre los perros y la réplica para los parámetros evaluados en estas muestras. La variabilidad individual observada en la calidad seminal a la descongelación sugiere que cada individuo puede tener diferente susceptibilidad a la crioconservación del esperma, como previamente señalaron (Batista et al., 2006; Silva et al., 2006).

La selección del esperma canino crioconservado mediante el gradiente de densidad PureSperm® mejoró significativamente ($P < 0,01$) la mayoría de los parámetros cinéticos evaluados (PMOT, VCL, VSL, LIN, STR, ALH y BCF) con el

sistema CASA. Hasta donde sabemos, este es el primer intento de utilizar PureSperm® para separar espermatozoides crioconservados de perro. La centrifugación del esperma descongelado a través del gradiente de densidad PureSperm® seleccionó una población de espermatozoides de calidad mejorada en lo que se refiere a un aumento de la motilidad (valores medios de PMOT, VCL, VSL, LIN, STR, ALH y BCF elevados). Esta mejora del movimiento y de la velocidad del espermatozoide canino coincide con estudios previos que emplearon PureSperm® en otras especies (Maxwell et al., 2007; Underwood et al., 2009) y también con aquellos que usaron otras técnicas de selección mediante gradientes de densidad como Percoll® (Valcárcel et al., 1996; Suzuki et al., 2003; Samardzija et al., 2006; Brum et al., 2008; Garcia et al., 2010), BoviPure® (Samardzija et al., 2006; Underwood et al., 2009) y Androcoll-E™ (Johannisson et al., 2009; Macías García et al., 2009; Morrell et al., 2009).

No hubo diferencias significativas ($P < 0,05$) entre perros para los parámetros de cinéticos, tanto en el semen fresco como en las muestras congeladas-descongeladas, así como observamos un efecto significativo ($P < 0,001$) de la réplica, lo que sugiere que los perros, de forma individual, pueden tener diferente susceptibilidad a la crioconservación del esperma, como anteriormente han sugerido (Batista et al., 2006; Silva et al., 2006). Además, observamos una interacción significativa ($P < 0,001$) entre el perro y la réplica.

El procedimiento FASTCLUS detectó cuatro subpoblaciones cinéticas a partir de los datos de movimiento. La Subpoblación 1 representó aquellos espermatozoides con baja velocidad (VCL, VSL y VAP bajas), con escaso movimiento progresivo (VAP, LIN y STR bajas) y que cubrieron una distancia muy corta (VSL muy baja). El total de espermatozoides móviles incluido en esta subpoblación fue 20,97%. La Subpoblación 2 incluyó aquellos espermatozoides cuyo movimiento se definió como menos vigoroso, indicado por valores más bajos de VCL y ALH. Sus trayectorias mostraron una baja linealidad mayor que los de la Subpoblación 1 ($P < 0,001$). Esta subpoblación incluyó al 18,24% del total de espermatozoides móviles. La Subpoblación 3 incluyó espermatozoides con gran actividad cinética pero no progresivos, como indicaron los altos valores de VCL y BCF junto a valores bajos de LIN y STR. Su escasa progresividad fue evidente por su alto valor de ALH. El total de espermatozoides móviles incluidos en esta subpoblación fue 20,75%. La Subpoblación 4 representó a

aquellos espermatozoides con el mayor grado de movimiento progresivo (VSL, VAP, LIN y STR muy altos junto a valores bajos de ALH). Estos exhibieron una vigorosa actividad flagelar (BCF alta). El total de espermatozoides móviles incluidos en esta subpoblación fue 40,03%.

Nuestro estudio reveló la presencia de cuatro subpoblaciones espermáticas en el semen fresco, no seleccionado y seleccionado de perro, que pueden ser fácilmente definidas por la combinación de ocho parámetros cinéticos CASA. La identificación de subpoblaciones cinéticas se ha realizado previamente en semen fresco y congelado-descongelado de perro (Rigau et al., 2001; Núñez Martínez et al., 2006). Debido a que tanto el sistema CASA como los parámetros cinéticos usados para definir las subpoblaciones espermáticas en estos estudios previos fueron diferentes de los utilizados en el presente estudio, es difícil comparar los patrones de movimiento de las subpoblaciones espermáticas identificadas. A pesar de ello, una simple interpretación de esta estructura de subpoblaciones de espermatozoides es que estos cuatro grupos representan espermatozoides en diferentes estados fisiológicos (Abaigar et al., 1999), reflejo de las grandes diferentes existentes en las características del comportamiento natatorio de estos espermatozoides.

La presencia de tres o cuatro subpoblaciones cinéticas definidas ha sido demostrado en numerosas especies (Holt, 1996; Abaigar et al., 1999; Quintero-Moreno et al., 2003; Miró et al., 2005; Muiño et al., 2008; Dorado et al., 2010). Como indican nuestros resultados y resultados previos (Núñez Martínez et al., 2006), la especie canina parece no ser una excepción. Sin embargo, según la bibliografía consultada, la existencia de subpoblaciones cinéticas definidas en muestras de semen canino seleccionado no ha sido previamente investigada.

Aunque, el porcentaje de espermatozoides móviles incluido en cada subpoblación experimentó importantes cambios tras el proceso de crioconservación y tras la centrifugación en gradiente de densidad, la estructura de las subpoblaciones se mantuvo perfectamente tras ambos procedimientos. De acuerdo con estudios previos (Cremades et al., 2005; Flores et al., 2008; Muiño et al., 2008; Flores et al., 2009; Muiño et al., 2009), la criopreservación tuvo un efecto significativo tanto en la frecuencia de distribución de los espermatozoides dentro de las subpoblaciones como en

las características cinéticas de cada subpoblación. Ésta indujo una disminución significativa ($P < 0,001$) en el porcentaje de espermatozoides móviles incluidos en las Subpoblaciones 3 (espermatozoides muy activos pero no progresivos; de 24,1% a 13,2%) y 4 (espermatozoides altamente activos y progresivos; de 47,0% a 24,9%). Esta disminución fue compensada por un aumento concomitante ($P < 0,001$) en los porcentajes de espermatozoides incluidos en las Subpoblaciones 1 (de 15,9% a 31,1%) y 2 (de 12,9% a 30,8%). Las Subpoblaciones 3 y 4 pudieron perder su actividad flagelar, probablemente como consecuencia de una lesión inducida por el proceso de congelación y descongelación. La crioconservación también modificó la mayoría de los parámetros cinéticos evaluados en cualquiera de las subpoblaciones obtenidas. En general, los parámetros de velocidad espermáticas (VCL, VSL y VAP) se redujeron en todas las subpoblaciones. La trayectoria del movimiento espermático cambió de una manera diferente en función de la subpoblación espermática. Así, en las Subpoblaciones 3 y 4, las trayectorias fueron más progresivas tras la criopreservación (mayor LIN), mientras que en las Subpoblaciones 1 y 2, las trayectorias fueron menos progresivas tras la congelación y descongelación. En este contexto, la simple aplicación de un análisis multivariante sobre los patrones cinéticos de los espermatozoides móviles para asignarlos a subpoblaciones parece ser completamente informativo, abriendo así la posibilidad de utilizar este tipo de análisis para mejorar en gran medida el análisis estándar de la calidad del semen de perro en cuanto a su capacidad de resistir a la crioconservación. De hecho, Flores et al. (2009) señalaron que la resistencia a la criopreservación del semen de verraco está relacionado con cambios específicos tanto en la estructura de las subpoblaciones cinéticas como en las características de movimiento de cada subpoblación.

La centrifugación mediante gradiente de densidad también enriqueció las muestras de esperma tratado en aquellas subpoblaciones con mayores velocidades. La subpoblación predominante en las muestras no seleccionadas fue la Subpoblación 1, una subpoblación caracterizada por un escaso movimiento no progresivo, representando más del 30% de los espermatozoides en la muestra. El tratamiento de las muestras crioconservadas a través de PureSperm® modificó significativamente la distribución de los espermatozoides dentro de las subpoblaciones. Por lo tanto, la Subpoblación 4 (espermatozoides muy activos y con movimiento progresivo) representó ahora más del 31% de los espermatozoides obtenidos tras la centrifugación en el gradiente

PureSperm®. Por otra parte, la Subpoblación 4 se observó con mayor frecuencia en el semen fresco en comparación con las muestras no seleccionadas. Tras la centrifugación del semen descongelado a través de PureSperm®, hubo un aumento significativo ($P < 0,001$) en los valores medios de STR en las Subpoblaciones 1-3, como de los valores medios de BCF fueron significativamente ($P < 0,001$) mayores en las Subpoblaciones 3 y 4. Nuestros resultados son consistentes con los que anteriormente obtuvo Macías García et al. (2009) tras la centrifugación de espermatozoides congelados-descongelados de caballo en una sola capa de coloide, lo que nos permite concluir que PureSperm® es un método que mejora la calidad de las muestras de esperma en términos de estructura de las subpoblaciones de espermatozoides. En este sentido, el significado de calidad hace referencia a que los espermatozoides poseen los atributos básicos de importancia crítica para la fertilidad de un eyaculado dado.

Es de destacar que la estructura general de espermatozoides móviles presente en el perro se mantiene constante a pesar del efecto causado tanto por la crioconservación como por la separación en el gradiente PureSperm®. Esto indica que el mantenimiento de la estructura global de las subpoblaciones podría ser importante a la hora de mantener la función general del eyaculado, independientemente del estado funcional específico. Estos resultados señalaron hacia la importancia de mantener una estructura de subpoblaciones específica en los eyaculados de mamíferos, independientemente de la especie en la que los estudios fueron realizados. En este sentido, la existencia de una estructura de subpoblaciones específica ha sido descrita en diferentes especies mamíferas como el cerdo (Abaigar et al., 1999), el caballo (Quintero-Moreno et al., 2003), el toro (Muiño et al., 2008), el macho cabrío (Dorado et al., 2010), el ciervo (Martinez-Pastor et al., 2005), la gacela (Abaigar et al., 1999), el perro (Rigau et al., 2001), el hámster dorado (Holt, 1996) y el conejo (Quintero-Moreno et al., 2007), indicando que es una característica común del eyaculado de mamíferos. Estudios recientes indican que los cambios en el movimiento asociados con procesos como la capacitación *in vitro* en el semen de verraco (Ramió et al., 2008) no modifican la estructura general de las subpoblaciones de estas muestras y que los cambios de movimiento vinculados a estos procesos inducen principalmente cambios concomitantes en el porcentaje de movimiento incluido en cada subpoblación específica. Por lo tanto, el mantenimiento de esta estructura subpoblacional específica sería importante en el mantenimiento de la función general del esperma en mamíferos.

La mayoría de los parámetros de movimiento utilizados en el presente estudio para la identificación de subpoblaciones espermáticas han sido identificados previamente como los marcadores de la calidad seminal más fiables (Olds-Clarke, 1996; Holt et al., 1997; De Geyter et al., 1998; Larsen et al., 2000; Dorado et al., 2009) y asociados con la fertilidad (Amann, 1989; Farrell et al., 1993; Macleod and Irvine, 1995; Moore and Akhondi, 1996; Holt et al., 1997; Farrell et al., 1998; Vidament et al., 2000). Estudios en perros (Silva et al., 2006) y cerdos (Holt et al., 1997) han demostrado que altas velocidades son puntos de referencia de fertilidad tanto *in vivo* como *in vitro*. También se ha informado anteriormente de que la VCL es de vital importancia para la formación de depósitos de espermatozoides en la unión útero-tubárica en ratones (Olds-Clarke, 1996), que la VCL y VAP están relacionados con la capacidad de los espermatozoides de carnero para penetrar el moco cervical (Robayo et al., 2008), y que tras la descongelación, la VSL está relacionado con la fertilidad de espermatozoides de toro (Gillan et al., 2008) y humanos (Thys et al., 2009). Además, el predominio de una subpoblación particular de espermatozoides móviles en muestras con una mejor crioresistencia o fertilidad has sido demostrado en otros estudios (Quintero-Moreno et al., 2003; Martinez-Pastor et al., 2005). Por lo tanto, teniendo en cuenta esta evidencia experimental, y puesto que la técnica de selección mediante gradientes de densidad ha sido estudiada en este estudio, se puede suponer que las preparaciones de esperma tratadas con esta técnica obtiene dosis seminales con espermatozoides más veloces y, por tanto, son las alícuotas con aquellos espermatozoides de mayor potencial fecundante. Sin embargo, la implicación en la fertilidad del esperma de las diferentes subpoblaciones de espermatozoides móviles y su distribución en éste requieren una futura investigación.

Por otra parte, se identificó una variabilidad individual para cada una de las cuatro subpoblaciones espermáticas ($P < 0,001$) tanto en el semen fresco como en el descongelado o en el seleccionado tras la centrifugación en gradiente de densidad. Así, se encontraron diferencias significativas ($P < 0,05$) entre los tratamientos para las Subpoblaciones 1 y 2 del perro 2, para la Subpoblación 3 de los perros 1 y 5 y para la Subpoblación 4 de los perros 2-5.

Finalmente, la tasa de recuperación espermática obtenida dependió de la calidad de la muestra original tratada y fue del 40,83% tras la centrifugación del semen descongelado a través del gradiente PureSperm®. El volumen de la suspensión de esperma recuperado osciló entre 0,5 y 1 ml tras la centrifugación coloidal.

- Tercera Publicación: ***Single-layer centrifugation through PureSperm® 80 selects improved quality spermatozoa from frozen-thawed dog semen*****.** *J. Dorado, L. Alcaraz, M.J. Gálvez, D. Acha, I. Ortiz, M. Urbano, M. Hidalgo.* Animal Reproduction Science 2013, 140: 232-240.

El objetivo de este estudio fue evaluar la centrifugación coloidal usando PureSperm® 80 para la selección de espermatozoides crioconservados de perro. La centrifugación del esperma congelado-descongelado de perro a través de una sola capa del coloide PureSperm® 80 mejoró significativamente ($P < 0,001$) los parámetros cinéticos evaluados (MOT, PMOT, VCL, VSL, VAP, LIN, STR, WOB, BCF y ALH) con el sistema CASA. Los valores medios de MOT y ALH fueron significativamente mayores ($P < 0,001$) en el semen fresco que en las muestras congeladas-descongeladas. Además, los valores medios de VS y DS mejoraron significativamente ($P < 0,001$) tras la centrifugación coloidal, mientras que observamos lo contrario para los valores de VS-AR, DS-AR y ASM ($P < 0,001$). No hubo diferencias individuales ($P > 0,05$) para los parámetros de calidad del esperma, como tampoco observamos interacción ($P > 0,05$) entre los perros y la réplica de los parámetros espermáticos evaluados tras la selección espermática. Sin embargo, un efecto significativo ($P < 0,001$) de la réplica fue observado en la calidad seminal.

