TESIS DE LA UNIVERSIDAD
DE ZARAGOZA

2021

248

Cristina Álvarez San Martín

Nuevas estrategias para mejorar la calidad espermática en la criopreservación de la especie equina

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ISSN 2254-7606

Prensas de la Universidad
Universidad Zaragoza

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Servicio de Publicaciones

ISSN 2254-7606

Tesis Doctoral

NUEVAS ESTRATEGIAS PARA MEJORAR LA CALIDAD ESPERMÁTICA EN LA CRIOPRESERVACIÓN DE LA ESPECIE EQUINA

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UNIVERSIDAD DE ZARAGOZA
Escuela de Doctorado

Programa de Doctorado en Medicina y Sanidad Animal

2020

Repositorio de la Universidad de Zaragoza – Zaguan http://zaguan.unizar.es

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Universidad de Zaragoza

Año 2020

La Dra. LYDIA GIL HUERTA, Profesora Titular del Departamento de Patología Animal de la Facultad de Veterinaria y la Dra. NOELIA GONZALEZ ORTI, Profesora Contratado Doctor del Departamento de Patología Animal de la Facultad de Veterinaria, ambas de la Universidad de Zaragoza, tienen el honor de informar como directoras de su Tesis Doctoral que:

Dña. CRISTINA ALVAREZ SAN MARTIN, ha trabajado bajo nuestra dirección durante los años comprendidos entre 2016 y 2020 en la preparación y puesta a punto de su Tesis Doctoral la cual coincide con el proyecto original sobre: Nuevas estrategias para mejorar la calidad espermática en la criopreservación de la especie equina.

Así mismo, certificamos que el material bibliográfico, experiencias y casuística presentados han sido seleccionados y que tanto su elaboración como los resultados y conclusiones obtenidas hacen estimar a las que suscriben como directoras de la Tesis Doctoral, que cumple los requisitos para su defensa, pudiendo ser sometida al Tribunal que sea nombrado por la Dirección del Departamento.

La presente **Tesis Doctoral**, presentada por Cristina Alvarez San Martín, de acuerdo con el informe correspondiente, autorizado por los Directores de Tesis y el Organo Responsable del Programa de Doctorado, se presenta como un compendio de trabajos previamente publicados. La unidad temática de todas ellas está directamente relacionada con la preservación seminal en la especie equina, por lo que está justificada su presentación por compendio de publicaciones.

Las referencias completas de los artículos que constituyen el cuerpo de la tesis son los siguientes:

Publicaciones en revistas incluidas en "Journal of Citation Reports "(JCR)

Alvarez, C., Luño, V., González, N., Guerra, P., Gil, L. (2019). Effect of mare colostrum in extenders for freezing stallion semen. *J Equine Vet Sci, 77,* 23-17**.**
https://doi.org/10.1016/j.jevs.2019.02.010

Alvarez, C., Luño, V., González, N., Gil, L. (2019). A preliminary study on the use of jenny colostrum to improve quality in extenders for freezing donkey semen**.** *Cryobiology*, *87,* 110-114.
https://doi.org/10.1016/j.cryobiol.2019.01.009

Alvarez, C., González, N., Luño, V., Martínez, F., Gil, L. (2019). Alternatives in donkey semen cryopreservation: Mare vs. Jenny Colostrum*. Repr Dom Anim, 54* (4), 94-97.
https://doi.org/ 10.1111/rda.13516

Alvarez, C., Luño, V., González, N., Gil, L. (2019). Ejaculated compared with epididymal stallion sperm vitrification. *Anim Reprod Sci, 211 (106205),* 1-7.
https://doi.org/10.1016/j.anireprosci.2019.106205

Publicaciones en libros:

Gil, L., **Alvarez, C.**, Luño, V. (2020). Part II: Equine. Chapter 8: Equine semen preservation: Current and future trends. *Biotechnologies applied to animal reproduction, current trends and practical applications for reproductive management.* Apple Academic Press. (*In Press*)

Publicaciones previas al desarrollo de la tesis, iniciación a estudios sobre mejora de calidad seminal en equinos:

Lozano, D., Gil, L., **Alvarez, C.** (2011). Efecto de la adición de plasma seminal en el semen equino descongelado. *Sanid Mil, 67* (3), 284-290.
http://dx.doi.org/10.4321/S1887-85712011000400005

Gil, L., Galindo-Cardiel, I., Malo, C., González, N., **Alvarez, C.** (2013). Effect of cholesterol and Equex-STM addition to an egg yolk extender on Pure Spanish Stallion cryopreserved sperm, *ISRN Vet Sci,* 21–23.
http://dx.doi.org/10.1155/2013/280143

Alvarez, C., Gil, L., González, N., Olaciregui, M., Luño, V. (2014). Equine sperm post-thaw evaluation after the addition of different cryoprotectants added to INRA 96® extender. *Cryobiology*, *69*, 144–148.
https://doi.org/10.1016/j.cryobiol.2014.06.008

Este trabajo ha sido financiado por la Universidad de Zaragoza, el Fondo Social Europeo (2014-2020) y el Gobierno de Aragón a través de la dotación económica al Grupo de Referencia RAySA (A17_17R).

A mi madre

A mi marido Erik

A mis hijos Iago, Iker e Iciar

El primer agradecimiento y más importante de todos es a mi amiga y compañera la **Dra. Lydia Gil**. Nos conocimos en el año 2005 cuando fui destinada al Centro Militar de Cría Caballar de Zaragoza, y desde ese momento nos unió una amistad. Ella me alentó a hacer el "Máster de Iniciación a la Investigación en Ciencias Veterinarias" y, gracias a su constante apoyo, hizo que no sólo empezara, sino que acabara escribiendo la Tesis Doctoral. Confió plenamente en mí y logró que me apasionase el campo de la investigación.

También quiero agradecer a mi codirectora de tesis, la **Dra**. **Noelia González** su dedicación, tiempo, apoyo y esfuerzo, gracias a los cuales conseguimos llegar al final de este maravilloso camino.

Por supuesto, un agradecimiento muy especial a la **Dra**. **Victoria Luño**, por su entrega incondicional, su ayuda e ilusión en el desarrollo de los trabajos de investigación de esta tesis. Su ánimo, positividad y alegría hicieron que en los momentos más duros y en los que parecía que no lográbamos avanzar, saliésemos triunfantes hacia delante. Ha sido una grata satisfacción personal el haberte conocido y haber trabajado juntas.

Al **Centro Militar de Cría Caballar de Zaragoza**, el cual ha cedido el material biológico de sus sementales para poder desarrollar el estudio de investigación y al que me siento orgullosa de haber pertenecido durante 13 años como Veterinaria Militar. Agradecimientos a su equipo de especialistas, por cierto, grandes compañeros, por su profesionalidad y disponibilidad en todo momento.

Agradecimientos también a todo el equipo de la **Unidad de Reproducción** de la Facultad de Veterinaria de Zaragoza (FVZ) por su predisposición y ayuda constante.

A mis amigos **Jo During** y **David McDowell** por su ayuda en la traducción de textos y redacción en inglés académico.

Y finalmente, como no puede ser de otra manera, a mi familia, a mi marido **Erik**, por su comprensión y sobre todo su paciencia al tenerme que aguantar mientras yo me absorbía en mi trabajo. Gracias por tu cariño incondicional, tu ánimo y tu apoyo, ¡te quiero! Gracias a mis hijos (**Iago, Iker** e **Iciar**), por transmitirme siempre alegría y cariño, a mi hermano, **Dr. Pablo Alvarez San Martín** especialista en Estadística e Investigación Operativa, por su colaboración en el desarrollo y análisis de datos estadísticos, quitando tiempo de donde no lo tenía y animándome continuamente durante estos años, a mi hermana **Iciar** por ser mi mejor amiga y dejarme ahogar mis penas en ella, y , para acabar, una mención muy especial a mi madre **Mariví**, que, aunque ahora ya no sea capaz de compartir con ella esta inmensa satisfacción, se que en sus "momentos", estará muy orgullosa de mí.

A todos, ¡¡¡¡GRACIAS!!!!

.

Informe de la comisión ética asesora para la experimentación animal de la universidad de Zaragoza

Comisión ética asesora para la experimentación animal
Universidad Zaragoza

Fecha: 5 de febrero de 2019
Ref. PI58/18NE
Destinatario/s:
Lydia Gil Huerta y Noelia González Ortí
Patología Animal
Facultad de Veterinaria

Asunto: Remitiendo informe emitido por la Comisión Ética Asesora para la Experimentación Animal sobre actividad de experimentación.

Adjunto le remito el informe que la Comisión Ética Asesora para la Experimentación Animal de la Universidad de Zaragoza acordó emitir en su reunión de 1 de febrero de 2019 respecto a la actividad experimental: "Criopreservación seminal equina".