En relación a las subpoblaciones espermáticas, el presente estudio identificó 3 poblaciones cinéticas definidas tanto en semen fresco, congelado-descongelado (control) como en las muestras tratadas, tras la clasificación de 26.051 espermatozoides motiles en base a ocho parámetros cinéticos (VCL, VSL, VAP, LIN, STR, WOB, ALH y BCF). Este resultado coincide con lo mostrado en estudios previos en perros (Rigau et al., 2001; Núñez-Martínez et al., 2006; Núñez Martínez et al., 2006; Dorado et al., 2011b; Dorado et al., 2011c). La Subpoblación 1 (sP1) incluyó espermatozoides muy activos pero no progresivos, como lo indican los valores altos de VCL y BCF junto a valores bajos de LIN y STR. Su escasa progresividad se evidenció mediante sus valores de ALH. Los bajos valores de LIN indican que estas células muestran movimientos circulares o irregulares. Asimismo, los valores elevados de VCL y ALH indican que sP1 se caracteriza por un amplio desplazamiento lateral de la cabeza del espermatozoide. Esta subpoblación podría ser considerada como espermatozoides con movimiento similar al hiperactivo. El total de espermatozoides móviles incluidos en esta

subpoblación fue 40,3%. La Subpoblación 2 (sP2) representó aquellos espermatozoides con el mayor grado de motilidad progresiva (valores muy elevados de VSL, VAP, LIN y STR junto a valores bajos de ALH), probablemente con el patrón de movimiento más adecuado para formar parte de la población de fertilización. Estos exhiben una vigorosa acción flagelar (BCF elevada). El total de espermatozoides incluidos en esta subpoblación fue 30,0%. La Subpoblación 3 (sP3) incluyó aquellos espermatozoides cuyo movimiento fue menos vigoroso, como lo indican los valores más bajos de VCL, VSL, VAP y ALH. Poseyeron escasa progresividad (valores bajos de VAP, LIN y STR) y recorrieron distancias muy cortas (VSL muy baja). Esta población, con valores bajos de VCL y LIN, podría representar al grupo de espermatozoides metabólicamente comprometidos, los cuales rápidamente pierden su movilidad por completo. Además, poseerían alteraciones estructurales o cambios bioquímicos importantes que afectan al espermatozoide. El total de espermatozoides incluidos en esta subpoblación fue 29,7%. Los espermatozoides con escasa motilidad, como los pertenecientes a sP1 y sP3, tienen menor probabilidad de progresar hasta áreas particulares del tracto reproductivo femenino y por lo tanto es menos probable que estén envueltos en la fertilización (Cremades et al., 2005).

La centrifugación coloidal indujo un aumento significativo ($P < 0,001$) del porcentaje de espermatozoides de sP2 (valores elevados de VCL, VSL y VAP), de un 36,8% en las muestras congeladas-descongeladas a un 44,1% en las muestras congeladas-descongeladas y tratadas mediante centrifugación coloidal. Por el contrario, la proporción de espermatozoides asignados a sP3 (valores bajos de VCL, VSL y VAP) se redujo significativamente ($P < 0,001$) del 35,2% en las muestras congeladas-descongeladas control al 16,0% en las muestras seleccionadas tras la descongelación. Por otra parte, la variabilidad individual se identificó para cada una de las subpoblaciones espermáticas ($P < 0,001$), ya sea en semen fresco, congelado-descongelado o seleccionado.

Nuestros resultados proporcionan, por primera vez, una prueba concluyente de que PureSperm® 80 mejora la calidad del esperma canino crioconservado en lo que se refiere a la integridad de acrosoma, motilidad, morfología y viabilidad, y además modifica la distribución de las subpoblaciones espermáticas en favor a espermatozoides con mayor movimiento progresivo (sP2). Esto coincide en términos generales con

estudios previos sobre la centrifugación en una sola capa de coloide de esperma crioconservado de semental (Macías García et al., 2009) y de macho cabrío (Jiménez-Rabadán et al., 2012). Del mismo modo, usando la centrifugación a través de una sola capa de PureSperm® 80 fue posible preparaciones de esperma con calidad seminal equivalente a las obtenidas mediante la técnica de centrifugación en gradiente de densidad (Dorado et al., 2011b; Dorado et al., 2011c; Phillips et al., 2012).

La mayoría de los parámetros CASA usados en el presente estudio han sido previamente identificados como marcadores fiables de la calidad del esperma (Holt et al., 1997; Larsen et al., 2000; Dorado et al., 2009). En relación a esto, la centrifugación coloidal usando una sola capa de PureSperm® 80 mejoró significativamente todos los parámetros de motilidad evaluados en el esperma canino congelado-descongelado, lo que en términos generales coincide con estudios previos para la centrifugación coloidal con Androcoll-C™ (Morrell et al., 2008b), Androcoll-E™ (Johannisson et al., 2009)o EquiPure® (Gutiérrez-Cepeda et al., 2011), y para otras técnicas de centrifugación en gradiente de densidad como PureSperm® (Hernández-López et al., 2005; Maxwell et al., 2007; Dorado et al., 2011b; Nicolas et al., 2012; Phillips et al., 2012), Percoll® (Samardzija et al., 2006; Brum et al., 2008; Garcia et al., 2010) y BoviPure® (Samardzija et al., 2006; Brum et al., 2008; Underwood et al., 2009; Garcia et al., 2010). De especial interés fueron las mejoras en la velocidad de los espermatozoides. Los tres parámetros de velocidad espermática proporcionados por el equipo CASA son las principales características cinemáticas relacionadas con la fertilidad. Estudios en el perro (Silva et al., 2006) y el cerdo (Holt et al., 1997) han demostrado que las altas velocidades del espermatozoide son puntos de referencia de la fertilidad *in vivo* e *in vitro*. También ha sido descrito previamente que la VCL es de vital importancia para la formación del reservorio de espermatozoides en la unión útero-tubárica en ratones (Olds-Clarke, 1996), que la VCL y VAP están vinculados a la capacidad del espermatozoide de carnero para penetrar el moco cervical (Robayo et al., 2008), y que la VSL a la descongelación es relacionada con la fertilidad del toro (Gillan et al., 2008) y humanos (Van den Bergh et al., 1998). Así, se puede suponer que las preparaciones de esperma tratadas mediante centrifugación en una sola capa de PureSperm® son las alícuotas de aquellos espermatozoides con mayor fertilidad potencial. Futuros estudios sobre la correlación de los parámetros cinéticos del esperma canino crioconservado y seleccionado a través de coloides con la fertilidad de los perros deberán realizarse.

El predominio de una subpoblación particular en las muestras con mejor resistencia al proceso de crioconservación o fertilidad ha sido demostrado en otros estudios (Quintero-Moreno et al., 2003; Martinez-Pastor et al., 2005). En este sentido, la centrifugación en una sola capa de PureSperm® 80 modificó la distribución de los espermatozoides dentro de las subpoblaciones. Así, sP2 (espermatozoides con alta velocidad y movimiento progresivo) representó más del 44% de espermatozoides recuperados tras la centrifugación en PureSperm® 80. Nuestros resultados son consistentes con los obtenidos previamente por Macías García et al. (2009) tras la centrifugación de espermatozoides equinos congelados a través de una sola capa de coloide. En un estudio previo observamos también que el gradiente de densidad PureSperm® enriqueció la subpoblación con velocidades más altas en el esperma canino crioconservado (Dorado et al., 2011b), lo que nos permite concluir que el gradiente de densidad mejoró la calidad seminal de las muestras procesadas en términos de estructura subpoblacional. En este sentido, el término de calidad hace referencia a que los espermatozoides poseen los atributos básicos de importancia crítica para la fertilidad de un eyaculado dado. Por lo tanto, teniendo en cuenta ésta evidencia experimental, y puesto que la centrifugación coloidal usando PureSperm® 80 selecciona los espermatozoides con mayor velocidad en la dosis de inseminación, se puede suponer que las dosis de esperma obtenidas tras éste procedimiento son las alícuotas de espermatozoides con mayor potencial fecundante. En este sentido, somos conscientes de que futuras investigaciones deben realizarse para estudiar la implicación de las diferentes subpoblaciones cinéticas y su distribución en el semen descongelado en la fertilidad *in vivo* o *in vitro.*

El objetivo de la selección de los espermatozoides tras la congelación y descongelación debe ser mejorar la calidad de la muestra seminal, reduciendo el riesgo de usar espermatozoides no funcionales en la fertilización y optimizando así los resultados de las TRA (Underwood et al., 2009). Nuestros resultados muestran que la población seleccionada mediante la centrifugación coloidal tras la descongelación del esperma canino mostró una mejor morfología, vitalidad e integridad de acrosoma frente a las muestras crioconservadas (control). Estos resultados se corresponden con los obtenidos por diversos estudios en otras especies animales (Macías García et al., 2009; Jiménez-Rabadán et al., 2012), cuando la centrifugación coloidal se realiza tras la

descongelación de las muestras. Estudios previos demuestran que la centrifugación en gradientes de densidad no mejora la morfología del espermatozoide canino criopreservado (Kim et al., 2010; Dorado et al., 2011a; Phillips et al., 2012). Para concluir, parece claro que la centrifugación coloidal del esperma canino crioconservado mediante PureSperm® 80 puede ser un procedimiento adecuado para la mejora de la calidad a la descongelación de las dosis seminales congeladas.

La mayoría de los sistemas de selección de espermatozoides resultan en la pérdida de espermatozoides y, por tanto, puede ser que la proporción de células funcionales seleccionadas sea su factor crítico de estas técnicas. Se ha demostrado la eficacia de la centrifugación coloidal en la mejora de la calidad seminal de muestras frescas de esperma canino, recuperándose hasta un 47% de espermatozoides tras el procesado (Morrell et al., 2008a). Por lo tanto, la centrifugación en una sola capa de coloide podría ser un método adecuado para mejorar la calidad de los eyaculados para la IA. Sin embargo, el presente estudio adapta la técnica de centrifugación en una sola capa de coloide al semen canino congelado-descongelado, usando PureSperm® 80 como coloide. La tasa de recuperación varió entre un 32,2 y un 84,5% (tasa media de recuperación del 61,5%), la cual fue mayor que la obtenida por Morrell et al. (2008b). Esta discordancia entre estudios podría reflejar diferencias entre las muestras procesadas, lo que podría afectar al número de espermatozoides funcionales que atraviesan el coloide (Martinez-Alborcia et al., 2012). Además, resulta difícil comparar estudios que usan métodos diferentes (formulación del coloide, volúmenes de esperma y de coloide, fuerzas de centrifugación, tamaño y diámetro de los tubos de centrifugación) o especies, porque la dinámica física puede afectar a las tasas de recuperación, como ha sugerido previamente por Nicolas et al. (2012). De todos modos, se necesitan más estudios con vistas a optimizar el rendimiento del procedimiento de centrifugación coloidal en una sola capa.

En un estudio previo, recuperamos un menor porcentaje de espermatozoides (40,8%) tras la centrifugación del esperma canino crioconservado a través del gradiente de densidad PureSperm® (Dorado et al., 2011a; Dorado et al., 2011b). De acuerdo a estos hallazgos, y desde una perspectiva práctica y clínica, la centrifugación a través de una sola capa de PureSperm® 80 ofrece un método alternativo, más práctico que el

gradiente de densidad para la selección de espermatozoides caninos de buena calidad, ahorrando así tiempo y esfuerzo en la preparación de las muestras.

Varios estudios demuestran que los resultados de la IA intrauterina son generalmente buenos cuando dosis de 50-100 × 10^6 espermatozoides de buena calidad son depositados en perras sanas y en el momento adecuado (Pretzer et al., 2006; Thomassen and Farstad, 2009). En nuestro estudio, el rendimiento medio obtenido fue de aproximadamente 60 × 10^6 espermatozoides en cada muestra de semen procesado (0,5 ml por pajuela × 100 millones de espermatozoides por ml x tasa de recuperación/100), lo cual es suficiente para inseminar una perra si empleáramos la IA intrauterina. Además, la eliminación de los espermatozoides indeseables de la muestra de esperma canino crioconservado usando la centrifugación a través del coloide PureSperm® 80 hace posible la preparación de dosis seminales enriquecidas en características espermáticas específicas para la IA intrauterina y poder obtener así mayores tasas de concepción. Por otra parte, de acuerdo con estudios anteriores (Hollinshead et al., 2004b; Maxwell et al., 2007), observamos que la centrifugación en una sola capa de coloide fue un método eficaz para eliminar la yema de huevo de la muestra congelada-descongelada original, lo que podría tener un impacto positivo en la evaluación de la calidad del semen.

La criopreservación indujo una disminución significativa ($P < 0,001$) de los valores medios de MOT, PMOT, VCL, ALH y BCF. Los valores de ASM, DS, DS-AR y VS-AR aumentaron significativamente ($P < 0,001$) tras el proceso de congelación y descongelación, mientras que lo opuesto fue observado para VS. No existió diferencias ($P > 0,05$) individuales para los parámetros de calidad seminal, tanto en muestras de semen fresco como congelado-descongelado, mientras que se encontró un efecto significativo ($P < 0,001$) de la réplica. Además, hubo interacción ($P < 0,001$) entre los perros y la réplica para todos los parámetros evaluados. Nuestros resultados mostraron que la mayoría de parámetros de calidad espermática evaluados (motilidad, morfología, vitalidad e integridad de acrosoma) se deterioraron tras la descongelación en comparación con el semen fresco. En cambio, la calidad seminal obtenida tanto tras la descongelación como tras la selección mediante PureSperm® 80 es comparable a la obtenida por otros estudios (Yildiz et al., 2000; Peña et al., 2003; Silva et al., 2003; Batista et al., 2006).

A pesar de que el porcentaje de espermatozoides incluidos en cada subpoblación experimentó cambios importantes tras el proceso de crioconservación y de selección, la estructura subpoblacional se mantuvo perfectamente tras ambos procedimientos. La criopreservación indujo una disminución significativa ($P < 0,001$) en el porcentaje de espermatozoides móviles incluidos en sP1 (de 44,6% a 28,1%). Esta disminución fue compensada por el consiguiente aumento significativo ($P < 0,001$) de los porcentajes de espermatozoides incluidos en sP2 (de 22,5% a 36,8%) y sP3 (de 32,9% a 35,2%). Diferencias individuales fueron observadas para cada una de las tres subpoblaciones ($P < 0,001$) tanto en muestras de semen fresco, congelado-descongelado como seleccionado mediante centrifugación coloidal. Es sabido que los cambios en la motilidad espermática inducidos por la crioconservación están vinculados a cambios concomitantes en la frecuencia de distribución de los espermatozoides dentro de las subpoblaciones (Flores et al., 2008). Nuestros resultados confirman estos hallazgos y también establecen claramente, en concordancia con estudios previos de otras especies de mamíferos (Cremades et al., 2005; Flores et al., 2008; Muiño et al., 2008), que la estructura general de los espermatozoides móviles presentes en el esperma canino se mantuvo constante a pesar del efecto causado por la crioconservación. Esto estuvo relacionado con cambios simétricos en el porcentaje específico de cada subpoblación. Por lo tanto, el aumento global en la mayoría de las características cinéticas medias del esperma canino tras la crioconservación, especialmente la velocidad de los espermatozoides, se relacionó con una disminución en el porcentaje de espermatozoides en sP1 (espermatozoides muy activos pero no progresivos) y un con un aumento concomitante de espermatozoides de sP2 (velocidad alta y movimiento progresivo) y sP3 (espermatozoides lentos y poco lineales) en las muestras de control (congeladas-descongeladas), cuando son comparadas con el semen fresco. La reducción en el porcentaje de espermatozoides en sP1 indicó que muchos espermatozoides muestran un movimiento similar al hiperactivo que los hace incapaces de sobrevivir a la congelación y descongelación, probablemente como consecuencia de la lesión sub-letal inducida por este proceso, y que quedarían excluidos del posterior análisis. Como resultado, la proporción de espermatozoides incluidos en sP2 y sP3 aumentaron parcialmente en el esperma crioconservado. Además, algunos de los espermatozoides de sP1 y sP2 fueron asignados a sP3, posiblemente a causa de la alteración de su funcionalidad (Quintero-Moreno et al., 2003). Por lo tanto, es posible que el aumento en el porcentaje de sP3 sea

una señal precoz de los cambios en la función del espermatozoide, los cuales también podrían ser detectados por las alteraciones en otros parámetros de calidad seminal (p. ej. movimiento total, vitalidad o alteración de acrosoma). El aumento del número de espermatozoides en sP2 podría haber sido debido al efecto de la dilución o de la adición de fructosa antes de su valoración. Verstegen et al. (2005) describieron cómo los parámetros de motilidad de espermatozoides refrigerados se volvieron a estimular mediante la centrifugación y la adición de más diluyente. También se ha demostrado que la fructosa induce un aumento del movimiento progresivo (Rigau et al., 2001).

Conclusiones

Conclusiones

De acuerdo a los resultados obtenidos en la presente Tesis Doctoral, se pueden extraer las conclusiones que se enumeran a continuación:

- Primera Publicación: ***Centrifugation on PureSperm® density-gradient improved quality of spermatozoa from frozen-thawed dog semen*****.** *J. Dorado, L. Alcaraz, N. Duarte, J.M. Portero, D. Acha, S. Demyda, A. Muñoz-Serrano, M. Hidalgo*. Theriogenology 2011, 76: 381-385.

1. La centrifugación a través del gradiente de densidad PureSperm® puede ser un método eficaz para mejorar la calidad del esperma canino dañado por el proceso de congelación-descongelación, y presumiblemente con la mayor capacidad fecundante potencial, debido a que la mayoría de parámetros de calidad espermática explorados aparecen significativamente mejorados con respecto a la suspensión original de esperma congelado-descongelado.

2. El lavado del pellet resultante de la centrifugación en gradiente de densidad redujo drásticamente la calidad seminal a la descongelación, indicando que la inclusión de este paso en el proceso de selección es innecesario.

- Segunda Publicación: ***Changes in the structutes of motile sperm subpopulations in dog spermatozoa after both cryopreservation and centrifugation on PureSperm® gradient*****.** *J. Dorado, L. Alcaraz, N. Duarte, J.M. Portero, D. Acha, M. Hidalgo***.** Animal Reproduction Science 2011, 125: 211-218.

1. Los resultados del presente estudio mostraron la existencia de cuatro subpoblaciones cinéticas tanto en el semen fresco, en las muestras no seleccionadas como en las seleccionadas de perro.

2. La crioconservación modificó significativamente tanto los parámetros específicos como la distribución de los espermatozoides entre las subpoblaciones.

3. El gradiente discontinuo PureSperm® es un método simple para mejorar la calidad del esperma canino dañado por el proceso de congelación-descongelación y, aunque esto debería ser confirmado experimentalmente, presumiblem**e**nte con la mayor capacidad fecundante potencial, ya que la Subpoblación 4 (espermatozoides muy veloces y con movimiento progresivo) fue la subpoblación más frecuentemente observada tras la preparación en el gradiente.

4. Finalmente, este estudio demostró que la estructura general de subpoblaciones cinéticas en el perro se mantuvo constante a pesar del efecto causado tanto por el proceso de crioconservación como por el de separación a través del gradiente PureSperm®.

- Tercera Publicación: ***Single-layer centrifugation through PureSperm® 80 selects improved quality spermatozoa from frozen-thawed dog semen*****.** *J. Dorado, L. Alcaraz, M.J. Gálvez, D. Acha, I. Ortiz, M. Urbano, M. Hidalgo.* Animal Reproduction Science 2013, 140: 232-240.

1. La centrifugación en una sola capa del coloide PureSperm® 80 puede ser un método alternativo y eficaz para la mejora de la calidad del esperma canino dañado por el proceso de congelación-descongelación, y aunque esto debería ser confirmado experimentalmente, presumiblemente con la mayor capacidad fertilizante potencial, ya que la mayoría de los parámetros de calidad espermática explorados fueron significativamente mejores que los obtenidos en las muestras originales de esperma congelado-descongelado.

2. Además, la Subpoblación 2 (espermatozoides con alta velocidad y movimiento progresivo) se observó con mayor frecuencia tras la selección a través del coloide PureSperm® 80.

3. Por último, este estudio también demostró que la crioconservación modifica significativamente la distribución de los espermatozoides dentro de las subpoblaciones; sin embargo, la estructura general de las subpoblaciones

espermáticas en perros se mantuvo constante a pesar del efecto causado por la crioconservación o selección a través de PureSperm® 80.

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Lista de Publicaciones

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Technical note

Centrifugation on PureSperm® density-gradient improved quality of spermatozoa from frozen-thawed dog semen

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Abstract

The main objective of this study was to investigate if centrifugation through PureSperm® density-gradient can improve the post-thaw semen quality of dog semen. Semen from 5 dogs was collected and cryopreserved following a standard protocol. After thawing, semen samples were selected by centrifugation on PureSperm®. Assessments of sperm motility (assessed by computerized-assisted semen analysis), morphology (Diff-Quick staining) and viability (triple fluorescent stain of Propidium iodine/isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin/Rhodamine 123), were performed on aliquots of fresh semen, unselected samples and selected preparations. Cryopreservation had a significant ($P < 0.001$) effect on all studied semen parameters. PureSperm® centrifugation yielded sperm suspensions with improved motility and viability ($P < 0.001$). The washing step significantly reduced ($P < 0.001$) all of the kinematics parameters evaluated as well as reduced the proportion of viable spermatozoa with intact acrosomes ($P < 0.05$). We concluded that PureSperm® centrifugation is a successful method for improving the quality of frozen-thawed dog spermatozoa. However, washing after density-gradient centrifugation dramatically reduces the post-thaw semen quality, indicating that the inclusion of such a washing step is unnecessary.
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Keywords: Sperm separation; CASA; Sperm viability; Cryopreservation; Dog spermatozoa

1. Introduction

Cryopreservation induces a series of osmotic, chemical and mechanical stresses to sperm, causing death of some sperm and severe post-thaw damage in surviving cells, reducing fertilizing ability [1]. Therefore, current pregnancy and live birth success rates of assisted reproduction techniques (ART) are not completely satisfactory with frozen-thawed dog semen [2]. The selection of the most viable spermatozoa (potentially fertile) from a population where the majority has been damaged or is dead might be one of the prerequisites for achieving optimal conception rates after ART with frozen-thawed dog semen.

Centrifugation through layers of colloid has been satisfactorily used to separate motile, chromatin-intact and morphologically normal spermatozoa from the extended semen [3]. Recently, some species-specific formulations have been developed for use in human ART and also in animal ART [3], including

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dogs [4]. PureSperm®, one of those commercial products, has also been used inter-species with acceptable results [5,6]. However, to our knowledge, studies on the effect of PureSperm® centrifugation on frozen-thawed dog semen have not been reported.

The main objective of this study was therefore to investigate if centrifugation through PureSperm® density-gradient can improve the post-thaw quality (motility, morphology and viability) of dog semen.

2. Materials and methods

2.1. Semen collection and processing

Semen was obtained from 5 dogs of different breeds, ranging between 5 and 6 years of age. Two ejaculates from each dog were collected by digital manipulation. Semen volume was measured in a calibrated tube. Sperm concentration was determined with a photometer (SpermaCue, Minitüb, Tiefenbach, Germany). Only ejaculates with volume $\geq$ 0.8 mL, sperm concentration $\geq 200 \times 10^6$ spermatozoa/mL, motility $\geq$ 70% and normal morphology $\geq$ 70% were included in the study.

Semen was cryopreserved using a standard commercial extender (CaniPRO™ Freeze A & B, Minitüb, Tiefenbach, Germany). In brief, semen was diluted 1:1 (v:v) with Tris-based extender (Biladyl A, Minitüb, Tiefenbach, Germany) and centrifuged at 700 g for 10 min at 22 °C. The sperm pellet was suspended to a final concentration of 300 to 400 $\times 10^6$ sperm/mL with CaniPRO™ Freeze A plus 20% egg yolk at 22 °C. Extended semen was cooled to 5 °C within an hour and then diluted to a final concentration of 150 to 200 $\times 10^6$ sperm/mL in CaniPRO™ Freeze B plus 20% egg yolk at 5 °C. Finally, the spermatozoa were loaded in 0.5 mL plastic straws, frozen horizontally in ranks placed 4 cm above the surface of liquid nitrogen (LN_2) for 10 min and placed into LN_2. After 24 to 48 h of storage, straws were thawed in a water bath at 37 °C for 30 sec, for analyses.

2.2. Sperm separation procedure

Sperm selection on PureSperm® gradients was accomplished according to producer's directions (Nidacon International AB, Gothenburg, Sweden). In brief, 2 mL of PureSperm® 80 was pipetted into a 15 mL centrifuge tube and then carefully layered with 2 mL of PureSperm® 40. An aliquot of the frozen-thawed semen (three 0.5 mL straws containing 150 to 200 sperm/mL) was layered (1.5 mL) on top of the gradient before centrifugation (300 × g/20 min at 22 °C). The sperm pellet was diluted with 5 mL PBS (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and washed by further centrifugation at 500 × g for 10 min at 22 °C for later examinations.

2.3. Sperm quality parameters assessment

Sperm quality were immediately assessed (1) post-thawing (unselected sperm samples), (2) after centrifugation of thawed samples through PureSperm® (selected sperm samples after DGC) and (3) after washing of the sperm pellet resulting from the DGC (selected sperm samples after washing step).

2.3.1. Sperm morphology

At least 200 spermatozoa were counted on slides stained with Diff-Quick® (Baxter DADE Diagnostics AG, Düdingen, Switzerland) to determine the percentage of spermatozoa with abnormal morphology (ASM, %).

2.3.2. Objective sperm motility

Motility was measured using a CASA system (Sperm Class Analyzer, Microptic SL, Barcelona, Spain), as described by Núñez-Martínez et al [7]. The following parameters were recorded: curvilinear velocity (VCL, μm/sec), straight line velocity (VSL, μm/sec), average path velocity (VAP, μm/sec), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz), total motility (MOT, %) and progressive motility (PMOT, %).

2.3.3. Plasma membrane integrity, mitochondrial function and acrosomal integrity

Sperm viability was assessed in terms of plasma membrane integrity, mitochondrial membrane potential and acrosome membrane integrity. These characteristics were analyzed simultaneously using a modification of a triple fluorescent procedure described by Graham et al [8], which includes the fluorochromes propidium iodide (PI), Rhodamine 123 (R123) and isothiocyanate-labeled peanut *(Arachis hypogaea)* agglutinin assay (FITC-PNA). All reagents were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Briefly, 200 μL of sperm sample (25 to 50 million cells approximately) were incubated at 38 °C in the dark for 25 min with 5 μL of R123 (0.1 mg/mL). Excess probe was removed from the spermatozoa by centrifugation (400 × g / 6 min), and spermatozoa were re-suspended in 200 μL of PBS. A mixture of 10 μL of PI (0.1 mg/mL) and 5 μL of FITC-PNA (0.1 mg/mL) was added to the samples, and the cells were then incubated at 38 °C for 10 min. A total of 300 spermatozoa were evaluated under 100 × magnification with epifluorescence mi-

Table 1
Means values of the semen quality analysis of fresh dog semen and frozen-thawed samples.

Parameter	Fresh semen	Original frozen-thawed samples
Total abnormalities (%)	10.23 ± 0.08[b]	35.07 ± 0.06[a]
Viable sperm with an intact acrosome (%)	71.62 ± 0.09[a]	42.65 ± 0.23[b]
Viable sperm with an acrosome reaction (%)	3.93 ± 0.02[a]	2.95 ± 0.01[b]
Nonviable sperm with an intact acrosome (%)	14.10 ± 0.07[b]	26.20 ± 0.17[a]
Nonviable sperm with an acrosome reaction (%)	10.31 ± 0.06[b]	28.20 ± 0.21[a]
Total motility (%)*	97.13 ± 0.03[a]	76.49 ± 0.18[b]
Progressive motility (%)†	78.92 ± 0.09[a]	35.30 ± 0.14[b]
Curvilinear velocity (VCL, μm/sec)	108.37 ± 0.39[a]	66.00 ± 0.67[b]
Straight line velocity (VSL, μm/sec)	67.65 ± 0.37[a]	40.04 ± 0.57[b]
Average path velocity (VAP, μm/sec)	85.46 ± 0.35[a]	49.92 ± 0.58[b]
Linearity (LIN, %)	58.27 ± 0.23[a]	47.09 ± 0.40[b]
Straightness (STR, %)	73.18 ± 0.23[a]	63.89 ± 0.41[b]
Wobble (WOB, %)	75.98 ± 0.14[a]	67.80 ± 0.27[b]
Amplitude of lateral head displacement (ALH, μm)	3.17 ± 0.01[a]	2.84 ± 0.02[b]
Beat cross frequency (BCF, Hz)	8.99 ± 0.03[a]	6.39 ± 0.06[b]

* Total motility is defined as the percentage of spermatozoa with a mean velocity > 15 μm/sec.
† Rapid progressive motility is defined as the percentage of spermatozoa with a mean velocity > 50 μm/sec and straightness > 75%.
Semen quality parameters shown here are described in Section 2.3. Each ejaculate was diluted for freezing in CaniPRO™ Freeze A & B containing 20% egg yolk.
[a,b] Different superscripts in the same row indicate significant differences ($P < 0.05$).

croscopy (Olympus BX40, Tokyo, Japan) in each smear, using a U-ND25-2 filter (a 460 to 490 nm excitation filter). Four staining patterns could be discerned: 1) viable sperm with an intact acrosome (VS), 2) viable sperm with an acrosome reaction (VS-AR), 3) nonviable sperm with an intact acrosome (DS) and 4) nonviable sperm with an acrosome reaction (DS-AR). Values were expressed as percentages.