El código de identificación asignado a la actividad es el PI58/18NE. Este código puede serle requerido en la solicitud de prestación de servicios del Centro donde prevé realizar la experimentación y puede figurar entre los datos identificativos de las jaulas o sistemas de confinamiento de los animales.

En caso de que la Comisión haya considerado que la actividad presentada, no cumple en su presente forma con los requisitos exigidos, el investigador responsable podrá corregir los aspectos necesarios, atendiendo a lo indicado, pudiendo ser presentada nuevamente para su evaluación por esta Comisión.

Aprovecho la ocasión para mandarle un cordial saludo.

El Secretario de la Comisión

Comisión ética asesora para la experimentación animal Universidad Zaragoza

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Comisión ética asesora para la experimentación animal
Universidad Zaragoza

Ref. PI58/18NE

CONTROL DE BUENA PRÁCTICA EN EXPERIMENTACIÓN ANIMAL

Título del procedimiento de experimentación:

Criopreservación seminal equina

Título del proyecto en el que se incluye:

Criopreservación seminal equina

Centro de realización: --

Investigador responsable: Lydia Gil Huerta y Noelia González Ortí

Animales o muestras que implica:

especie (s): Caballos, asnos e híbridos (*Equidae*)

peculiaridades: Muestras seminales de Centro de Cría Caballar y testículos de matadero.

número: No procede

Fecha de presentación: 26 de noviembre de 2018

Reunida la Comisión Ética Asesora para la Experimentación Animal* el día 1 de febrero de 2019, y una vez revisada la documentación disponible en relación a la actividad de experimentación descrita, considera:

Que queda excluida del ámbito de aplicación del RD53/2013, de 1 de febrero, por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia.

Que el diseño cumple los principios éticos y de protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia, que se ha impuesto la Universidad de Zaragoza.

Por todo ello, se emite **informe FAVORABLE****.

Este informe será válido únicamente para esta actividad. Deberá ser presentada nuevamente a revisión en caso de modificación sustancial de la actividad presentada o cuando la normativa en vigor así lo requiera.

*Órgano habilitado para realizar la evaluación y evaluación retrospectiva (Resolución de 6 de agosto de 2013 de la Dirección General de Alimentación y Fomento Agroalimentario del Gobierno de Aragón).

****Observaciones:** Actividad con muestras seminales excedentarias procedentes de animales no utilizados expresamente para el estudio y muestras de órganos de matadero.

En Zaragoza, a 5 de febrero de 2019

EL SECRETARIO DE LA COMISIÓN	EL PRESIDENTE DE LA COMISIÓN
Jorge Palacio Liesa	Luis Miguel García Vinuesa

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ADN: Acido desoxirribonucleico

ALH: Amplitud media del desplazamiento lateral de la cabeza

AO: Naranja de Acridina (*acridine orange*)

BCF: Frecuencia de batida

BSA: Albúmina sérica bovina

CASA: Computer-Assisted Sperm Analysis

CMCCZ: Centro Militar de Cría Caballar de Zaragoza

CP: Crioprotectores

°C: Grados centígrados

DMF: Dimetilformamida

EDTA: Acido etilendiaminotetraacético

EPT: Vitrificación con trealosa con muestras de epidídimo

EYT: Vitrificación con trealosa con muestras de eyaculado

EPL: Vitrificación con lactosa con muestras de epidídimo

EYL: Vitrificación con lactosa con muestras de eyaculado

FICT: Fluorocromo de Isotiocianato de Fluoresceína

FVZ: Facultad de Veterinaria de Zaragoza

g: Gramos

g: Fuerza centrífuga relativa

G: Glicerol

HOST: Test hipoosmótico

IA: Inseminación artificial

Ig: Inmunoglobulinas

IgA: Inmunoglobulina A

IgG: Inmunoglobulina G

IP: Isocianato de fluoresceína

LIN: Indice de linealidad

µl: Microlitros

µm: Micrómetros

M: Molar

mg: Miligramos

MHz: Megahercios

min: Minuto

ml: Mililitros

µM: Micromoles

mM: Milimoles

MP: Motilidad progresiva

MT: Motilidad total

N_2L: Nitrógeno líquido

PI: Ioduro de propidio

PNA: Aglutinina de maní (*Arachis hypogaea agglutinin*)

PMNN: Polimorfonucleares neutrófilos

PS: Plasma seminal

seg: Segundos

SCD: Dispersión de la Cromatina

SDFI: Porcentaje de células espermáticas fragmentadas

Spmtz: espermatozoide

STR: Indice de rectitud

tª: Temperatura

TB: Azul de Toluidina

TL: Trealosa

TR: Test de Resistencia

VA: Vagina artificial

VAP: Velocidad de la trayectoria media

VCL: Velocidad curvilínea

VSL: Velocidad rectilínea

WOB: Indice de oscilación

Cabeza de caballo. Boceto para "Guernica" (Pablo Ruiz Picasso)

1. Introducción

1. Introducción

Las primeras citaciones sobre inseminación artificial (IA) en el caballo aparecieron en textos árabes, y su uso fue descrito por primera vez en 1322, sin embargo, no existe evidencia de que estas prácticas se extendieran. Aunque en 1590 Zacharias Janssen inventó el microscopio, no fue hasta 1677, cuando el científico holandés Leeuwenhoek (precursor de la biología experimental) lo utilizó, desarrollando microscopios simples de fabricación propia, para estudiar los espermatozoides por primera vez, llamándolos "animáculos". A finales de 1700 el fisionomista italiano Lazzaro Spallanzani, tras demostrar que debía existir contacto físico entre el ovocito y el semen para que se desarrollara un embrión (hasta ese momento se creía que el embrión era "producto de la semilla masculina, nutrido en el suelo de la mujer"), empezó a experimentar técnicas de IA en peces y anfibios Tras el éxito obtenido, trasladó esos trabajos a otras especies animales superiores. Así, en 1784 reportó la primera IA en una perra que tras una gestación normal parió tres cachorros totalmente sanos. A partir de ahí aplicó esta técnica también a los equinos, describiendo ya en estos estudios el efecto de la congelación sobre los espermatozoides.

A finales del siglo XIX el Dr. Pearson, profesor de medicina veterinaria en la facultad de Pennsylvania, empezó a utilizar IA en yeguas obteniendo notorios resultados.

En Europa, en 1890, el Veterinario francés Repiquet, trabajó con esta técnica en la especie equina. Fue el primero en recomendar el procedimiento como medio de lucha contra la esterilidad. Paralelamente, Heape (1897) utilizó esta técnica en otras especies domésticas además de en caballos y propuso el nombre de IA en vez de fecundación artificial. Unos años después, Sand *y* Stribolt (1902) describieron este método como un método muy adecuado para aquellos caballos de alto valor económico. En 1914 Amantea desarrolló la primera vagina artificial para recolección de semen de perros.

Posteriormente, gracias a la financiación del Studbook de la Raza Rusa Equina, I.I. Ivanov (1870-1932) investigó el uso de IA en esta raza, haciendo selección de los mejores sementales para llegar a obtener en sus estudios un alto índice de preñez en las yeguas. Demostró que mediante esta técnica se podía inseminar un número mucho mayor de yeguas en comparación con la monta natural. Fue el primero en obtener híbridos interespecíficos. El primero de ellos, el cebroide, resultó de la hibridación entre el burro y la cebra. Sin embargo, sus estudios fueron polémicos al intentar crear un híbrido hombre-mono. En 1912 vio la posibilidad de almacenar semen bovino y transportarlo a 2 ºC para después usarlo en potocolos de IA. Los estudios posteriores fueron encaminados a congelar semen y almacenarlo a temperaturas por debajo de 0 ºC, así como a crear nuevos medios diluyentes capaces de proteger a los espermatozoides ante estos descensos importantes de temperatura. Milovanov, científico ruso que continuó los trabajos de Ivanov, en 1934 describió un protocolo de congelación que sería la base de la actual criopreservación de semen. También diseñó una vagina artificial (VA) para la especie equina, basada en la de Amantea en 1914, siendo muy similar a las usadas en la actualidad.

Durante esa época, también otros descubrimientos fueron determinantes en el desarrollo de las técnicas de criopreservación de semen. Phillips y Lardy (1939) fueron los primeros en usar la yema de huevo para proteger las células espermáticas de toro contra el shock por frío; Salisbury y cols. (1941) mejoraron estos medios incluyendo citrato de sodio para garantizar el equilibrio físico-químico y con ello el pH y el equilibrio iónico. Polge y cols. en 1949 descubrieron el efecto crioprotector del glicerol (G), publicando en 1950 el primer artículo sobre congelación de semen equino, utilizando una solución acuosa con Glicerol, congelando a -79ºC y obteniendo unos índices de supervivencia del semen de un 25%. Foote y Bratton también en 1950, descubrieron el efecto beneficioso de los antibióticos al ser

Figura 1. La revista "Canadian Journal of Comparative Medicine" (Vol. XXI, nº 2, febrero 1957), publica el primer trabajo sobre IA en una yegua con espermatozoides congelados de epidídimo

agregados a los diluyentes seminales, crearon el diluyente "Cornell" que contenía una mezcla de antibióticos consistente en penicilina, polimixina B y estreptomicina. En 1957 destacaron las investigaciones llevadas a cabo por Barker y Gandier, al conseguir la primera gestación en una yegua utilizando semen congelado equino obtenido a partir del epidídimo (*Figura 1*). En esa misma década, además, el nitrógeno líquido (N_2L) se convirtió en el refrigerante de elección para la conservación de semen de toro.

En España, el primer veterinario en introducir las técnicas de IA fue Marcelino Montón Cardós, veterinario bilbilitano. Sus trabajos se centraron en la especie equina y su primera experiencia la realizó en 1915 en la Yeguada Militar y Sementales de Córdoba. Desarrolló un recolector de esperma al que denominaron "Espermocaptor". Este aparato consistía en una funda de goma muy fina, larga y cilíndrica con uno de sus extremos cerrado y el otro engarzado a un aro metálico, que a su vez estaba unido a un mango de madera. Antes de utilizarlo, la goma se introducía en leche, y a continuación se colocaba enrollada en la vulva de la yegua, de modo que cuando el semental la cubriera, la goma se arrastrara hacia el interior de la vagina y el eyaculado quedara recogido y pudiera recuperarse tirando del mango. Este artilugio fue la base para el desarrollo posterior de vaginas artificiales. Sin embargo, Dionisio Sanjuan Jaraula fue considerado el primer veterinario español que aplicó científicamente las técnicas de IA en el ganado caballar. En 1933 desarrolló una vagina artificial para aplicar su técnica y en 1934 reportó con éxito la primera gestación y parto de una yegua inseminada con estos procedimientos.

A pesar de la gran evolución en el desarrollo de técnicas de IA, en el caballo no hubo demasiada aceptación. Muchas asociaciones de ganaderos se opusieron a su uso como medida para evitar la pérdida de diversificación de sus líneas de sangre, incluso hoy en día es una técnica que sigue sin aceptarse para algunas razas, como por ejemplo para el Pura Sangre Inglés. Esta baja aceptación, unido a una carencia de fondos para la investigación, junto con un número de yeguas reducido y en las que se necesita mayor especialización, cuidado y control que en otras especies, hicieron que los avances en el desarrollo de estas técnicas de reproducción asistida se retrasasen con respecto a otras especies (Amann y Pickett, 1987). Sin embargo, dichas consideraciones poco a poco han ido cambiando y actualmente cada vez son más frecuentes los programas de Reproducción Asistida en los planes de cubrición de la

especie equina, con todas las variantes que la técnica contempla, adaptándose a las necesidades que surjan en cada momento.

Las investigaciones en la criopreservación de semen equino han tenido que enfrentarse a obstáculos, tales como la variabilidad del semen de esta especie para tolerar el proceso de congelación-descongelación. Además, se ha sumado el complejo ciclo estral de las yeguas, que ha obligado a hacer seguimientos ecográficos muy estrictos sobre el ciclo para determinar el momento exacto de la ovulación y así realizar la inseminación en el momento más adecuado. Todo ello se refleja en unos índices de gestación bastante reducidos (<60% de preñez).

En base a esta gran variabilidad, Arman y Picket (1987) postularon que establecer un único protocolo de congelación para todos los sementales era poco realista y disminuía la capacidad de éxito. De hecho, con los procedimientos de crioconservación utilizados tradicionalmente sólo en un 25% de los sementales se consiguen unos porcentajes de fertilidad aceptables, mientras que más del 50% de los sementales son considerados moderadamente aptos para el proceso de crioconservación de semen, lo que demuestra la gran variabilidad individual de los espermatozoides en la especie equina para resistir el proceso de congelación-descongelación (Aurich, 2008; Tischner, 1979). Fueron Loomis y Graham los que en el año 2008 propusieron la utilización de distintos protocolos de congelación, así como distintos diluyentes y crioprotectores para hacer el proceso de forma individualizada y maximizar con ello la crio-supervivencia de los espermatozoides. Actualmente, muchos laboratorios realizan "pruebas de congelación" individualizadas para los sementales que van a destinar los eyaculados a la criopreservación, y aunque la determinación de estas pruebas resulta costosa, a largo plazo es evidente el efecto beneficioso que tienen, puesto que suponen poder descartar sementales "malos congeladores".

En las yeguas, a parte del complicado manejo y control del ciclo estral, hay otro factor que se debe considerar, en el endometrio post-cubrición o tras la inseminación, aparece de forma natural una reacción como respuesta fisiológica para facilitar la fagocitosis y eliminación de contaminantes, tales como bacterias, plasma seminal, diluyentes y espermatozoides no viables. El fin de esta reacción es restablecer el ambiente endometrial óptimo para maximizar

las posibilidades de fecundación y viabilidad de la gestación. En condiciones normales, se produce una reacción endometrial que comienza a los 30 min tras la inseminación, alcanzando su pico a las 4-8 h, pero desaparece completamente a las 48 h (Kotilainen y cols., 1994; Troedsson y Woodward, 2016). Sin embargo, en algunas yeguas esta endometritis persiste, siendo incompatible con la gestación. Muchos diluyentes presentan en su composición determinadas sustancias para mejorar la capacidad de preservación de los espermatozoides (glicerol, yema de huevo, etc.), que van a incrementar la migración de polimorfos nucleares (PMN) al endometrio, favoreciendo aún más los procesos inflamatorios en el útero. Este proceso fisiológico no solo se da en yeguas, también se ha visto en burras, siendo la reacción inflamatoria aún mayor (Miró y Papas, 2018). Además, en el caso de las burras encontramos una dificultad añadida, ya que cuando se trabaja con semen congelado, la calidad del mismo tras la descongelación cuenta con una calidad aún inferior que la de los caballos (Acha y cols., 2016; Canisso y cols., 2011).

Se considera que el porcentaje de preñez por ciclo con semen congelado está sobre el 30-40% siempre y cuando éste se use de forma correcta, pero hay un gran margen, por ello no es raro encontrar rangos que van desde el 0% hasta más del 70% (Samper, 2009). Además de toda esta variabilidad, existe una falta de estandarización en la información disponible en los diferentes puntos del protocolo de inseminación, por ejemplo, sobre cuál es el tiempo más adecuado para inseminar, cuales son las pautas exactas a seguir en el proceso de congelación y de descongelación, o incluso el propio manejo de semen. Todo esto implica que aquellos que empleen esta técnica deban saber los factores que afectan la fertilidad del semen congelado para ser realistas en las expectativas deseadas.

Sin embargo, no todo son inconvenientes, existen también ventajas importantes al usar semen congelado en los programas reproductivos de la especie equina, que hacen que cada día se investigue más sobre el tema, con el objeto de intentar mejorar estos porcentajes de preñez y concretar o definir mejor los protocolos que se deben seguir.

Dentro de las ventajas encontramos:

a) Aumento del acervo genético, con acceso a la genética de los mejores sementales del mundo, pudiendo disponer de dosis incluso tras el fallecimiento del animal.
b) Bajo coste de transporte (tanques de nitrógeno en lugar del semental).

c) Disponibilidad continua de dosis.
d) Posibilidad de ajustar la inseminación al momento justo de la ovulación.
e) Disminución de enfermedades venéreas si los animales son evaluados correctamente.
f) Continuidad de la temporada de cubrición del semental incluso cuando está realizando entrenamientos, competiciones o se está recuperando de una enfermedad.
g) Desaparición de la estacionalidad reproductiva entre el hemisferio norte y sur.
h) Almacenamiento de la dosis durante un periodo de tiempo ilimitado.
i) Disminución del riesgo y del estrés en la yegua y el potro.
j) Disminución de accidentes (traumatismos principalmente) derivados del acto de la monta natural.

Actualmente continúan en auge los trabajos de investigación sobre criopreservación espermática, con objeto de optimizar la fertilidad tras su descongelación. No obstante, todavía sigue siendo necesario obtener conclusiones más precisas sobre las causas que originan el daño celular, producidas durante los proceso de criopreservación/descongelación, encontrar los diluyentes y/o crioprotectores que mejor se adecúen y protejan a los espermatozoides durante este complejo proceso, así como evaluar todas las posibles causas de fallo en la conservación (Alvarenga y cols., 2016).