2.4. Statistical analysis

Results are expressed as mean ± SEM. Data were studied using a general linear model (PROC GLM) in which the effects of males and replica within male were considered as random effects, as well as the interaction between them. Dependent variables expressed as percentages were arcsine-transformed before the analysis. Differences between mean values were analyzed by the Duncan method. All analyses were performed with SAS statistic package v9.0 (SAS Institute Inc., Cary, NC, USA). The level of significance was set at $P < 0.05$.

3. Results

3.1. Comparison between mean quality parameters of fresh semen and frozen-thawed samples

Cryopreservation induced a significant ($P < 0.001$) increase in mean values of ASM, DS and DS-AR, whereas the opposite was found for mean VS and VS-AR values. CASA-derived parameters were significantly ($P < 0.001$) higher in fresh semen than in frozen-thawed samples (Table 1).

There were significant ($P < 0.05$) differences between dogs in sperm quality parameters, both in fresh semen and frozen-thawed samples, as well as we found a significant ($P < 0.001$) effect of the replica (data not shown). Moreover, there was interaction ($P < 0.001$) among dogs and replica for sperm parameters.

3.2. Effect of PureSperm selection on post-thaw semen quality

Centrifugation of frozen-thawed spermatozoa through PureSperm® yielded a significant ($P < 0.001$) increase in mean PMOT, VSL, STR, ALH and BCF values (Table 2). No significant ($P > 0.05$) differences between unselected samples and those selected by density-gradient centrifugation (DGC) were found for VCL, VAP and LIN. Mean MOT and WOB values were significantly ($P < 0.001$) higher in the original spermatozoa than PureSperm-separated spermatozoa. Washing of the sperm pellet after DGC reduced significantly ($P < 0.001$) all CASA measures (Table 2). As shown in Table 3, mean VS and VS-AR values improved significantly ($P < 0.001$) after DGC, whereas the reverse was found for DS. No significant ($P > 0.05$) differences between unselected and selected samples were found for mean ASM and DS-AR values. Higher values of ASM, VS-AR and DS ($P < 0.001$) and lower values of VS and DS-AR ($P < 0.05$) were found after

Table 2
Motility parameters for dog sperm samples before PureSperm® gradient centrifugation, immediately after density-gradient centrifugation (DGC) and after washing of the sperm pellet resulting from the density-gradient centrifugation.

Sperm treatment	Sperm parameter									
	MOT (%)	PMOT (%)	VCL (μm/sec)	VSL (μm/sec)	VAP (μm/sec)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
Unselected	74.71 ± 0.19[a]	37.09 ± 0.21[b]	66.18 ± 0.67[a]	40.87 ± 0.59[b]	50.64 ± 0.60[a]	47.32 ± 0.41[a]	64.53 ± 0.41[b]	68.20 ± 0.27[a]	2.25 ± 0.02[b]	6.51 ± 0.07[b]
Selected										
After DGC	68.48 ± 0.48[b]	40.78 ± 0.41[a]	69.10 ± 1.22[a]	44.27 ± 1.04[a]	49.66 ± 1.04[a]	49.51 ± 0.78[a]	74.11 ± 0.76[a]	62.31 ± 0.61[b]	2.38 ± 0.04[a]	8.19 ± 0.16[a]
After washing step	55.45 ± 0.63[c]	20.47 ± 0.47[c]	54.14 ± 1.74[b]	32.80 ± 1.48[c]	37.31 ± 1.47[b]	40.33 ± 1.21[b]	66.15 ± 1.27[b]	55.92 ± 0.99[c]	2.13 ± 0.06[c]	5.88 ± 0.21[c]

Results are expressed as means ± SEM for 10 semen samples from 5 dogs.
MOT, total motility; PMOT, progressive motility; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.
[a,b,c] Different superscript letters in the same column indicate significant differences ($P < 0.05$).

washing of the sperm pellet resulting from the DGC (Table 3).

There were no dog differences ($P > 0.05$) for sperm quality parameters, nor was there any interaction ($P > 0.05$) among dogs and replica for sperm parameters assessed after semen processing (cryopreservation or sperm selection). However, a significant ($P < 0.001$) effect of the replica was observed in sperm quality.

The yield was 40.83% after PureSperm® centrifugation and 17.31% after washing of the sperm pellet resulting from DGC. The volume of the sperm suspension recovered ranged from 0.5 to 1 mL after DGC and below 0.5 mL after the washing step.

4. Discussion

We observed mainly decreases in the quality of sperm kinematics probably because the sperm mitochondria are the sperm structures more sensitive to cryodamage in dogs [9]. Nevertheless, post-thaw semen quality of both unselected and selected samples is comparable with that from other studies [9–11]. There was a significant male effect on post-thaw semen quality, suggesting that individual dogs may have different susceptibility to sperm cryopreservation, as previously reported [10,11].

This is the first attempt to use PureSperm® centrifugation to separate cryopreserved canine sperm. In agreement with findings in previous studies [5,6,12], the PureSperm® protocol selected a sperm population with improved quality in regard to higher motility, viability and acrosome integrity. Although there was a tendency for morphologically normal spermatozoa to improve after gradient selection, these differences did not reach statistical significance. Because of different factors such as force of centrifugation, volume of gradients and volume of spermatozoa loaded on gradients, the sperm parameters results in that protocol should be interpreted with caution.

Additionally, PureSperm-selected spermatozoa had higher velocity than unselected spermatozoa. The biologic relevance of this finding in dogs has been recently established by Silva et al [10], demonstrating that high sperm velocities are landmarks of fertility both *in vivo* and *in vitro*. Therefore, it can be surmised that sperm

Table 3
Parameters of the sperm morphology and viability assay for dog sperm samples before PureSperm® gradient centrifugation, immediately after density-gradient centrifugation (DGC) and after washing of the sperm pellet resulting from the density-gradient centrifugation.

Sperm treatment	Sperm parameter				
	ASM (%)	VS (%)	VS-AR (%)	DS (%)	DS-AR (%)
Unselected	35.07 ± 0.06[b]	42.65 ± 0.23[b]	2.95 ± 0.01[c]	26.20 ± 0.17[b]	28.20 ± 0.21[a]
Selected					
After DGC	34.84 ± 0.09[b]	44.46 ± 0.37[a]	3.14 ± 0.04[b]	24.02 ± 0.28[c]	34.64 ± 0.28[a]
After washing step	36.58 ± 0.23[a]	34.85 ± 0.65[c]	3.66 ± 0.05[a]	28.66 ± 0.27[a]	26.82 ± 0.57[b]

Results are expressed as means ± SEM for 10 semen samples from 5 dogs.
ASM, abnormal sperm morphology; VS, viable sperm with an intact acrosome; VS-AR, viable sperm with an acrosome reaction; DS, nonviable sperm with an intact acrosome; DS-AR, nonviable sperm with an acrosome reaction.
[a,b,c] Different superscript letters in the same column indicate significant differences ($P < 0.05$).

preparations following DGC are the aliquots of those spermatozoa with highest potential fertilizing ability. Further researches should be performed to determine if modifications in that protocol (canine-sperm-specific formulation, single-layer centrifugation, colloid density, centrifugation regime, etc) are required to improve the fertilizing capacity of cryopreserved and gradient-selected dog semen, including its use for artificial insemination of bitches.

It was also interesting to note that washing after PureSperm® centrifugation of the sperm pellet was harmful for cryopreserved dog spermatozoa, resulting in a decrease in the percentage of motile, progressive, viable and acrosome intact spermatozoa. These results indicate that there was no need to include a washing step in the protocol, thus saving time and effort in preparation.

In conclusion, DGC using PureSperm® may be a successful method for improving canine sperm quality damaged by the freezing-thawing process, and presumably with the highest potential fertilizing ability, since the majority of the parameters of sperm quality explored hereby appeared significantly better than the counterpart original suspension of frozen-thawed spermatozoa. However, washing after DGC of the sperm pellet reduces dramatically the post-thaw semen quality, indicating that the inclusion of such a washing step is unnecessary.

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Changes in the structures of motile sperm subpopulations in dog spermatozoa after both cryopreservation and centrifugation on PureSperm® gradient

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ABSTRACT

The aims of the present study were to: (1) determine if discrete motile sperm subpopulations exist and their incidence in fresh dog ejaculates, (2) evaluate the effects of cryopreservation on the distribution of spermatozoa within the different subpopulations, and (3) determine the effect of the discontinuous PureSperm® gradient on the sperm subpopulation structure of frozen–thawed dog spermatozoa. Semen from 5 dogs were collected and cryopreserved following a standard protocol. After thawing, semen samples were selected by centrifugation on PureSperm®. Sperm motility (assessed by computerized-assisted semen analysis, CASA) was assessed before freezing, just after thawing and after preparation on the PureSperm® gradients. Cryopreservation had a significant ($P < 0.001$) effect on CASA-derived parameters. PureSperm® centrifugation yielded sperm suspensions with improved motility ($P < 0.01$). A multivariate clustering procedure separated 19414 motile spermatozoa into four subpopulations: Subpopulation 1 consisting of poorly active and non-progressive spermatozoa (20.97%), Subpopulation 2 consisting of slow and low-linear spermatozoa (18.24%), Subpopulation 3 consisting of highly active but non-progressive spermatozoa (20.75%), and Subpopulation 4 consisting of high speed and progressive spermatozoa (40.03%). Although, cryopreservation had a significant ($P < 0.001$) effect on both the frequency distribution of spermatozoa within subpopulations and the motion characteristics of each subpopulation, the sperm subpopulation structure was perfectly maintained after freezing and thawing. The selected sperm samples was enrich in Subpopulation 4, reaching a proportion of 31.9% of the present spermatozoa, in contrast with the unselected sperm samples, where this sperm subpopulation accounted for 24.9% of the total. From these results, we concluded that four well-defined motile sperm subpopulations were present either in fresh semen, in unselected sperm samples or in selected preparations from dogs. The discontinuous PureSperm® gradient is a simple method to improve the quality of canine frozen–thawed semen samples, since Subpopulation 4 (high-speed and progressive spermatozoa) was more frequently observed after preparation on the gradient. Finally, this study also demonstrated that the general motile sperm structure present in dog remains constant despite the effect caused by either cryopreservation or separation on PureSperm® gradient.

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1. Introduction

Sperm cryopreservation is an integral part of assisted conception programs and has its application in many circumstances, such as situations involving impaired male fertility, assisted reproduction techniques (ART), and donor semen banking (Kim et al., 2010). However, cryopreservation induces both a loss of sperm viability and an impairment of functionally of the spermatozoa surviving the thawing, thus reducing fertilizing ability (Watson, 2000). Therefore, the selection of suitable spermatozoa from processed semen (frozen–thawed) where the majority has been damaged or is dead should be one of the prerequisites for achieving optimal conception rates after artificial insemination or other ART with frozen–thawed dog semen. In this sense, the PureSperm® density-gradient centrifugation (DGC) technique has been designed to select viable and morphologically intact human spermatozoa and to purify them for ART (Morrell et al., 2004; Mousset-Siméon et al., 2004; Sonderland and Lundin, 2000). PureSperm® gradients have also been satisfactorily applied to ejaculates and processed semen from many separate species, such as primates (O'Brien et al., 2003), marmosets (Hernández-López et al., 2005), bulls (Hollinshead et al., 2004b; Maxwell et al., 2007; Underwood et al., 2009) and rams (Hollinshead et al., 2004a; O'Brien et al., 2003), in order to clean the spermatozoa from seminal plasma or extenders and also to enrich the amount of cells with normal morphology and/or motility to be then used in ART.

Sperm motility is important for some steps of sperm transport within the female reproductive tract, primarily, for the penetration of the zona pellucida. The classical approach, considering the whole ejaculate as a homogeneous population with a normal statistical distribution, and the use of mean values to classify the ejaculates, or to asses the effect of a treatment or a biotechnological procedure is, nowadays, considered erroneous (Mortimer, 1997). Currently, the identification of subpopulations within the mammalian ejaculate has become an issue of utmost interest for the sound evaluation of the ejaculates. Although, there is no consensus about the physiological role for these motile sperm subpopulations in the ejaculate, it is widely known that the ejaculates of many mammalian species contain different subpopulations of spermatozoa (Abaigar et al., 1999; Dorado et al., 2010; Holt, 1996; Miró et al., 2005; Quintero-Moreno et al., 2003, 2007; Rigau et al., 2001), which are characterized by precise values of the motion parameters obtained after a computer-assisted sperm analysis (CASA). In this way, the presence of discrete motile sperm subpopulations has been related to resistance to cryopreservation (Flores et al., 2009; Martinez-Pastor et al., 2005; Núñez-Martínez et al., 2006a,b), presence of stimulants (Abaigar et al., 1999), storage and fertility (Quintero-Moreno et al., 2003, 2004). However, to our knowledge, studies on the effect of PureSperm® density-gradient centrifugation on frozen–thawed dog semen have not been reported as well as there are no available references relative to the influence of the centrifugation of thawed semen through PureSperm® on the structure of dog motile sperm subpopulation.

The aims of this study were therefore to (1) identify the presence of discrete motile sperm subpopulations, as well as their incidence, in fresh dog ejaculates, (2) evaluate the effects of cryopreservation in a used commercial extender on the distribution of spermatozoa within the different subpopulations, and (3) determine the effect of the discontinuous PureSperm® gradient on the sperm subpopulation structure of frozen–thawed dog spermatozoa.

2. Materials and methods

2.1. Animals

Semen was collected from 5 clinically healthy experimental dogs (3 Spanish Greyhounds, 1 German Pointer and 1 Crossbreed) ranging between 5 and 6 years and of unknown fertility. The study was carried out according to the Spanish laws for animal welfare and experimentation.

2.2. Semen collection and evaluation

Two ejaculates from each dog were obtained by digital manipulation on different and nonconsecutive experimental days, once or twice per week, and the sperm-rich second fraction of the ejaculates was collected (Linde-Forsberg, 1991). Immediately after collection sperm characteristics (volume, sperm concentration, objective sperm motility and sperm morphology) were assessed. Sperm concentration was determined with a photometer (Spermacue, Minitüb, Tiefenbach, Germany), as described by Peña et al. (2003). Sperm morphology was examined by light microscopy evaluation (Olympus BH-2, Olympus Optical Co., LTD, Tokyo, Japan) of smears stained with Diff-Quick® (Baxter DADE AG 3186, Düdingen, Sweden) staining (Kim et al., 2010). At least 200 spermatozoa per slide were counted to determine the percentage of spermatozoa with abnormal morphology (ASM, %). Only ejaculates with volume $\geq$0.8 ml, sperm concentration $\geq 200 \times 10^6$ spermatozoa/ml, motility $\geq$70% and normal morphology $\geq$70% were included in the study.