Los métodos para la conservación de semen están basados en la disminución de la temperatura para de ese modo reducir o detener el metabolismo de los espermatozoides mientras mantienen sus propiedades fecundantes.

Existen dos métodos de preservación seminal en función de la temperatura con la que se procese el semen: la refrigeración (0-15 ^{o}C) y la criopreservación en N_2L (-196 ^{o}C). El semen refrigerado, transcurridas 24 horas de almacenamiento, empieza a reducir su índice de fertilidad aproximadamente entre un 10% y un 35% al día. Se considera semen de "calidad aceptable" si éste, tras 24-48 h de refrigeración mantiene al menos un 30% de motilidad progresiva (McKinnon y cols., 2011). Sin embargo, aunque resulte contradictorio, la fertilidad del semen congelado es más alta en relación con el tiempo de almacenaje (pudiendo ser almacenado de forma indefinida) que la del semen refrigerado, pero es peor en términos de viabilidad post-descongelación. En cualquier caso, los principales efectos nocivos que sufren los espermatozoides no son debidos al almacenamiento, si no al proceso de congelación y de

descongelación. Los daños sufridos durante la criopreservación espermática se han clasificado según su origen en daños como consecuencia de cambios drásticos de temperatura, daños como consecuencia de cambios de osmolaridad y daños debidos al estrés oxidativo debido a agentes oxidantes, si bien, todos estos daños se encuentran correlacionados. Los trabajos que investigan mejorar de calidad seminal tras la descongelación están encaminados a reducir estos daños, ya que una mayor viabilidad del semen se traduce en un mayor índice de preñez.

Otro factor conocido como negativo sobre los resultados de preservación es el plasma seminal, por ello es necesario preparar los eyaculados para su preservación. Estos deben ser sometidos primero a una centrifugación con el objeto de retirar la mayor parte del plasma seminal (PS), ya que éste afecta negativamente a los espermatozoides durante el almacenamiento (Lozano y cols., 2011). A pesar de que el proceso no es totalmente inocuo para los espermatozoides, se pueden minimizar los efectos adversos utilizando baja fuerza de centrifugación (intensidad variable según distintos autores (Aurich, 2008, Alvarenga y cols., 2016) y diluyendo el eyaculado (1:1 de forma general) antes de someterlo al proceso (McKinnon, 2011), para conferirle cierta protección. El pellet resultante tras la centrifugación se debe resuspender en un diluyente apto para la conservación, el cual además llevará sustancias crioprotectoras.

Podemos confirmar que son numerosos los factores que afectan a los resultados de la calidad seminal tras el proceso de criopreservación, por ello, previo al desarrollo de esta Tesis Doctoral, ya planteamos tres trabajos, que hemos incluido al final de la tesis por su vinculación directa con el tema de nuestra investigación, enfocados a estudiar el efecto de distintos compuestos sobre los diluyentes de congelación de semen equino, con objeto de mejorar la calidad espermática.

En el primero de estos estudios, se evaluó la adición de PS a diferentes concentraciones, sobre espermatozoides equinos descongelados, concluyendo que el PS podía frenar la reacción acrosómica, reflejado este hecho en los bajos porcentajes de espermatozoides que sufrieron la verdadera reacción acrosómica. En el segundo estudio, evaluamos el efecto del colesterol y Equex-STM al ser añadidos a diluyentes con yema de huevo, y concluimos que EquexSTM al 0,5% mezclado con colesterol 125 mM, obtuvo una calidad espermática mejor que el diluyente Martin original. Finalmente, en nuestro tercer estudio, evaluamos la adición de distintos

agentes crioprotectores permeables: Glicerol (G), Dimetilformamida (DMF) y Acetal (A), a distinta concentración adicionados en el diluyente INRA 96® para su uso en la congelación de espermatozoides procedentes de eyaculados y de epidídimo, concluyendo que la mejor calidad seminal tras la descongelación, se obtenía incorporando un 5% de DMF en diluyentes para congelación de eyaculado y una combinación de 2,5% DMF con 2,5% G en diluyentes para congelación de muestras de semen de epidídimo.

Como hemos visto, la composición de los diluyentes es muy variable y ha constituido uno de los retos principales para mejorar la calidad seminal tras la descongelación ya que de su composición depende la función protectora sobre los espermatozoides frente al estrés por frío. La leche desnatada se ha utilizado y se sigue utilizando por sus propiedades protectoras como uno de los principales componentes en muchos diluyentes, aunque el diluyente "ideal" aún no ha sido establecido.

¿Podría plantearse el uso del calostro como parte de los diluyentes seminales? La composición no es la misma, ya que el calostro, como primera forma de leche producida por la glándula mamaria, a diferencia de la leche madura, presenta un mayor número de inmunoglobulinas (Ig) y otros componentes con propiedades antibióticas, antiinflamatorias, inmunomoduladoras y antioxidantes (lisozimas, lactoperoxidasa, lactoferrinas, oligosacáridos, etc.). Esto nos podría hacer plantear que quizá sería una opción muy interesante, pero conviene analizarlo más en profundidad, viendo sus características y de que manera podría resultar beneficioso.

El calostro se produce bajo influencia hormonal durante las últimas semanas de gestación. Durante la preñez, las glándulas mamarias de la yegua están expuestas a altos niveles de estrógenos y progestágenos. Se cree que la lactancia está restringida por el aumento de progestágenos hasta aproximadamente el día 310 de la gestación, para disminuir de 2 a 3 días antes del parto, mientras que los niveles de prolactina aumentan durante la última semana de gestación (Worthy y cols., 1986; McKinnon y cols.,2011). El aumento de tamaño de la ubre suele comenzar entre 2 y 3 semanas antes del parto. Durante este tiempo se produce una secreción de líquido grisáceo y acuoso, que poco a poco se va volviendo más amarillento, denso y viscoso al incluir una incorporación selectiva de anticuerpos humorales (Ig) en su composición. La glándula mamaria se llena de calostro entre las 24 a 48 horas antes

del parto. Tras el parto, los niveles de progesterona en suero disminuyen drásticamente, pudiendo ser ésta la causa de la transición de calostro a leche. Además, se hace presente la α-lacto albúmina, que hace que una gran cantidad de agua sea atraída hacia los alveolos, de manera que la producción de leche es más copiosa. 24 horas después del parto, el nivel de Ig ha disminuido en un 40% (McKinnon y cols., 2011).

El calostro está compuesto por un complejo de hidratos de carbono, grasas, proteínas y electrolitos. La glándula mamaria no es capaz de sintetizar ninguna proteína inmunológica excepto la inmunoglobulina A (IgA) bajo la influencia hormonal. Así, todas las Ig se agregan selectivamente desde la sangre en las primeras 2-3 semanas de gestación. Este cambio puede ser detectado en la yegua como una caída en la concentración de las globulinas circulantes (Knottenbelt y cols., 2004). La Ig más abundante en el calostro es la inmunoglobulina G (IgG), aunque su cantidad es muy variable (Kenzig y cols., 2009) dependiendo de varios factores, como la raza (se ha demostrado que en Pura Raza Arabe la concentración es mayor) o factores individuales (García y cols., 2010). En la *tabla 1* se muestra las distintas concentraciones de Ig.

Tabla 1. Concentraciones de las distintas Ig en el calostro y leche de la yegua (Tizard, 2018).

	CONCENTRACIONES DE LAS DISTINTAS Ig (Mg/Dl)						
	Ig A	Ig M	Ig G	Ig G(T)	Ig G(B)	Ig G3	IgG6
Calostro	500-1500	100-350	1500-5000	500-2500	50-150	500-2500	50-150
Leche	50-100	5-10	20-50	5-20	0	20-50	0

La composición del calostro y la leche de las yeguas varían a lo largo de la lactación (*Tabla 2*). Los niveles de proteína y materia seca disminuyen considerablemente a lo largo de las semanas, en cambio, los niveles de lactosa son mucho menores en el calostro que en la leche entre 3 y 6 semanas (Pecka y cols., 2012).

En relación con la composición de macro y micro-elementos, cabe destacar que los niveles de calcio y hierro no alcanzan su máximo hasta los 2-5 días de lactación (*Tabla 3*).

Tabla 2. Composición básica del calostro y de la leche de las yeguas (Pecka y cols., 2012).

COMPOSICIÓN	CALOSTRO POSTPARTO	3 SEMANAS	6 SEMANAS
Grasa %	1,68±0,76	1,49±0,59	1,78±0,33
Proteína %	15,21±7,01	2,07±0,43	2,05±0,29
Lactosa %	2,46±1,82	6,64±0,67	6,64±0,57
Materia seca %	19,34±4,87	11,10±1,11	11,41±0,92

Tabla 3. Contenidos proteicos, fracciones proteicas, cenizas, macro-elementos y micro-elementos del calostro y de la leche de la yegua (Csapò-Kiss y cols., 1995).