2.3. Semen processing

Semen was frozen using the Uppsala method (Peña and Linde-Forsberg, 2000) modified by Núñez-Martínez et al. (2006a) as follows: After collection, semen was diluted 1:1 (v:v) with Tris-based extender (Biladyl A, Minitüb, Tiefenbach, Germany) and centrifuged at $700 \times g$ for 10 min at room temperature (22 °C). The resulting sperm pellet was suspended to a final sperm concentration of $300–400 \times 10^6$ spermatozoa/ml with CaniPRO™ Freeze A added to 20% centrifuged egg yolk at room temperature. Extended semen was slowly cooled to 5 °C within an hour and then diluted to a final sperm concentration of $150–200 \times 10^6$ spermatozoa/ml in cooled (5 °C) CaniPRO™ Freeze B added to 20% centrifuged egg yolk. Finally, the spermatozoa were loaded in 0.5 ml plastic straws and frozen horizontally in ranks placed 4 cm above the surface of liquid nitrogen (LN_2) for 10 min, after which they were

directly placed in LN_2. After 24–48 h of storage, straws were thawed in a water bath at 37 °C for 30 s, for analyses.

2.4. Sperm separation procedure

PureSperm® 80 and PureSperm® 40 gradients were prepared in 15 ml Falcon tubes (BDFalcon™Tubes, BD Biosciences, Erembodegem, Belgium) according to producer's directions (Nidacon International AB, Gothenburg, Sweden). PureSperm® works at room temperature. In brief, a 2 ml of PureSperm® 80 was pipetted into a conical tube and then carefully layered with 2 ml of PureSperm® 40. Three straws of an individual ejaculate containing 150–200 spermatozoa/ml, were thawed at the same time and their contents were pooled. Frozen–thawed semen was gently loaded (1.5 ml) onto the top of the gradient before centrifugation ($300 \times g$/20 min). The upper layers (egg yolk and dead sperm) were removed, and the remaining pellet (separated sperm population) re-suspended in a new conical tube to obtain a final sperm concentration of 30×10^6 spermatozoa/ml with PBS (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) for later examinations.

2.5. Sperm motility

Motility was measured before freezing, just after thawing and after preparation on the PureSperm® gradients, using a CASA system (Sperm Class Analyzer, Microptic SL, Barcelona, Spain), as described by Núñez-Martínez et al. (2006a) for dog semen. The analysis was based on the analysis of 25 consecutive, digitized images obtained from a single field. Images were taken in a time lapse of 1 s, corresponding to a velocity of image-capturing of 1 photograph every 40 ms. Before the analysis an aliquot of semen was diluted with TRIS-based extender (Biladyl A, Minitüb, Tiefenbach, Germany) (Peña et al., 2003) to reach a sperm concentration of approximately 25×10^6 spermatozoa/ml and incubated for 5 min at 38 °C. Two consecutive 5 μl drops of each semen sample were then evaluated using a phase contrast microscope (Eclipse 50i, Nikon, Tokyo, Japan) with a prewarmed stage at 38 °C at 100× magnification. Three microscopic fields per drop were filmed at random. Objects incorrectly identified as spermatozoa were minimized on the monitor by using the playback function. The measured parameters of sperm motion were curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz). Definitions of these descriptors of sperm movement can be found in a previous publication (Dorado et al., 2007). Total motility was defined as the percentage of spermatozoa with a mean average path velocity (VAP) >15 μm/s, while objects with a VAP <10 μm/s were considered immobile.

2.6. Statistical analysis

The data matrix consisted of 12,826 observations of individual spermatozoa for fresh semen, 5154 observations for unselected sperm samples and 1434 for selected sperm samples. The FASTCLUS clustering procedure was used to separate the spermatozoa into their different motility subpopulations (Martinez-Pastor et al., 2005). A general linear model (PROC GLM) was used to evaluated significant differences ($P < 0.05$) and the Duncan's Multiple Range test was applied to list these differences, after arcsine transformation of percentage data. The summary statistics of the relative frequencies of spermatozoa belonging to each subpopulation were calculated and compared by ANOVA and chi-square test (FREQ procedure). All analyses were performed with SAS statistic package v9.0 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Changes in mean motility parameters after cryopreservation and after selection by PureSperm® density-gradient centrifugation

As expected, cryopreservation induced great changes in the values of the mean motility parameters of dog spermatozoa. As shown in Table 1, frozen–thawed samples (unselected and selected sperm samples) showed a significant ($P < 0.001$) decrease in the percentages of CASA-derived parameters, with the exception of STR in selected sperm samples (75.36 ± 0.22 versus 74.11 ± 0.76; $P > 0.05$). Selection by PureSperm® density-gradient centrifugation significantly ($P < 0.01$) improved the majority of sperm

Table 1
Means values (±SEM) of the semen motility analysis of fresh semen, unselected sperm samples and selected preparations from dogs.

Parameter	Fresh semen	Unselected sperm samples	Selected sperm samples
Total motility (%)[a]	97.03 ± 0.03[a]	76.77 ± 0.18[b]	69.57 ± 0.51[c]
Progressive motility (%)[b]	78.89 ± 0.09[a]	35.30 ± 0.14[c]	41.86 ± 0.43[b]
Curvilinear velocity (VCL, μm/s)	105.77 ± 0.39[a]	66.18 ± 0.67[c]	69.10 ± 1.22[b]
Straight line velocity (VSL, μm/s)	69.54 ± 0.38[a]	40.87 ± 0.59[c]	44.27 ± 1.04[b]
Average path velocity (VAP, μm/s)	85.33 ± 0.37[a]	50.64 ± 0.60[b]	49.66 ± 1.04[b]
Linearity (LIN, %)	60.55 ± 0.23[a]	47.32 ± 0.41[c]	49.51 ± 0.78[b]
Straightness (STR, %)	75.36 ± 0.22[a]	64.53 ± 0.41[b]	74.11 ± 0.76[a]
Wobble (WOB, %)	77.24 ± 0.14[a]	68.20 ± 0.27[b]	62.31 ± 0.61[c]
Amplitude of lateral head displacement (ALH, μm)	3.00 ± 0.01[a]	2.25 ± 0.02[c]	2.38 ± 0.04[b]
Beat cross frequency (BCF, Hz)	9.14 ± 0.04[a]	6.51 ± 0.07[c]	8.19 ± 0.16[b]

Different superscripts (a–c) in the same row indicate significant differences ($P < 0.05$).

[a] Total motility (MOT, %) was defined as the percentage of spermatozoa with a mean average path velocity (VAP) >15 μm/s.

[b] Progressive motility (PMOT, %) was defined as the percentage of spermatozoa with a mean velocity >50 μm/s and straightness >75%.

motility parameters assessed (PMOT, VCL, VSL, LIN, STR, ALH and BCF) by CASA.

There were significant ($P < 0.05$) differences between dogs in sperm motility parameters, both in fresh semen and frozen–thawed samples, as well there was a significant ($P < 0.001$) effect of the replicate (data not shown). Moreover, there was an interaction ($P < 0.001$) among dog and replicate for sperm parameters.

3.2. Identification of motile sperm subpopulations

The FASTCLUS procedure detected four motile sperm subpopulations from the motility data. Summary statistics for the motility characteristics of the subpopulations are shown in Table 2.

Subpopulation 1 represented those spermatozoa with low velocity (low VCL, VSL and VAP). They were poorly progressive (low VAP, LIN and STR) and covering very short distances (very low VSL). The total motile spermatozoa included in this subpopulation was 20.97%.

Subpopulation 2 included those spermatozoa whose movement was defined as less vigorous, as indicated by the lowest values of VCL and ALH. Trajectories show low linearity although it was higher than Subpopulation 1 ($P < 0.001$; Table 2). This population included 18.24% of the total motile spermatozoa.

Subpopulation 3 included highly active but non-progressive spermatozoa, as indicated by the high values of VCL and BCF together with low LIN and STR values. Their poor progressiveness characteristics were evident from their high values of ALH. The total motile spermatozoa included in this subpopulation was 20.75%.

Subpopulation 4 represented spermatozoa showing the highest degree of progressive motility (as inferred from the very high VSL, VAP, LIN and STR values together with low ALH values). They exhibited a vigorous flagellar action (high BCF). The total motile spermatozoa included in this subpopulation was 40.03%.

3.3. Changes in the motile sperm subpopulations structure after cryopreservation and after selection by PureSperm® density-gradient centrifugation

Although, the percentage of motile sperm included in each subpopulation experienced important changes after cryopreservation and after DGC, the sperm subpopulation structure was perfectly maintained after both procedures (Table 3). Cryopreservation induced a significant ($P < 0.001$) decrease in the percentage of motile sperm included in Subpopulations 3 (from 24.1% to 13.2%) and 4 (from 47.0% to 24.9%). This decrease was compensated for by concomitant, significant ($P < 0.001$) increases in the percentages of sperm included in Subpopulations 1 (from 15.9% to 31.1%) and 2 (from 12.9% to 30.8%). On the other hand, Subpopulation 4 was more frequently observed in fresh semen compared to unselected sperm samples (Table 3). Moreover, while the Subpopulation 1 predominated in the unselected sperm samples, after the centrifugation of the thawed semen through PureSperm® the predominant subpopulation became again 4. Hereby, Subpopulation 1 was characterized by low values of VSL and VAP, whereas Sub-

Table 2
Motility parameters for the four sperm subpopulations defined after pattern analysis in fresh semen, unselected sperm samples and selected preparations from dogs.

Subpopulation	*n* (%)	Sperm motility parameters							
		VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
1	4072 (20.97)	44.06 ± 0.46^{c}	9.98 ± 0.17^{d}	24.21 ± 0.31^{d}	18.80 ± 0.20^{c}	37.74 ± 0.35^{d}	50.70 ± 0.25^{c}	2.16 ± 0.02^{c}	4.33 ± 0.05^{d}
2	3542 (18.24)	40.65 ± 0.46^{d}	22.38 ± 0.32^{c}	30.55 ± 0.39^{c}	48.28 ± 0.30^{b}	67.60 ± 0.33^{c}	71.05 ± 0.22^{b}	1.71 ± ± 0.01^{d}	4.98 ± 0.06^{c}
3	4029 (20.75)	130.72 ± 0.35^{a}	62.46 ± 0.32^{b}	93.28 ± 0.35^{b}	48.02 ± 0.21^{b}	68.76 ± 0.33^{b}	71.13 ± 0.15^{b}	4.30 ± 0.01^{a}	10.00 ± 0.05^{b}
4	7771 (40.03)	121.84 ± 0.35^{b}	102.25 ± 0.31^{a}	108.61 ± 0.32^{a}	83.70 ± 0.11^{a}	94.62 ± 0.07^{a}	88.38 ± 0.08^{a}	2.74 ± 0.01^{b}	11.20 ± 0.04^{a}

Results are expressed as means ± SEM for 10 semen samples from 5 dogs. The total number of spermatozoa analyzed was 19414.
VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.
Different letters (a–d) in the same column indicate significant differences ($P < 0.05$).

Table 3
Changes in the sperm subpopulation structure of dog spermatozoa in fresh semen, unselected sperm samples and selected sperm samples.

Subpopulation	Fresh semen		Unselected sperm samples		Selected sperm samples	
	n	%	*n*	%	*n*	%
1	2037	15.9[b]	1604	31.1[a]	431	30.1[a]
2	1660	12.9[c]	1586	30.8[a]	296	20.6[b]
3	3097	24.1[a]	682	13.2[c]	250	17.4[b]
4	6032	47.0[a]	1282	24.9[c]	457	31.9[b]

Different superscript letters (a–c) in the same row indicate significant differences ($P < 0.05$, Chi-square test).

population 4 was still composed of spermatozoa depicting high values for all sperm velocities (Table 2).

Cryopreservation also modified the majority of motility parameters evaluated in any of the obtained subpopulations (Table 4). In general, the sperm velocity parameters (VCL, VSL and VAP) decreased in all subpopulations. The trajectory of the sperm movement changed in a different way depending on the sperm subpopulation. In Subpopulations 3 and 4, the sperm trajectories were more progressive after cryopreservation than fresh semen (higher LIN), whereas in Subpopulations 1 and 2, the sperm trajectories were less progressive after freezing and thawing. After the centrifugation of the thawed semen through PureSperm®, there was a significant ($P < 0.001$) increase in the mean values of STR in Subpopulations 1–3, as well as mean BCF values were significantly ($P < 0.001$) higher in Subpopulations 3 and 4.

Individual variability was identified for each of the four sperm subpopulations ($P < 0.001$) either in fresh semen, just after thawing or after DGC. Thus, significant ($P < 0.05$) differences between treatments were found in Subpopulations 1 and 2 for dog 2, in Subpopulation 3 for dogs 1 and 5 and in Subpopulation 4 for dogs 2–5 (data not shown).

3.4. Sperm yield after density-gradient centrifugation

The yield depended on sperm quality of the original frozen–thawed samples and was 40.83% after the centrifugation of thawed semen through the PureSperm® gradient. The volume of the sperm suspension recovered ranged from 0.5 to 1 mL after DGC.

4. Discussion

Cryopreservation implies osmotic stress and the formation or reshaping of intracellular ice during freezing and again during thawing, resulting in alterations of the cell physiology. In the present study, instead of a high percentage of immotile sperm after thawing we observed mainly decreases in the quality of sperm kinematics, prob-

Table 4
Effects of two-layer centrifugation through PureSperm® gradient on motility parameters of the motile-sperm subpopulations determined in dog semen samples.