COMPOSICIÓN	CALOSTRO POSTPARTO	LECHE 2-5 DÍAS	LECHE 8-45 DÍAS
Proteínas totales (g/100g)	16,41	4,13	2,31
Proteínas en suero (g/100g)	13,46	2,11	1,11
Caseína (g/100g)	2,95	2,02	1,2
Nitrógeno no proteico (%)	0,052	0,043	0,031
Cenizas %	0,592	0,526	0,405
Calcio (mg/kg)	747,7	953,7	822,9
Potasio (mg/kg)	928,6	709	517,2
Sodio (mg/kg)	320	177	166,6
Fósforo (mg/kg)	741,7	638	498,8
Magnesio (mg/kg)	139,7	86	65,87
Zinc (mg/kg)	2,95	2,08	1,99
Hierro (mg/kg)	1,00	1,58	1,21
Cobre (mg/kg)	0,61	0,25	0,23
Manganeso (mg/kg)	0,045	0,053	0,0544

Los niveles de γ-caseína, β-caseína, albúmina sérica y α-lactoalbúmina son menores en el calostro que en la leche a las 6 semanas, habiendo alcanzado su máximo la tercera semana de lactación (*Tabla 4*). En cambio, los niveles de IgG disminuyen considerablemente a lo largo de la lactación, siendo el nivel máximo el observado en el calostro (Pecka y cols., 2012).

El valor biológico de la proteína de la leche tras el parto es de 132,3 debido a los altos niveles de treonina y lisina. A los 5 días es de 119,7 y a los 45 días de 107,9. Destacan la composición de aminoácidos esenciales y el valor biológico de la proteína de la leche al ser mucho mayores que los niveles de vacuno.

Tabla 4. Contribución porcentual de las fracciones proteicas seleccionadas en el calostro y leche de yaguas (Pecka y cols., 2012).

COMPOSICIÓN	CALOSTRO POSTPARTO	3 SEMANAS	6 SEMANAS
Albúmina sérica	10,83±2,67	13,34±4,03	13,23±4,28
Ig G	55,64±3,38	38,98±8,86	41,14±7,62
β-caseína	11,66±1,29	14,46±7,05	13,16±4,47
γ-caseína	9,77±0,92	14,63±5,43	12,10±3,08
α-lactoalbúmina	12,09±0,99	14,66±4,08	14,24±2,68

La suma de los niveles de aminoácidos esenciales -treonina, valina, cistina, tirosina y lisina- decrecen a lo largo de la lactación de 26,4 a 21,3 g/100g de proteína. En cambio, la suma de los niveles de ácido glutámico y prolina aumentan de 21,9 a 28,5 g/100 g de proteína (*Tabla 5*).

Tabla 5. Composición de aminoácidos de las proteínas de calostro y de la leche de las distintas Ig en del calostro y leche (Csapò y cols., 2009)

COMPOSICIÓN (/100g PROTEÍNA)	CALOSTRO	LECHE
Threonina	4,6	11,5
Valina	14,5	5.4
Cistina	0,8	0,2
Tyrosina	2,2	0,9
Lysina	9,9	2,8
Ácido glutámico	14,5	31,8
Prolina	3,9	5,2

Todas estas características que hacen diferente al calostro de la leche convierten al calostro en una opción interesante si, además, también es capaz de ejercer un efecto protector preservando a los espermatozoides frente el shock térmico.

En base a esto, una parte de los trabajos de investigación desarrollados en esta tesis se ha dedicado al estudio del calostro, tanto de burra como de yegua, como un nuevo

componente para incrementar esta calidad espermática en semen de caballos o de asnos sometidos a procesos de crioconservación, en concreto de congelación. En la primera experiencia se evaluó la adición de calostro de yegua en diluyentes para congelación de semen de caballos, obteniendo buenos resultados y considerándolo válido como agente crioprotector en la preservación de los espermatozoides. Para ello se recogieron muestras de calostro de cuatro yeguas justo tras el nacimiento del potro y se determinó su composición y su calidad. Se recogieron eyaculados de nueve sementales fértiles con buena calidad seminal. Tras llevar las muestras a la FVZ y realizar otra evaluación, fueron agrupadas y criopreservadas en tres grupos experimentales: diluyentes a base de lactosa complementada con calostro de yegua (20%), diluyentes a base de lactosa complementada con yema de huevo (20%), y BotuCrioÒ. Después de la descongelación se hizo una contrastación para determinar la calidad de las muestras. La motilidad de los espermatozoides se evaluó mediante el sistema computerizado ISAS ProiserÒ, la viabilidad mediante SYBR-14 y tinción con PI, la integridad del acrosoma mediante FITC-PNA/PI, la funcionalidad de la membrana plasmática mediante el HOST y la desnaturalización del ADN mediante tinción de naranja de acridina (AO). En cuanto a los resultados obtenidos se observó que entre los diluyentes no hubo diferencias significativas en los porcentajes de motilidad total, integridad del acrosoma y fragmentación de ADN después de la descongelación. Los parámetros cinemáticos, sin embargo, sí mostraron valores significativamente más altos en BotuCrioÒ que en los diluyentes a base de lactosa (P<0,05). Tanto BotuCrioÒ como los diluyentes de calostro presentaron tasas significativamente mejores para HOST, linealidad, rectitud y oscilación que el diluyente de yema de huevo (P<0,05). Sin embargo, en relación con la viabilidad de los espermatozoides, los diluyentes con yema de huevo mostraron significativamente mejores resultados en comparación con los otros medios experimentales (P<0,05). En conclusión, la incorporación del calostro de yegua en medios de crioconservación protegió a los espermatozoides contra el shock térmico; por lo tanto, su utilización puede ser una buena alternativa como agente crioprotector en los diluyentes para congelar semen equino.

En la segunda experiencia enfocamos nuestro trabajo a valorar la calidad espermática del semen congelado de burros (*Equus asinus),* ya que actualmente, después de ser descongelado todavía sigue dando pobres resultados, inferiores a los de otros animales, incluidos los caballos. Con los buenos resultados de la adición de calostro de yeguas en el caso de semen

equino, decidimos testar la utilidad del calostro igualmente en la congelación de semen de burro. Para ello, añadimos calostro de burra en vez de calostro de yegua en los diluyentes de congelación, con el objeto de intentar reducir al máximo cualquier reacción interespecífica que pudiese influir. Por tanto, el objetivo de este estudio fue valorar el efecto protector de la adición de calostro de burra en los diluyentes para la congelación de semen asnal. Se recogió los eyaculados de cinco burros fértiles (dos eyaculados por burro en diferentes días). Las muestras se agruparon, diluyeron y criopreservaron en tres grupos experimentales: BotuCrio®, diluyente de lactosa suplementado con yema de huevo (20%) y diluyente de lactosa suplementado con calostro de burra (20%). Después de descongelar evaluamos la motilidad de los espermatozoides mediante sistema computerizado ISAS Proiser®, la viabilidad mediante SYBR-14 y PI, la integridad funcional de la membrana mediante HOST e integridad del acrosoma por FITC-PNA/PI. Los resultados demostraron que las muestras con calostro de burra fueron significativamente mejores (*P<0,05*) en casi todos los parámetros evaluados (Motilidad total, viabilidad, HOST, VCL, VSL y VAP) en comparación con los otros dos diluyentes después de la descongelación (BotuCrio® y diluyente a base de yema de huevo). Los valores obtenidos en la integridad del acrosoma, LIN, STR y WOB a pesar de ser más bajos, no fueron estadísticamente significativos (P>0,05). En conclusión, el diluyente a base de calostro de burra puede usarse con éxito para la criopreservación del semen de burro y podría mejorar eficazmente la fertilidad de los espermatozoides de burro después de la descongelación.