Sperm motility parameters	Subpopulation 1			Subpopulation 2		
	Fresh semen	Unselected sperm samples	Selected sperm samples	Fresh semen	Unselected sperm samples	Selected sperm samples
VCL (μm/s)	55.24 ± 0.67[a]	34.91 ± 0.58[b]	25.28 ± 1.00[c]	49.27 ± 0.74[a]	32.70 ± 0.56[bc]	34.88 ± 1.33[b]
VSL (μm/s)	12.86 ± 0.25[a]	7.71 ± 0.23[b]	4.79 ± 0.40[c]	28.43 ± 0.51[a]	16.62 ± 0.40[bc]	19.27 ± 0.94[b]
VAP (μm/s)	31.14 ± 0.47[a]	19.05 ± 0.41[b]	10.71 ± 0.60[c]	38.17 ± 0.63[a]	23.83 ± 0.47[b]	23.88 ± 1.00[b]
LIN (%)	21.34 ± 0.29[a]	17.10 ± 0.31[b]	13.10 ± 0.53[c]	51.91 ± 0.43[a]	44.30 ± 0.44[b]	49.26 ± 0.96[a]
STR (%)	38.87 ± 0.47[b]	34.75 ± 0.53[c]	43.61 ± 1.41[a]	70.60 ± 0.47[b]	63.33 ± 0.50[c]	73.67 ± 1.06[a]
WOB (%)	54.62 ± 0.32[a]	49.69 ± 0.38[b]	35.97 ± 0.88[c]	73.03 ± 0.29[a]	69.87 ± 0.13[b]	66.34 ± 0.72[c]
ALH (μm)	2.57 ± 0.02[a]	1.83 ± 0.02[b]	1.48 ± 0.04[c]	1.94 ± 0.02[a]	1.50 ± 0.02[b]	1.59 ± 0.04[b]
BCF (Hz)	5.29 ± 0.07[a]	3.69 ± 0.08[b]	2.14 ± 0.12[c]	5.75 ± 0.09[a]	4.07 ± 0.07[b]	5.56 ± 0.26[a]

	Subpopulation 3			Subpopulation 4		
	Fresh semen	Unselected sperm samples	Selected sperm samples	Fresh semen	Unselected sperm samples	Selected sperm samples
VCL (μm/s)	133.14 ± 0.40[a]	126.69 ± 0.77[b]	111.74 ± 1.02[c]	124.34 ± 0.39[a]	114.55 ± 0.88[b]	109.27 ± 1.17[c]
VSL (μm/s)	61.78 ± 0.38[bc]	66.28 ± 0.71[a]	60.45 ± 0.98[c]	103.99 ± 0.35[a]	98.85 ± 0.84[b]	88.84 ± 1.07[c]
VAP (μm/s)	96.80 ± 0.40[a]	85.34 ± 0.66[b]	71.28 ± 0.90[c]	110.71 ± 0.35[a]	104.88 ± 0.81[b]	91.27 ± 1.07[c]
LIN (%)	46.56 ± 0.25[c]	52.40 ± 0.46[b]	54.11 ± 0.69[b]	83.34 ± 0.12[b]	86.19 ± 0.26[a]	81.48 ± 0.47[c]
STR (%)	65.31 ± 0.37[d]	78.46 ± 0.67[c]	84.92 ± 0.85[b]	94.15 ± 0.09[c]	95.90 ± 0.13[b]	97.23 ± 0.12[a]
WOB (%)	72.58 ± 0.17[a]	67.27 ± 0.29[b]	63.72 ± 0.52[c]	88.42 ± 0.08[b]	89.79 ± 0.22[a]	83.77 ± 0.45[c]
ALH (μm)	4.23 ± 0.02[c]	4.56 ± 0.04[b]	4.47 ± 0.06[b]	2.81 ± 0.01[a]	2.49 ± 0.03[bc]	2.59 ± 0.04[b]
BCF (Hz)	9.71 ± 0.06[c]	10.70 ± 0.12[b]	11.71 ± 0.23[a]	11.09 ± 0.04[c]	10.85 ± 0.11[c]	13.68 ± 0.18[a]

Results are expressed as means ± SEM for 10 semen samples from 5 dogs. The total number of spermatozoa analyzed was 12826 (fresh semen), 5154 (after thawing) and 1434 (after density-gradient centrifugation).
VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.
Different superscript letters (a–c) between rows in the same subpopulation or between the mean values indicate significant differences ($P < 0.05$).

ably because as has been previously proposed in dogs, the sperm mitochondria are the sperm structures more sensitive to cryodamage (Peña et al., 2003). Nevertheless, values obtained in semen frozen are comparable to those from other studies (Batista et al., 2006; Peña et al., 1998, 2003; Rota et al., 1999; Silva et al., 1996, 2003; Ström et al., 1997; Yildiz et al., 2000). On the other hand, there was a significant effect of the individual dog on post-thaw sperm quality, suggesting that individual dogs may have different susceptibility to sperm cryopreservation, as previously reported (Batista et al., 2006; Silva et al., 2006).

Our study also reveals the presence of four discrete sperm subpopulations either in fresh semen, in unselected sperm samples or in selected preparations of dogs, which can be easily defined by the combination of eight CASA kinematic parameters. Sperm subpopulations have previously been identified in fresh semen and frozen–thawed dog semen (Núñez-Martínez et al., 2006a,b; Rigau et al., 2001). Since either the CASA system or the kinematic parameters used to define the sperm subpopulations in these studies were different from the one used in the present study, it is difficult to compare the movement patterns of the sperm subpopulations identified. In spite of this, a simple interpretation of this sperm subpopulation structure is that these four groups represent spermatozoa in different physiological states (Abaigar et al., 1999), reflected their very different swimming behaviors.

The presence of three or four well-defined motile sperm subpopulations has been demonstrated in numerous species (Abaigar et al., 1999; Dorado et al., 2010; Holt, 1996; Miró et al., 2005; Muiño et al., 2008; Quintero-Moreno et al., 2003). As indicated by our current and previous results (Núñez-Martínez et al., 2006a), the canine species does not seem to be an exception. However, to our knowledge, the existence of separate motile subpopulations in selected canine semen has not been previously investigated.

In agreement with previous studies (Cremades et al., 2005; Flores et al., 2008, 2009; Muiño et al., 2008, 2009), cryopreservation had a significant effect on both the frequency distribution of spermatozoa within subpopulations and the motion characteristics of each subpopulation. A significant decrease in the proportion assigned to the Subpopulations 3 (highly active but non-progressive spermatozoa) and 4 (highly active and progressive spermatozoa) was seen after cryopreservation. Subpopulations 3 and 4 might loose their flagellar activity, probably as consequence of injury induced by the freezing and thawing process. In this context, a simple application of a multivariate pattern analysis to allocate motile spermatozoa into subpopulations on the basis of their kinematic parameters seems to be fully informative, thus opening the possibility to use this type of analysis to greatly improve the standard dog-semen quality analysis when ability to resist cryopreservation is concerned. In fact, Flores et al. (2009) pointed out that boar ejaculate resistance to cryopreservation is related to specific changes in both the structures of motile sperm subpopulations and the motion characteristics of each subpopulation.

It is noteworthy that the general motile sperm structure present in the dog remains constant despite the effect caused by either cryopreservation or separation on PureSperm® gradient. This indicates that the maintenance of an overall subpopulations structure could be important in order to maintain the general function of the ejaculate, regardless of the specific functional status. These results pointed to a specific, important role for the maintenance of a specific subpopulational structure in mammalian ejaculates, regardless of the species in which the studies were carried out. In this way, the existence of an specific subpopulational structure has been described in very different mammals like boar (Abaigar et al., 1999), horse (Quintero-Moreno et al., 2003), bull (Muiño et al., 2008), buck (Dorado et al., 2010), red deer (Martinez-Pastor et al., 2005), gazelle (Abaigar et al., 1999), dog (Rigau et al., 2001), golden hamster (Holt, 1996) and rabbit (Quintero-Moreno et al., 2007), indicating that it is a common feature of all mammalian ejaculates. Recent studies indicate that motility changes associated with processes like “in vitro” capacitation in boar semen (Ramió et al., 2008) do not modify the overall subpopulational structure of these samples, the mean motility changes linked to these processes being induced mainly to concomitant changes in the percentage of motile sperm included in each specific subpopulation. Therefore, the maintenance of this specific subpopulational structure would be important in the maintenance of the overall semen function in mammals.

To our knowledge, this is the first attempt to use PureSperm® centrifugation to separate cryopreserved canine sperm. The centrifugation of thawed semen through PureSperm® gradient selected a sperm population with improved quality in regard to higher motility (high mean values of PMOT, VCL, VSL, LIN, STR, ALH and BCF). The improvement in sperm motility and velocity is generally consistent with previous studies for PureSperm® gradient (Maxwell et al., 2007; Underwood et al., 2009) and for other DGC techniques such us Percoll® (Brum et al., 2008; García-Álvarez et al., 2010; Samardzija et al., 2006; Suzuki et al., 2003; Valcárcel et al., 1996), BoviPure® (Samardzija et al., 2006; Underwood et al., 2009), and Androcoll-E™ (Johannisson et al., 2009; Macías García et al., 2009b; Morrell et al., 2009).

Likewise, the DCG also enriched the subpopulations depicting higher velocities in the processed frozen–thawed semen sample. The predominant subpopulation present in the unselected sperm samples was the Subpopulation 1; a subpopulation characterized by poorly active and non-progressive movement, representing more than 30% of the spermatozoa in the sample. Treatment of the sperm suspension by PureSperm® density-gradient significantly modified the distribution of spermatozoa within subpopulations. Therefore, Subpopulation 4 (highly active and progressive spermatozoa) represented now more than 31% of the spermatozoa harvested after centrifugation through PureSperm® gradient. Our results are consistent with those reported previously by Macías García et al. (2009a) for single-layer centrifugation of frozen–thawed stallion spermatozoa, and this leads to the conclusion that the DGC process improved the quality of the sperm sample in terms of the sperm subpopulation structure. Quality in this respect meant that spermatozoa had the basic attributes of critical relevance for the fertility of a given ejaculate.

The majority of the CASA motion parameters used in the present study for the identification of sperm subpopulations have been previously identified as the most reliable markers of sperm quality (De Geyter et al., 1998; Dorado et al., 2009; Holt et al., 1997; Larsen et al., 2000; Olds-Clarke, 1996) and associated with fertility (Amann, 1989; Farrell et al., 1993, 1998; Holt et al., 1997; MacLeod and Irvine, 1995; Moore and Akhondi, 1996; Vidament et al., 2000). Studies in dogs (Silva et al., 2006) and boars (Holt et al., 1997) have demonstrated that high sperm velocities are landmarks of fertility both *in vivo* and *in vitro*. It has also been reported previously that VCL is of key importance for formation of the sperm reservoir at the utero-tubal junction in mice (Olds-Clarke, 1996), that VCL and VAP are linked to the ability of ram spermatozoa to penetrate cervical mucus (Robayo et al., 2008), and that post-thaw VSL is related to the fertility of bull (Gillan et al., 2008) and human (Thys et al., 1998) spermatozoa. Furthermore, the predominance of a particular motile subpopulation in samples with better cryoresistance or fertility has been demonstrated in other studies (Martinez-Pastor et al., 2005; Quintero-Moreno et al., 2003). Therefore, taking into account this experimental evidence, and since the DGC technique herein studied, particularly harvested those spermatozoa with highest velocity in the insemination dose, it can be surmised that sperm preparations following DGC are the aliquots of those spermatozoa with highest potential fertilizing ability. However, the implications for that the different motile sperm subpopulations and their distribution in thawed semen can have on subsequent *in vivo* or *in vitro* fertility require further investigation.

In conclusion, the results of the present study show that four well-defined motile sperm subpopulations were present either in fresh semen, in unselected sperm samples or in selected preparations from dogs. Cryopreservation significantly modified both the specific parameters and the distribution of spermatozoa within subpopulations. The discontinuous PureSperm® gradient is a simple method to improve canine sperm quality damaged by the freezing–thawing process, and although this should be experimentally confirmed, presumably with the highest potential fertilizing ability, since Subpopulation 4 (high-speed and progressive spermatozoa) was more frequently observed after preparation on the gradient. Finally, this study also demonstrated that the general motile sperm structure present in dog remains constant despite the effect caused by either cryopreservation or separation on PureSperm® gradient.

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Single-layer centrifugation through PureSperm® 80 selects improved quality spermatozoa from frozen-thawed dog semen

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ABSTRACT

The aim of this study was to investigate whether single-layer centrifugation (SLC) with PureSperm® 80 could select good quality spermatozoa, including those with specific motility patterns, from doses of frozen dog semen. Semen from 5 dogs was collected and cryopreserved following a standard protocol. After thawing, semen samples were divided into two aliquots: one of them was used as control and the other one processed by SLC. Assessment of sperm motility (assessed by computer-assisted semen analysis), morphology (Diff-Quick staining) and viability (triple fluorescent stain of propidium iodine/isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin/Rhodamine 123), were performed on aliquots of fresh semen, frozen-thawed control and frozen-thawed SLC treated samples. A multivariate clustering procedure separated 26,051 motile spermatozoa into three subpopulations (sP): sP1 consisting of highly active but non-progressive spermatozoa (40.3%), sP2 consisting of spermatozoa with high velocity and progressive motility (30.0%), and sP3 consisting of poorly active and non-progressive spermatozoa (29.7%). SLC with PureSperm® 80 yielded sperm suspensions with improved motility, morphology, viability and acrosome integrity ($P < 0.001$). The frozen-thawed SLC treated samples were enriched in sP2, reaching a proportion of 44.1% of the present spermatozoa. From these results, we concluded that SLC with PureSperm® 80 may be an alternative and successful method for improving the quality of frozen-thawed dog spermatozoa. Moreover, sP2 (high-speed and progressive spermatozoa) was more frequently observed after SLC. Finally, this study also demonstrated that the general motile sperm structure present in dogs remained constant despite the effect caused by either cryopreservation or separation by SLC through PureSperm® 80.

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1. Introduction

Sperm cryopreservation is an integral part of assisted conception programs and has its application in many circumstances, such as situations involving impaired male fertility, assisted reproduction techniques (ART), and donor semen banking (Kim et al., 2010). However, cryopreservation induces a series of osmotic, chemical, and mechanical stresses to sperm, causing death of some sperm and severe post-thaw damage in surviving cells, reducing fertilizing

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ability (Watson, 1995, 2000). Moreover, damage caused by semen processing procedures is accumulative, and small injuries may result in important deleterious changes at the end of the process (Nicolas et al., 2012). Therefore, current pregnancy and live birth success rates of ART are not completely satisfactory with frozen-thawed dog semen (Kim et al., 2010). For all these reasons, the selection of suitable spermatozoa from processed semen (frozen-thawed) where the majority has been damaged or is dead should be one of the prerequisites for achieving optimal conception rates after artificial insemination (AI) or other ART with frozen-thawed dog semen.

There are several methods (*i.e.* migration, filtration, and colloid centrifugation) available for selecting functional spermatozoa on the basis of certain sperm attributes (Morrell and Rodriguez-Martinez, 2011). Density gradient centrifugation (DGC) has been suggested as a means of selecting spermatozoa for artificial breeding (Morrell et al., 2009). This technique has been satisfactorily used to separate motile, chromatin-intact and morphologically normal spermatozoa from the extended semen (Morrell and Rodriguez-Martinez, 2009). Specially, the PureSperm® (Nidacon International AB, Gothenburg, Sweden) density-gradient centrifugation technique has been designed to select viable and morphologically intact human spermatozoa and to purify them for ART (Morrell et al., 2004; Mousset-Siméon et al., 2004). Moreover, PureSperm® gradients have also been satisfactorily applied to ejaculates and processed semen from many species, such as primates (O'Brien et al., 2003), marmosets (Hernández-López et al., 2005), bulls (Underwood et al., 2009), rams (Hollinshead et al., 2004), bears (Nicolas et al., 2012) and dogs (Dorado et al., 2011a, 2011b; Phillips et al., 2012), in order to clean the spermatozoa from seminal plasma or extenders and also to enrich the amount of cells with normal morphology and/or motility to be then used in ART.