En la tercera experiencia, puesto que ambos tipos de calostro mostraron buenos resultados en los trabajos anteriores, comparamos el efecto protector del calostro de yegua y de burra en la preservación espermática en la especie asnal, viendo si el calostro de las distintas especies equinas (*Equus equus y Equus asinus*), el cual presenta variaciones en su composición, podría interferir en la calidad post congelación de la especie asnal. El calostro se obtuvo de cuatro yeguas y cuatro burras justo después del nacimiento del potro. Los eyaculados fueron recogidos de cinco burros fértiles. Las muestras de semen se agruparon, diluyeron y criopreservaron en tres grupos experimentales de diluyentes: lactosa suplementada con de yema de huevo (20%) como grupo control, lactosa suplementada con calostro de burra (20%) y lactosa suplementada con calostro de yegua (20%). Después de descongelar, evaluamos la motilidad de los espermatozoides y parámetros de velocidad

mediante el sistema informático ISAS Proiser®, la viabilidad mediante SYBR-14 y PI, la funcional de membrana mediante HOST y la integridad del acrosoma por FITC-PNA/PI. Los resultados obtenidos en las muestras de calostro de burra adicionada a diluyentes para congelación de semen de burro fueron significativamente superiores ($P<0,05$) en casi todas las pruebas evaluadas (Motilidad total, Viabilidad, HOST, y todos los parámetros de velocidad VCL, VSL, VAP, LIN, STR y WOB), en comparación con el calostro de yegua y el diluyente control de yema de huevo después de la descongelación. En conclusión, al comparar calostro de burras y de yeguas en diluyente de congelación de semen de burros, los resultados son significativamente mejores cuando se utiliza calostro de burra, quizás asociado este efecto protector a la distinta composición de ambos calostros.

Los crioprotectores constituyen también un elemento esencial en la protección de los espermatozoides frente al daño celular durante la exposición de los espermatozoides a las bajas temperaturas, siendo necesarios para permitir su supervivencia durante el proceso de congelación-descongelación (Moore y cols., 2006). Se dividen en permeables y no permeables. Los permeables (glicerol, etilenglicol, dimetilsulfóxido, dimetilformamida…) actúan reduciendo la contracción celular durante el enfriamiento. Los no permeables (trealosa, lactosa, sucrosa, glucosa, proteínas y lípidos, como la yema de huevo) tienen acción protectora de la membrana plasmática, ayudando en el proceso de pérdida de agua celular durante el enfriamiento (Hammerstedt y cols., 1990).

A pesar de los avances en técnicas de IA, aún sigue sin establecerse un protocolo “ideal” para realizar el proceso de criopreservación, ya que las principales dificultades no derivan de la permanencia a bajas temperaturas, sino de los procesos de enfriamiento y calentamiento. Las técnicas de criopreservación tradicional están basadas en descensos lentos de la temperatura obteniendo con ello mejores resultados (Salazar Jr y cols., 2011). El semen, una vez disminuida la temperatura requerida, es envasado en pajuelas de congelación de distinta capacidad. Las pajuelas más utilizadas son las de 1 ml y 0,5 ml., pero también se puede guardar en otro tipo de contenedores, como bolsas de plástico hechas de polipropileno, pellets o paquetes de aluminio (McKinnon, 2011).

El semen congelado conlleva que su almacenamiento se realice utilizando temperaturas extremadamente bajas. Para ello se utiliza el nitrógeno líquido (-196ºC), y puesto que la actividad metabólica se considera nula a esta temperatura (Brinsko y cols., 2011), si los espermatozoides son capaces de soportar los protocolos de congelación-descongelación sin ver alterada su integridad, dichos espermatozoide se podrían mantener de manera indefinida.

Nuevas técnicas de conservación seminal investigan la viabilidad de los espermatozoides tras ser sometidos a distintos procesos. Entre estás técnicas novedosas, destacamos la liofilización y la vitrificación, que es una congelación ultrarrápida.

La liofilización es un método de preservación en el que el semen congelado es deshidratado por sublimación de hielo, lo que implica una transición directa de una fase sólida (hielo) a una fase de vapor (gas). El objetivo principal del proceso es eliminar el agua de las células evitando cualquier reacción química o biológica. Presenta ventajas importantes, ya que no precisa de N_2L para su almacenamiento y envío y requiere una conservación a temperatura ambiente o a 4 ° C. Aunque tras su reconstitución por hidratación, los espermatozoides no son capaces de fertilizar por si solos al ovocito, teniendo que recurrir siempre a la inyección intracitoplasmática (ICSI) para que la fecundación tenga éxito. Esta es una limitación importante, dados los equipos que se requieren para llevarla a cabo y la experiencia sobre la técnica de las personas que la desarrollen.

Otro protocolo de preservación es la vitrificación, y desde hace relativamente poco tiempo, se está investigando el efecto sobre los espermatozoides tras ser sometidos a una congelación ultra rápida con exposición directa a N_2L. Este tipo de proceso denominado vitrificación, permite que el agua pase a un estado vítreo como consecuencia de un incremento extremo de la viscosidad produciéndose una solidificación a bajas temperaturas sin la formación de cristales tan perjudiciales para las células (Jiménez-Rabadán y cols., 2015; Slabbert y cols., 2015) como pudiera ocurrir en la congelación tradicional.

Aunque la primera vitrificación data de 1938, fue realizada por Luyet y Hoddap, ésta tuvo un resultado muy pobre y no ha sido hasta las fechas actuales cuando se ha empezado a profundizar en el desarrollo de este método. En la especie humana está más estudiado, pero en animales aún no hay ningún protocolo establecido, especialmente en equinos, donde los estudios son aún más limitados (Pérez-Marín y cols., 2018).

El uso de crioprotectores permeables (glicerol, dimetilformamida, etc.) estaría justificado en esta técnica a fin de deshidratar rápidamente el espermatozoide y prevenir el hielo intracelular, aunque hay que tener en cuenta su elevada toxicidad de cara al éxito de la congelación, por ello es más frecuente el uso de crioprotectores no permeables (trealosa, lactosa o sucrosa) ya que, al actuar como una fuerza osmótica, evitan el daño celular tras la descongelación. No obstante, es común el uso de protocolos que combinan ambos tipos de crioprotectores. Actualmente, también se ha visto que los espermatozoides son capaces de recuperarse sin la adición de crioprotectores (Isachencko y cols., 2019). Los estudios relativos al uso o no de crioprotectores, tipo de crioprotectores añadidos, así como la cantidad necesaria de éstos, están aún en desarrollo. También se está trabajando en líneas de investigación basadas en la determinación de que medios de vitrificación serían los óptimos. De manera general, los mejores resultados se están obteniendo con crioprotectores no permeables tales como la trealosa, lactosa, o sucrosa a concentraciones de 0.15M (Pérez-Marín y cols., 2018), siendo el medio suplementado con albúmina sérica bovina (BSA) en proporción del 1% (Hidalgo y cols., 2018) el que está mostrando ser el más adecuado. Por supuesto, otra parte muy importante del proceso es la desvitrificación, en la que el aumento de la temperatura también puede afectar de manera negativa a la calidad de los espermatozoides (Hidalgo y cols., 2018). Sí bien, todas estas investigaciones continúan en desarrollo ya que son técnicas relativamente nuevas, y los resultados, aunque un poco limitados, son esperanzadores. Por tanto, el papel de la vitrificación de espermatozoides, así como su procedimiento están todavía por aclarar.

Basándonos en esta nueva línea de investigación, nuestra cuarta experiencia, la enfocamos en la vitrificación de espermatozoides equinos, evaluando el uso de crioprotectores no permeables como fueron la lactosa y la trealosa. Además del estudio en muestras de eyaculado, y como trabajo novedoso ya que no se he publicado nada al respecto en la especie equina, estudiamos el resultado de la vitrificación en muestras de origen epididimario recogidas de sementales sacrificados en matadero. Las muestras de semen de eyaculado se recogieron de siete sementales fértiles y las de epidídimo de diez sementales después de su sacrificio en el matadero local de Zaragoza (MZA). Tanto las muestras de eyaculado como las de epidídimo se diluyeron y vitrificaron usando INRA 96® y BSA como medio base, así como trealosa o lactosa como crioprotectores. Como control se

realizó una congelación por el método convencional con muestras epididimarias y de eyaculado. La vitrificación se realizó formando microgotas de esperma depositadas directamente en N2L, formándose pequeñas esferas. Después de descongelar (control) y desvitrificar, se evaluó la motilidad de los espermatozoides y parámetros de velocidad mediante el análisis computerizado ISAS Proiser®, la viabilidad se evaluó utilizando SYBR-14 y PI y la integridad del acrosoma mediante tinción fluorescente utilizando FITC-PNA/PI. En las muestras de epidídimo, la desvitrificación del grupo con trealosa (EPT) y la del grupo de lactosa (EPL) tuvo espermatozoides con mayor motilidad progresiva que en la muestra control (EPC). Después de la desvitrificación la motilidad de las muestras de EPT fue superior ($P<0,001$) a la de EPL (50,72 ± 5,09% vs 34,21 ± 3,02% respectivamente). Tras la desvitrificación de las muestras de eyaculado, los resultados obtenidos fueron peores que los del grupo control ($P<0,001$). En conclusión, la vitrificación con trealosa de espermatozoides obtenidos de epidídimo de sementales, podría ser una alternativa beneficiosa para el almacenamiento a largo plazo de muestras de semen con gran valor económico. Las muestras de eyaculado vitrificadas, sin embargo, tuvieron menores tasas de motilidad y viabilidad que las muestras sometidas a congelación convencional.