A new method, single-layer centrifugation (SLC) through a colloid, has been recently developed by Morrell et al. (2008a), which uses only one layer of colloid. This technique is simpler to use than DGC, while apparently being equally effective (Morrell et al., 2009). Therefore, using different colloids, SLC has successfully been used to improve the quality of sperm samples in humans (Zhou et al., 2010), stallions (Gutiérrez-Cepeda et al., 2011; Morrell et al., 2011), boars (Martinez-Alborcia et al., 2012), bulls (Thys et al., 2009), dogs (Morrell et al., 2008b) and cats (Chatdarong et al., 2010). A modification of the PureSperm® density gradient technique, centrifugation through a single-layer of colloid, has also been reported for human spermatozoa (Zhang et al., 2004). However, to our knowledge, studies on the effect of sperm selection by means of SLC through PureSperm® on sperm quality after cryopreservation of dog semen have not been reported. Likewise, there are no available references relative to the influence of the centrifugation of frozen-thawed semen through a single-layer of PureSperm® on the structure of dog motile sperm subpopulation.

The aim of this study was therefore to evaluate whether SLC through PureSperm® 80 improves the post-thaw sperm quality of dog spermatozoa, concerning its ability to separate the subpopulation of spermatozoa with good motility, morphology, viability and acrosome integrity.

2. Materials and methods

2.1. Animals

Semen was collected from 5 clinically healthy experimental dogs (4 Beagles and 1 German Pointer) ranging between 2 and 8 years and of unknown fertility. The study was carried out according to the Spanish laws for animal welfare and experimentation.

2.2. Semen collection and processing

A total of 10 ejaculates (2 ejaculates per dog) were obtained by digital manipulation on different and non consecutive experimental days, once or twice per week, and the sperm-rich second fraction of the ejaculates was collected (Linde-Forsberg, 1991). Semen volume was determined in a calibrated tube. Sperm concentration was determined with a photometer (SpermaCue, Minitüb, Tiefenbach, Germany), as described by Peña et al. (2003). Only ejaculates with volume $\geq$ 0.8 mL, sperm concentration $\geq 200 \times 10^6$ spermatozoa/mL, motility $\geq$ 70% and normal morphology $\geq$ 70% were included in the study.

Semen was then frozen using the Uppsala method (Peña and Linde-Forsberg, 2000) modified by Dorado et al. (2011a) as follows: after collection, semen was diluted 1:1 (v/v) with Tris-based extender (Biladyl A, Minitüb, Tiefenbach, Germany) and centrifuged at $700 \times g$ for 10 min at 22 °C. The resulting sperm pellet was suspended to a final sperm concentration of $300–400 \times 10^6$ spermatozoa/mL with CaniPRO™ Freeze A plus 20% centrifuged egg yolk at 22 °C. Extended semen was slowly cooled to 5 °C within an hour and then diluted to a final sperm concentration of $150–200 \times 10^6$ spermatozoa/mL in CaniPRO™ Freeze B plus 20% centrifuged egg yolk at 5 °C. Finally, the spermatozoa were loaded in 0.5 mL plastic straws and frozen horizontally in ranks placed 4 cm above the surface of liquid nitrogen (LN_2) for 10 min, after which they were directly placed in LN_2. After 24–48 h of storage, straws were thawed in a water bath at 37 °C for 30 s, for analyses.

2.3. Sperm separation procedure

The technique used for SLC was a modification of the procedure described by Morrell et al. (2012). In brief, 2 mL of PureSperm® 80, equilibrated to 22 °C, were poured into a 15 mL Falcon centrifuge tube (BDFalcon™ Tubes, BD Biosciences, Erembodegem, Belgium). Thereafter, an aliquot of the frozen-thawed semen (straws containing $80–100 \times 10^6$ spermatozoa/mL) was layered (1 mL) on top of the colloid. The tubes were centrifuged for 20 min at $300 \times g$ in a bench centrifuge (Eppendorf Centrifuge 5702RH, Eppendorf Ibérica SLU, Madrid, Spain) with a swing-out rotor. The sperm pellet was re-suspended in a new conical tube to obtain a final sperm concentration of 30×10^6 spermatozoa/mL with

PBS (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) for later examinations. Sperm concentration was finally measured to calculate the yield of the SLC procedure.

2.4. Sperm quality parameters assessment

Sperm quality was assessed (1) before freezing (fresh semen), (2) just after thawing (frozen-thawed control samples), and (3) after SLC through PureSperm® 80 (frozen-thawed SLC treated samples).

2.4.1. Sperm morphology

Sperm morphology was examined by light microscopy evaluation (Olympus BH-2, Olympus Optical Co., Ltd., Tokyo, Japan) of smears stained with Diff-Quick® (Medion Diagnostics AG, Düdingen, Switzerland) staining (Kim et al., 2010). At least 200 spermatozoa per slide were counted to determine the percentage of spermatozoa with abnormal morphology (ASM, %).

2.4.2. Objective sperm motility

Motility was measured using a CASA system (Sperm Class Analyzer, Microptic SL, Barcelona, Spain), as described by Núñez-Martínez et al. (2006a) for dog semen. The analysis was based on the analysis of 25 consecutive, digitized images obtained from a single field. Images were taken in a time lapse of 1 s, corresponding to a velocity of image-capturing of 1 photograph every 40 ms. Before the analysis an aliquot of semen was diluted with TRIS-based extender (Biladyl A, Minitüb, Tiefenbach, Germany) (Peña et al., 2003) to reach a sperm concentration of approximately 25×10^6 spermatozoa/mL and incubated for 5 min at 38 °C. Two consecutive 5 μL drops of each semen sample were then evaluated using a phase contrast microscope (Eclipse 50i, Nikon, Tokyo, Japan) with a prewarmed stage at 38 °C at 100× magnification. Three microscopic fields per drop were filmed at random. Objects incorrectly identified as spermatozoa were minimized on the monitor by using the playback function. Total motility (MT, %) was defined as the percentage of spermatozoa with a mean average path velocity (VAP) > 15 μm/s, while progressive motility (MP, %) was defined as the percentage of spermatozoa with VAP > 50 μm/s and straightness > 75%.

The measured parameters of sperm motion were curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz). Definitions of these descriptors of sperm movement can be found in a previous publication (Dorado et al., 2007).

2.4.3. Plasma membrane integrity, mitochondrial function and acrosomal integrity

Sperm viability was assessed in terms of plasma membrane integrity, mitochondrial membrane potential and acrosome membrane integrity. These characteristics were analyzed simultaneously using a modification of a triple fluorescent procedure described by Dorado et al. (2011a), which includes the fluorochromes propidium iodide (PI), Rhodamine 123 (R123) and isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin assay (FITC-PNA). All reagents were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Briefly, 200 μL of sperm sample (25–50 million cells approximately) were incubated at 38 °C in the dark for 25 min with 5 μL of R123 (0.1 mg/mL). Excess probe was removed from the spermatozoa by centrifugation (400 × *g*/6 min), and spermatozoa were resuspended in 200 μL of PBS. A mixture of 10 μL of PI (0.1 mg/mL) and 5 μL of FITC-PNA (0.1 mg/mL) was added to the samples, and the cells were then incubated at 38 °C for 10 min. A minimum of 300 spermatozoa were evaluated under 1000× magnification with epifluorescence microscopy (Olympus BX40, Tokyo, Japan) in each smear, using a U-ND25-2 filter (a 460–490 nm excitation filter). Four staining patterns could be discerned: (1) viable sperm with an intact acrosome (VS), (2) viable sperm with an acrosome reaction (VS-AR), (3) nonviable sperm with an intact acrosome (DS), and (4) nonviable sperm with an acrosome reaction (DS-AR). Values were expressed as percentages.

2.5. Experimental design

The collected ejaculate was immediately transported to the laboratory for evaluation and processing, as described previously. Thereafter, two straws from each individual ejaculate were thawed and their contents were pooled. An aliquot (1 mL) of pooled semen was treated with PureSperm® 80 (Nidacon International AB) and resulting sperm pellets were evaluated for sperm quality, and recovery rate. The remaining frozen-thawed semen was used as control.

2.6. Statistical analysis

Results are expressed as mean ± SEM. The data matrix consisted of 16,904 observations of individual spermatozoa for fresh semen, 5928 observations for frozen-thawed control samples and 5939 for frozen-thawed SLC treated preparations. Data were first studied using a general linear model (PROC GLM) in which the effects of males and replica within male were considered as random effects, as well as the interaction between them. Dependent variables expressed as percentages were arcsine-transformed before the analysis. Differences between mean values were analyzed by the Duncan method. Secondly, a clustering procedure was used to classify the spermatozoa of the data set into a reduced number of subpopulations according to their patterns of movement as previously described (Martinez-Pastor et al., 2005). The summary statistics of the relative frequencies of spermatozoa belonging to each subpopulation were calculated and compared by ANOVA and chi-square test (FREQ procedure). All analyses were performed with SAS statistic package v9.0 (SAS Institute Inc., Cary, NC, USA). The level of significance was set at $P < 0.05$.

Table 1
Mean values (±SEM) of the semen motility analysis of fresh semen, frozen-thawed control and frozen-thawed SLC treated samples from 5 dogs (10 ejaculates).

Parameter	Fresh semen	Frozen-thawed control samples	Frozen-thawed SLC treated samples
Total motility (MT, %)[a]	97.55 ± 0.01[a]	83.79 ± 0.13[c]	93.65 ± 0.05[b]
Progressive motility (MP, %)[b]	71.71 ± 0.04[b]	54.61 ± 0.16[c]	79.38 ± 0.07[a]
Curvilinear velocity (VCL, μm/s)	137.74 ± 0.52[b]	128.25 ± 1.10[c]	150.65 ± 0.78[a]
Straight line velocity (VSL, μm/s)	75.77 ± 0.44[c]	88.64 ± 0.97[b]	106.50 ± 0.78[a]
Average path velocity (VAP, μm/s)	103.78 ± 0.46[c]	106.77 ± 1.02[b]	124.72 ± 0.75[a]
Linearity (LIN, %)	49.72 ± 0.21[c]	56.26 ± 0.40[b]	64.92 ± 0.35[a]
Straightness (STR, %)	65.77 ± 0.21[c]	70.50 ± 0.38[b]	78.89 ± 0.34[a]
Wobble (WOB, %)	71.28 ± 0.13[c]	73.84 ± 0.27[b]	78.77 ± 0.22[a]
Amplitude of lateral head displacement (ALH, μm/s)	4.31 ± 0.01[a]	3.19 ± 0.02[c]	3.70 ± 0.02[b]
Beat cross frequency (BCF, Hz)	7.80 ± 0.03[b]	7.28 ± 0.06[c]	10.10 ± 0.06[a]

Different superscripts (a–c) in the same row indicate significant differences ($P < 0.05$).
[a] Total motility (MT, %) was defined as the percentage of spermatozoa with a mean average path velocity (VAP) > 15 μm/s.
[b] Progressive motility (MP, %) was defined as the percentage of spermatozoa with VAP > 50 μm/s and straightness > 75%.

3. Results

3.1. Comparison between mean quality parameters of fresh semen and frozen-thawed samples

As shown in Table 1, cryopreservation induced a significant ($P < 0.001$) decrease in the mean values of MT, MP, VCL, ALH and BCF. Table 2 shows that mean values of ASM, DS, DS-AR and VS-AR are significantly ($P < 0.001$) increased after freezing and thawing, whereas the opposite was found for mean VS values.

There were no differences ($P > 0.05$) in sperm quality parameters between dogs, both in fresh semen and frozen-thawed samples, whereas a significant ($P < 0.001$) effect of the replica was found (data not shown). Moreover, there was no interaction ($P < 0.001$) among dogs and replica for all sperm parameters.

3.2. Effect of PureSperm® selection on post-thaw semen quality

Centrifugation of frozen-thawed dog spermatozoa through a single-layer of PureSperm® 80 significantly ($P < 0.001$) improved the sperm motility parameters assessed (MT, MP, VCL, VSL, VAP, LIN, STR, WOB, BCF and ALH) by CASA (Table 1). Mean MT and ALH values were significantly ($P < 0.001$) higher in the fresh semen than selected frozen-thawed spermatozoa. As shown in Table 2, mean VS and DS values improved significantly ($P < 0.001$) after SLC, whereas the reverse was found for VS-AR, DS-AR and ASM ($P < 0.001$).

There were no individual dog differences ($P > 0.05$) for sperm quality parameters as well as there was no interaction ($P > 0.05$) among dogs and replica for sperm parameters assessed after sperm selection. However, a significant ($P < 0.001$) effect of the replica was observed in sperm quality (data not shown).

3.3. Changes in the motile sperm subpopulations structure after cryopreservation and after SLC through PureSperm® 80

Three sperm subpopulations were defined by the application of nonhierarchical and subsequent hierarchical classification of 26,051 individual motile spermatozoa using the eight motility parameters (VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF). Summary statistics for the motility characteristics of the subpopulations are shown in Table 3. Subpopulation 1 (sP1) included highly active but non-progressive spermatozoa, as indicated by the high values of VCL and BCF together with low LIN and STR values. Their poor progressiveness characteristics were evident from their values of ALH. This subpopulation might be considered as having an "hyperactivated-like" movement. The total motile spermatozoa included in this subpopulation was 40.3%. Subpopulation 2 (sP2) represented spermatozoa showing the highest degree of progressive motility (as inferred from the very high VSL, VAP, LIN and STR values together with low ALH values). They exhibited a vigorous flagellar action (high BCF). The total motile spermatozoa included in this subpopulation was 30.0%. Subpopulation 3 (sP3) included those spermatozoa whose movement was

Table 2
Mean values (±SEM) of the sperm viability and morphology parameters analysis of fresh semen, frozen-thawed control and frozen-thawed SLC treated samples from 5 dogs (10 ejaculates).

Parameter	Fresh semen	Frozen-thawed control samples	Frozen-thawed SLC treated samples
Viable sperm with an intact acrosome (%)	69.13 ± 0.06[a]	36.50 ± 0.17[c]	58.32 ± 0.04[b]
Viable sperm with an acrosome reaction (%)	9.45 ± 0.05[b]	9.74 ± 0.21[a]	2.81 ± 0.01[c]
Nonviable sperm with an intact acrosome (%)	11.00 ± 0.05[c]	23.24 ± 0.08[b]	24.15 ± 0.11[a]
Nonviable sperm with an acrosome reaction (%)	10.47 ± 0.05[c]	36.61 ± 0.18[a]	14.71 ± 0.11[b]
Total abnormalities (%)	15.45 ± 0.02[b]	16.49 ± 0.05[a]	13.55 ± 0.04[c]

Different superscripts (a–c) in the same row indicate significant differences ($P < 0.05$).

Table 3
Motility parameters for the sperm subpopulations defined after pattern analysis in fresh semen, frozen-thawed control and frozen-thawed SLC treated samples from 5 dogs (10 ejaculates).