Finalmente, en nuestra contribución al libro titulado "*Biotecnologies applied to animal reproduction, current trends and practical applications for reproductive managemen"* en su capítulo 8 "Equine semen preservation: Current and future trends" describimos de manera mas exhaustiva las nuevas técnicas y futura proyección de líneas de investigación para mejorar la calidad seminal en la preservación de semen equino, haciendo una descripción detallada de los diferentes procesos más actuales como son liofilización y vitrificación espermática.

Portada del libro "El Sanador de Caballos" (Gonzalo Giner)

2. Revisión Bibliográfica

Biotechnologies Applied to Animal Reproduction

Current Trends and Practical Applications for Reproductive Management

Editors

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BIOTECHNOLOGIES APPLIED TO ANIMAL REPRODUCTION

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4164 Lakeshore Road
Burlington ON L7L 1A4
Canada

Apple Academic Press Inc.
1265 Goldenrod Circle NE
Palm Bay, Florida 32905
USA

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Exclusive worldwide distribution by CRC Press, a member of Taylor & Francis Group

No claim to original U.S. Government works

International Standard Book Number-13: 978-1-77188-871-4 (Hardcover)

International Standard Book Number-13: 978-0-36781-752-7 (eBook)

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CHAPTER 8

Equine Semen Preservation: Current and Future Trends

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ABSTRACT

Nowadays, stallion semen processing and preservation for artificial insemination (AI) is a widely used tool in modern horse reproduction. During the 20th century, AI had a strong development in most domestic animal species except in equine species, due to economic interests, traditional aspects and mistrust of horse owners. Semen conservation technologies based on temperature decrease to reduce or stop sperm metabolism are necessary to increase the lifespan of sperm and preserving sperm functionality and fertility. Currently, AI is performed mainly with fresh, chilled or frozen semen. Sperm refrigeration at 5ºC has several advantages related to easy handling and shipping, low cost because it does not require special equipment and minor legal requirements for import and export. However, cryopreservation is the only viable method for spermatozoa storage during indefinite periods. The main problem of freezing semen is related to the low fertility rates obtained due to wide inter-individual sperm quality variability; therefore only some stallions produce suitable semen for cryopreservation. To solve these disadvantages, new methodologies have

been studied during recent years with different results. Vitrification and lyophilization are promising stallion semen preservation techniques that require further study to be applied routinely during long-term periods.

8.1 INTRODUCTION

Equine sperm preservation methods have been linked to the development of artificial insemination (AI). The first reports of AI in horses were documented from Arabian texts, in which semen samples were obtained from recently covered mares of rival tribes and then utilized to inseminate their own mares. However, until the end of the 19th century, AI did not obtain promising results in mares due to researches carried out by Ivanow in Russia and Dr. Pearson at Pennsylvania University.

The decrease of equine population after the Second World War and the restrictive regulations regarding the use of AI in several equine breed organizations delayed the progress of this technique in horses. First, studies about stallion semen collection and handling were described by Mckenzie et al. (1939) and Berliner (1942) with suitable sperm quality results. The discovery of glycerol as cryoprotectant agent in 1949 by Polge et al., was the beginning of the development of preservation techniques of biological materials including equine sperm. Next year, Barker and Gandier (1957) obtained the first foal from cryopreserved epidydimal stallion sperm. During the 1970s and 1980s, the utilization of cooled semen on AI programs increased due to the development of a transport container (Equitainer) (Douglas-Hamilton et al., 1984), the use of Kenney extender (Kenney et al., 1975) and the acceptance of reproductive biotechnology by several breeders.

Several advantages show frozen-thawed sperm in comparison to cooled sperm, such as the scheduled use of stallions outside the competition period, easy international transport and the centralized processing of frozen semen by specialized laboratories, which decrease the variability of the seminal quality. Despite the current advances, cryopreserved semen shows some disadvantages, such as low fertility rates, control of the mare cycle, and a high cost along cryopreservation process (Brinsko and Varner, 1992; Samper and Morris, 1998; Loomis and Graham, 2008). Furthermore, it has been estimated that only 30–40% of stallions produce semen that is suitable for cryopreservation, and a large interindividual variation on sperm survival during the freezing and thawing procedures has been also reported (Loomis and Graham, 2008).

Nowadays, AI is the most common biotechnology utilized around the world in the horse industry and it has enabled the development of other technologies, such as sperm sexing, regulation of the estrous cycle, embryo transfer, cloning as well as improvement of semen preservation methods as sperm refrigeration or freezing and, currently, sperm vitrification and lyophilization.

8.2 SEMEN COLLECTION

Semen collection is an essential part of preservation protocols and AI programs. The quality of collected semen depends on numerous factors such as libido, season, age, or breed (Samper, 2000). Semen can be collected using different methods; condom, pharmacological induction, manual manipulation or by the use of artificial vagina, the tool most commonly used (Samper, 2000). There are several models of artificial vagina available commercially. The Colorado model is the most wide-spread at the beginning, but currently the most used is the Missouri model due to the improvements and efficiency.

The ejaculate is a liquid suspension composed of sperm and seminal plasma, which comprised a complex mixture of secretions (fructose, sorbitol, ascorbic acid, lactic acid, citric acid, proteins, enzymes, vitamins, and hormones). Seminal plasma-derived primarily from the epididymis and accessory sex glands of the male. It participates in the final sperm maturation, modifies spermatic membrane surface, besides acts as a vehicle for the ejaculated sperm and protects the spermatozoa during the female reproductive tract transport (Töpfer-Petersen et al., 2000). In stallion, the seminal plasma is normally separated from the semen during the cryopreservation process, since it has proved to be a harmful medium because decrease the percentage of sperm with progressive motility (Pickett et al., 1975; Jasko et al., 1992). The ejaculate should not be exposed to mechanical damage, light, cold, or heat and the equipment in contact with the sperm must be tempered, dry, clean, and free of toxic residues. After ejaculation, the semen will be kept at 30/32ºC before the seminal evaluation and subsequent dilution (Brinsko and Varner, 1992).

Another effective way to recover genetic material in horses is to obtain it from the epididymis from castrated or slaughtered animals. This technique allows obtaining sperm from stallions of high genetic value that have suffered sudden death, major injuries, or castration. Different studies

have demonstrated the efficiency of epididymal sperm recovery (Tiplady et al., 2002, Monteiro et al., 2011). In addition, several researchers suggest that sperm can be harvested immediately after orchiectomy or after 24 h of storage at 4°C–5°C without any difference in terms of viability (Bruemmer et al., 2006; Neild et al., 2006). Different methods have been used to collect epididymal sperm, including percutaneous epididymal sperm aspiration, the flotation method, the retrograde flush technique, or the standard flush technique of epididymis and ductus deferens (Cary et al., 2004; Bruemmer, 2006). This technique allows recovery of a high number of sperms compared with the collection by artificial vagina (Bruemmer, 2006). The motility of epididymal and ejaculated sperm in stallions has been found comparable and no differences were observed in terms of morphological defects and sperm viability (Weiss et al., 2008; Guimarães et al., 2012).

8.3 SEMEN PROCESSING

The semen quality is assessed immediately after collection. Andrological evaluation verifies the reproductive potential of a stallion in the buying and selling process, before the beginning of the reproductive season and before semen preservation or AI. The general parameters used for the analysis of semen quality include total volume, sperm concentration, motility, and normal morphology. Other tests, such as acrosome and membrane integrity, hypo-osmotic swelling test (HOST), mitochondrial activity, or the thermal resistance test can be used for assessment of semen quality (Love et al., 2018). However, some animals with apparently normal semen quality show very poor fertility rates; in these cases, a more exhaustive evaluation is necessary.

After the semen quality evaluation, equine ejaculate is usually centrifuged. In some countries, it is a standard practice prior to preparing sperm doses before AI. Centrifugation increases sperm concentration of the ejaculate and also removes the seminal plasma (Parlevliet and Colenbrander, 1999; Hoogewijs et al., 2010). However, it might generate negative effects on the conservation of equine sperm, decreasing the motility and viability of sperm (Martin et al., 1979), due to the physical damage on the cells and ROS production (Hoogewijs et al., 2010).

Contradictory results have been obtained in relation seminal plasma utilization on sperm preservation. Different studies showed that the removal

of seminal plasma enhanced the survival spermatozoa (Parlevliet and Colenbrander, 1999; Aurich, 2005); however, the presence of a small amount of stallion seminal plasma (0.6–20%) increased sperm motility, plasma membrane integrity, and fertility (Jasko et al., 1992; Moore et al., 2006; Neuhauser et al., 2015). Beneficial effects may be related to antioxidant properties and the inhibitory effect on the binding of polymorphonuclear neutrophils on the female reproductive tract (Knop et al., 2005).