Subpopulation (sP)	*n* (%)	Sperm motility parameters							
		VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
1	10,498 (40.3)	176.03 ± 0.29^{b}	90.31 ± 0.33^{b}	131.33 ± 0.31^{b}	51.58 ± 0.18^{c}	69.52 ± 0.22^{c}	74.15 ± 0.10^{c}	5.39 ± 0.01^{a}	9.78 ± 0.03^{b}
2	7812 (30.0)	183.90 ± 0.43^{a}	160.55 ± 0.41^{a}	167.92 ± 0.42^{a}	87.38 ± 0.09^{a}	95.70 ± 0.05^{a}	91.23 ± 0.07^{a}	3.51 ± 0.01^{b}	10.90 ± 0.04^{a}
3	7741 (29.7)	54.48 ± 0.37^{c}	21.96 ± 0.24^{c}	35.08 ± 0.29^{c}	35.20 ± 0.24^{c}	54.20 ± 0.28^{c}	61.92 ± 0.18^{c}	2.45 ± 0.01^{c}	4.64 ± 0.03^{c}

Results are expressed as mean ± SEM. The total number of spermatozoa analyzed was 26,051.
VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.
Different superscripts (a–c) in the same column indicate significant differences ($P < 0.05$).

defined as less vigorous, as indicated by the lowest value of VCL, VSL, VAP and ALH. They were poorly progressive (low VAP, LIN and STR) and covering very short distances (very low VSL). The total motile spermatozoa included in this subpopulation was 29.7%.

Although, the percentage of motile sperm included in each subpopulation experienced important changes after cryopreservation and after SLC, the sperm subpopulation structure was perfectly maintained after both procedures (Table 4). Cryopreservation induced a significant ($P < 0.001$) decrease in the percentage of motile spermatozoa included in sP1 (from 44.6% to 28.1%). This decrease was compensated for by concomitant, significant ($P < 0.001$) increases in the percentages of spermatozoa included in sP2 (from 22.5% to 36.8%) and sP3 (from 32.9% to 35.2%). On the other hand, SLC induced a significant ($P < 0.001$) increase in the percentage of sP2 from 36.8% in frozen-thawed control samples to 44.1% in frozen-thawed SLC treated samples. In contrast, the proportion of spermatozoa assigned to sP3 significantly ($P < 0.001$) decreased from 35.2% in frozen-thawed control samples to 16.0% in samples selected by SLC after thawing. Hereby, sP3 was characterized by low values of VCL, VSL and VAP, whereas sP2 was still composed of spermatozoa depicting high values for all sperm velocities (Table 3).

Individual variability was identified for each of the three sperm subpopulations ($P < 0.001$) either in fresh semen, frozen-thawed control or frozen-thawed SLC treated samples (data not shown).

3.4. Sperm yield of the SLC procedure

The sperm yield varied from 32.2% to 84.5% (mean sperm recovery rate of 61.5%) depending on the sperm quality of the original frozen-thawed samples.

4. Discussion

The aim of this study was to evaluate for dog sperm whether SLC using PureSperm® 80 could select good quality spermatozoa, including motility patterns, after freezing and thawing. Our results provide, for the first time, conclusive evidence that the SLC with PureSperm® 80 of frozen-thawed dog spermatozoa improves the overall sperm quality of semen samples in regard to higher motility, morphology, viability and acrosome integrity, but also alter the relative size of the sperm subpopulations in favor of fast progressively motile spermatozoa (sP2). This is generally consistent with previous studies where SLC has been carried out on frozen-thawed stallion (Macías García et al., 2009a, b) and buck (Jiménez-Rabadán et al., 2012) semen samples. Similarly, using a single layer of PureSperm® 80, it was possible to produce sperm preparations of equivalent quality from DGC using frozen-thawed dog spermatozoa (Dorado et al., 2011a, 2011b; Phillips et al., 2012).

The majority of the CASA motion parameters used in the present study have been previously identified as the most reliable markers of sperm quality (Dorado et al., 2009; Holt et al., 1997; Larsen et al., 2000). In relation, SLC with PureSperm® 80 significantly improved all the sperm motility parameters evaluated in canine frozen-thawed

Table 4
Changes in the sperm subpopulation structure of dog spermatozoa in fresh semen, frozen-thawed control and frozen-thawed SLC treated samples.

Subpopulation (sP)	Fresh semen		Frozen-thawed control samples		Frozen-thawed SLC treated samples	
	n	%	*n*	%	*n*	%
1	6812	44.6[a]	1458	28.1[c]	2228	39.9[b]
2	3438	22.5[c]	1910	36.8[b]	2464	44.1[a]
3	5022	32.9[b]	1828	35.2[a]	891	16.0[c]

Different superscripts (a–c) in the same row indicate significant differences ($P < 0.05$, Chi-square test).

semen, which is generally consistent with previous studies for SLC methods with Androcoll-C™ (Morrell et al., 2008b), Androcoll-E™ (Johannisson et al., 2009; Macías García et al., 2009b; Martinez-Alborcia et al., 2012; Morrell et al., 2010) or EquiPure® (Gutiérrez-Cepeda et al., 2011), and for other DGC techniques such as PureSperm® gradient (Dorado et al., 2011a; Hernández-López et al., 2005; Maxwell et al., 2007; Nicolas et al., 2012; Phillips et al., 2012), Percoll® (Brum et al., 2008; Garcia et al., 2010; Samardzija et al., 2006), and BoviPure® (Samardzija et al., 2006; Underwood et al., 2009). Of special interest were the improvements in sperm velocity. The three sperm velocities provided by the CASA equipment are the major kinematic characteristics related to fertility. Studies in dogs (Silva et al., 2006) and boars (Holt et al., 1997) have demonstrated that high sperm velocities are landmarks of fertility both *in vivo* and *in vitro*. It has also been reported previously that VCL is of key importance for formation of the sperm reservoir at the utero-tubal junction in mice (Olds-Clarke, 1996), that VCL and VAP are linked to the ability of ram spermatozoa to penetrate cervical mucus (Robayo et al., 2008), and that post-thaw VSL is related to the fertility of bull (Gillan et al., 2008) and human (Van den Bergh et al., 1998) spermatozoa. Therefore, it can be surmised that sperm preparations following SLC are the aliquots of those spermatozoa with highest potential fertilizing ability. Further research should be performed to related motility parameters of cryopreserved and colloid-selected dog semen with the fertility of dogs.

With regard to sperm motile subpopulations, the present study identified three motile sperm subpopulations either in fresh semen, in frozen-thawed control or in frozen-thawed SLC treated samples of dogs, which is consistent with previous studies in dogs (Dorado et al., 2011b, 2011c; Núñez-Martínez et al., 2006a,b; Rigau et al., 2001). sP1 was characterized by non-linear but vigorous motion. The low LIN values indicate that these cells possibly show a form of circular or erratic movements. Likewise, high VCL values coupled with remarkably high ALH values indicated that sP1 is typified by wide lateral excursions of the sperm head. sP2 represents active and progressive spermatozoa, probably the pattern movement most suitable for being part of the fertilizing population. sP3 was characterized by low-speed, non-linear motion. This population, with low VCL and LIN values, might represent a subgroup of metabolically compromised spermatozoa, that were soon to lose their motility altogether. Structural alterations or major biochemical changes affected spermatozoa from sP3. Poorly motile spermatozoa, such as those from sP1 and sP3, are less likely to progress within particular areas of the female reproductive tract and are therefore less likely to be involved in fertilization (Cremades et al., 2005).

The predominance of a particular motile subpopulation in samples with better cryoresistance or fertility has been demonstrated in other studies (Martinez-Pastor et al., 2005; Quintero-Moreno et al., 2003). In relation, the SLC significantly modified the distribution of spermatozoa within subpopulations. Therefore, sP2 (high-speed and progressive spermatozoa) represented more than 44% of the spermatozoa harvested after centrifugation through a single-layer of PureSperm® 80. Our results are consistent with those reported previously by Macías García et al. (2009a) for single-layer centrifugation of frozen-thawed stallion spermatozoa. In a previous study we also observed that PureSperm® DGC enriched the subpopulation depicting higher velocities in the processed frozen-thawed semen sample (Dorado et al., 2011b), and this leads to the conclusion that the DGC process improved the quality of the sperm sample in terms of the sperm subpopulation structure. Quality in this respect means that spermatozoa had the basic attributes of critical relevance for the fertility of a given ejaculate. Therefore, taking into account this experimental evidence, and since the SLC technique herein studied, particularly harvested those spermatozoa with highest velocity in the insemination dose, it can be surmised that sperm preparations following SLC are the aliquots of those spermatozoa with highest potential fertilizing ability. In this regard, we note that the implications for that the different motile sperm subpopulations and their distribution in thawed semen can have on subsequent *in vivo* or *in vitro* fertility require further investigation.

The aim of sperm selection after the freezing and thawing should be to improve the quality of the semen sample, reducing the risk of non-functional spermatozoa being used for fertilization and thus optimize the results of ART (Underwood et al., 2009). Our results show that the population selected by SLC, after thawing, showed better morphology, viability and acrosomal integrity that the frozen-thawed control sample. These results correspond to those from other studies in other animals (Jiménez-Rabadán et al., 2012; Macías García et al., 2009b), when SLC is carried out after thawing. Several studies have reported that DGC separation did not significantly improve sperm morphology in frozen-thawed canine semen (Dorado et al., 2011a; Kim et al., 2010; Phillips et al., 2012). To conclude, it seems clear that SLC with PureSperm® 80 of frozen-thawed dog spermatozoa can be a suitable procedure for

improving the post-thaw sperm quality of frozen semen doses.

Most sperm selection systems result in loss of spermatozoa and therefore, it may be that the proportion of selected functional cells is a critical factor of these techniques. SLC has been shown to be effective in improving sperm quality of dog semen samples, besides reaching sperm recovery yields up to 47% (Morrell et al., 2008b). Therefore, SLC could be suitable for improving the sperm quality of fresh ejaculates for AI. However, the present study adapts the SLC technique to frozen-thawed canine semen using the colloid PureSperm® 80 as a single layer. Sperm yield varied from 32.2 to 84.5% (mean sperm recovery rate of 61.5%) in the current study, which was higher than that reported for SLC by Morrell et al. (2008b). The discordant recovery yields among studies would reflect differences in the initial semen traits of the samples, which could affect the number of functional spermatozoa passing through the colloid (Martinez-Alborcia et al., 2012). Moreover, it is difficult to compare assays developed with different methods (colloid formulation, volumes of sperm and colloid, centrifugation force and time or size and diameter of centrifuge tubes) or species because the physical dynamics may affect sperm recovery differently, as it has been previously suggested (Nicolas et al., 2012). Anyway, further studies aiming to optimize the yield of SLC procedure are needed.

In a previous study, we obtained a lower percentage (40.8%) of frozen-thawed dog spermatozoa after PureSperm® DGC (Dorado et al., 2011a, 2011b). According with these findings, from a practical and clinical point of view, SLC with PureSperm® 80 offers an alternative, more practical method than discontinuous PureSperm® DGC for selection of good quality canine spermatozoa, thus saving time and effort in preparation.

Some previous studies have reported that results from intrauterine AI generally are good when doses of $50–100 \times 10^6$ frozen-thawed spermatozoa of good quality is inseminated at the right time in healthy bitches (Pretzer et al., 2006; Thomassen and Farstad, 2009). In our study, the mean yield obtained was approximately 60×10^6 of spermatozoa in each semen sample processed (0.5 mL per straw × 100 million spermatozoa per mL × recovery rate/100), which is enough to inseminate a bitch if intrauterine AI is used. In addition, elimination of undesirable spermatozoa from frozen-thawed canine semen with SLC using PureSperm® 80 makes possible to prepare semen doses enriched in specific sperm characteristics for intrauterine AI and obtain the highest pregnancy rates. On the other hand, in agreement with previous studies (Hollinshead et al., 2004; Maxwell et al., 2007), we found that SLC was an effective method for removing egg yolk from the original frozen-thawed sample, which may have a beneficial impact on measures of semen quality.

Concerning the effects of cryopreservation on semen quality and motility patterns, the results showed that a number of sperm quality parameters (motility, morphology, viability and acrosome integrity) deteriorated after thawing in comparison to fresh semen. In contrast, all the sperm parameters evaluated in the frozen-thawed SLC treated samples were comparable to freshly ejaculated spermatozoa. Nevertheless, post-thaw semen quality of both frozen-thawed control and frozen-thawed SLC treated samples are comparable to those from other studies (Batista et al., 2006; Peña et al., 2003; Silva et al., 2003; Yildiz et al., 2000).

It is noteworthy that motility changes induced by cryopreservation are linked to concomitant changes in the frequency distribution of spermatozoa within subpopulations (Flores et al., 2008). Our results confirm these findings, but they also clearly establish, in accordance with those found in previous studies from other mammalian species (Cremades et al., 2005; Flores et al., 2008; Muiño et al., 2008), that the general motile sperm structure present in dogs remained constant despite the effect caused by cryopreservation. This was linked to symmetrical changes in the specific percentage of each subpopulation. Hence, the overall increase in most of the mean motility characteristics of dog semen after cryopreservation, especially sperm velocity, was linked to a decrease in the percentage of spermatozoa in sP1 (highly active but non-progressive spermatozoa) and a concomitant increase of spermatozoa from sP2 (high speed and progressive spermatozoa) and sP3 (low-speed and low-linear spermatozoa) in frozen-thawed control samples when compared with the fresh semen. The reduction in the percentage of spermatozoa in sP1 indicated that many of the spermatozoa showing hyperactivated-like movement were unable to survive to freezing and thawing, probably as consequence of sublethal injury induced by this process, and they would be excluded from the subsequent analysis. As a result, the proportion of spermatozoa included in sP2 and sP3 increased partially in cryopreserved samples. Moreover, some of the spermatozoa from sP1 and sP2 were being assigned to sP3, possibly through alteration of their functionality (Quintero-Moreno et al., 2003). Therefore, it is possible that the increase in the percentage of sP3 was an early signal of changes in sperm function, which would be further detected by alterations in other parameters of semen quality (*e.g.* total motility, viability or altered acrosomes). The increase of spermatozoa in sP2 could possibly have been due to the dilution effect or to the addition of more fructose prior to its assessing. Verstegen et al. (2005) report how motility parameters of cold-storage spermatozoa were restimulated by centrifuging and adding more of the extender. Fructose has also been shown to induce a higher progressive motility (Rigau et al., 2001).

5. Conclusions

The results of the present study show that SLC with PureSperm® 80 may be an alternative and successful method for improving canine sperm quality damaged by the freezing–thawing process, and although this should be experimentally confirmed, presumably with the highest potential fertilizing ability, since the majority of the parameters of sperm quality explored hereby appeared significantly better than the counterpart original suspension of frozen-thawed spermatozoa. Moreover, sP2 (high-speed and progressive spermatozoa) was more frequently observed after preparation on the gradient. Finally, this study also demonstrated that cryopreservation significantly modified the distribution of spermatozoa within

subpopulations; however, the general motile sperm structure present in dogs remained constant despite the effect caused by either cryopreservation or selection by SLC through PureSperm® 80.

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