A special extender based on dense isotonic compounds is utilized during centrifugation. The most common mediums are citrate-ethylene acid (EDTA) (Cochran et al., 1984), Tyrode medium (Ljaz and Ducharme, 1995), and glucose-EDTA (Martin et al., 1979). The extender function is to maintain motility and protect the sperm during the centrifugation process and in a dilution ratio of 1:1 or 1:2. The centrifugation influences on equine sperm by the strength and time of centrifugation, the kind of extender, the presence or absence of the extender before centrifugation, and the concentration of seminal plasma (Pickett et al., 1975; Aurich, 2005). Different protocols have been used as shown in (Table 8.1).

TABLE 8.1 Different Stallion Sperm Centrifugation Protocols.

Centrifugation	References
1000g/5 min	Martin et al. (1979)
600g/10 min	Palmer (1984)
400g/12 min	Brinsko et al. (2011)
400g/15 min	Cochran et al. (1984)

8.4 SEMEN PRESERVATION TECHNIQUES

8.4.1 SPERM COOLING

The utilization of fresh semen avoids the damage by thermal shock derived from the conservation techniques at refrigeration or freezing temperatures. Nowadays, sperm quality is preserved just a few hours, therefore, the sperm conservation in refrigeration is the technique more used for semen storage and transportation. The cooling temperature reduces sperm metabolism sufficiently to maintain sperm viability, functionality, and fertility (Gibb and Aitken, 2016) for up to 96 h. Several studies have determined that AI with cooling semen shows a similar fertility rate in comparison with

fresh semen (Jasko et al., 1992; Shore et al., 1998). In addition, the semen preserved in refrigeration at 5°C for 24 h maintains the same fertility rates as fresh semen (Aurich, 2005).

Semen preservation at low temperatures reduces sperm catabolism and cellular injury (Aurich, 2005). The metabolic rate restriction decreases the production of toxic metabolites (hydrogen peroxide, lipid aldehydes, or dioxide carbon), ATP and reactive oxygen species (ROS) (Vishwanath and Shannon, 1997; Gibb and Aitken, 2016). However, the cooling process involves a "cold-shock" that produces damage to the membrane, especially when the cooling rates are higher than 0.3°C/min (Aurich, 2005). This damage is characterized by abnormal patterns of movement, loss of motility during storage, acrosomal membrane damage, as well as a decrease of cellular metabolism due to the loss of enzymes and other intracellular components (Moran et al., 1992).

Several studies show that slow cooling rates (<0.3°C/min) maximize the motility and fertility of equine semen (Douglas-Hamilton et al., 1984; Varner et al., 1987), although equine semen can be refrigerated rapidly from 37ºC to 20ºC. Then, we should reduce to 0.1°C/min, preferably 0.05°C/min from 20°C to 5°C (Kayser et al., 1992). The time required for sperm cooling from 37ºC to 4ºC is between 2 and 4 h (Moran et al., 1992).

The extender most utilized in equine sperm refrigeration storage is INRA96®, a commercial media, made from purified fractions of milk that was developed by Batellier et al. in 1997. In this extender, the β-lactoglobulins and calcium phosphocaseins of milk were replaced by purified calcium phosphocaseins, which improve sperm membrane protection. In addition, it contains sugars and proteins that serve as an energy source and protect from thermal shock sperm, penicillin, and gentamicin to prevent bacterial growth and amphotericin B as a fungicide. The most common cooling extenders utilized are synthesized as given in Table 8.2 (Palmer, 1984; Aurich, 2004). The use of glucose (a source of energy), bicarbonate (buffer), and milk in the stallion cooling medium show the best results in progressive motility, viability, and fertility rates (Aurich, 2004). The inclusion of some antioxidants, such as taurine in INRA 82 and Kenney medium, improves motility and sperm viability (Ljaz and Ducharme, 1995). The addition of glutamine increases the fertility rates but not motility on equine chilled semen (Trimeche et al., 1999). Milk-based medium shows better semen analysis in comparison to egg yolk-based medium since the density of the milk does not interfere with the microscopic manipulations of semen. The

gelatin of some extenders produces plasma membrane stabilization but interfere with microscopic evaluation (Pickett et al.,1975).

TABLE 8.2 Stallion Sperm Chilled Extenders.

Extender	References
Skim-milk-glucose Kenney I	Kenney et al. (1975)
Skim-milk-glucose Kenney II	Kenney et al. (1975)
E-Z Mixin	Province et al. (1985)
INRA 82	Ljaz and Ducharme (1995)
Skim-milk-gelatin	Ljaz and Ducharme (1995)
INRA 96	Batellier et al. (1997)

8.4.2 SPERM CRYOPRESERVATION

Cryopreservation is the only viable method for sperm storage during indefinite periods of time (Gibb and Aitken, 2016). However, freezing-thawing process produces several detrimental effects on the gametes such us loss on sperm viability and motility due to membrane damage caused by the production of ROS, interruption of the membrane functionality as result of separation of lipid bilayers, changes in water transport properties, and alteration in calcium channels (Hammerstedt et al., 1990; Kodoma et al., 1996; Watson, 2000). It also causes damage to the cytoskeleton, in the flagellum, in the acrosome with reduction of the acrosomal integrity, modifications in the head and in the subacrosomal space, in the DNA status, in the genes essential for fertilization and in normal embryonic development (Amann and Pickett, 1987; Valcarce et al., 2013). As a result of these alterations and modifications on sperm, the response to osmotic stress changes and the survival life in the female genital tract might reduce (Flesch and Gadella, 2000). The osmotic stress is due to the extracellular ice crystals development during the cooling process that produces a great increase in the osmolarity of the remaining liquids surround or inside the sperm (Amann and Pickett, 1987).

8.4.2.1 EXTENDERS

The special sensitivity of equine sperm to heat shock and the interest into increase sperm storage time have been possible in the development of

methods to prevent cold-shock damage. Different extenders are added to ejaculate protecting the gametes from the effects of temperature changes. The main characteristics of a cooling medium are neutral pH or slightly acid, buffering power, isotonic osmotic pressure, an energy source for the metabolism of sperm, avoid or minimize the growth of microorganisms, stability against enzymatic degradation or oxidation, and protection against thermal shock using penetrating and nonpenetrating cryoprotectants (Aurich, 2005).

Egg yolk and skim milk are the most common protective nonpenetrating agents utilized against cold shock injury utilized on stallion sperm cryopreservation (Aurich, 2005). Milk is composed of caseins that are the proteins responsible for protecting the sperm during conservation (Lagares et al., 2012) and egg yolk exerts a protective effect due to its phospholipid components (Watson y Martin, 1974). However, several disadvantages are related to the utilization of these compounds, wide variability of composition, microbial contamination, and difficult manipulation (Aires et al., 2003). Different studies have determined other substances that might replace them, soy lecithin, pasteurized egg yolk, liposomes with high-density lipoproteins (LDL/HDL), or cyclodextrins saturated in cholesterol, with suitable results in the sperm samples after thawing (Marco-Jiménez et al., 2004; Papa et al., 2011; Pillet et al., 2011; Blommaert et al., 2016). On the other hand, different sugars have demonstrated the protective action on stallion sperm. Squires (2004) observed that raffinose and trehalose did not increase sperm motility or viability after the frozen-thawing process. However, methylcellulose improved stallion sperm quality.

Permeable cryoprotectants can pass across the membrane and rapidly enter and leave the cell reducing sperm osmotic stress (Alvarenga et al., 2005). Glycerol is one of the most widely used cryoprotectant for the stallion sperm cryopreservation (Gibb and Aitken, 2016). It is trivalent alcohol with low molecular weight and toxic effect because it produces disorders in the cytoplasm, in the permeability and stability of the plasma membrane, and in sperm metabolism (Amann and Pickett, 1987). This molecule passes across the sperm plasma membrane more slowly than other smaller molecular weight cryoprotective molecules, such as amides, dimethylsulfoxide, or ethylene glycol (Alvarenga et al., 2005). Dimethylformamide (or N, N-dimethylformamide, (CH3) 2-N-CHO) is a nonpenetrating cryoprotectant of the group of amides that show the best results in relation to stallion sperm quality after cryopreservation (Gomes et al., 2002;

Alvarenga et al., 2005; Gibb et al., 2013). The amides improve motility, viability, and fertility parameters due to its ability to maintain the structural integrity of the plasma membrane and the organelles (Vidament et al., 2002). Alvarenga et al., (2005) described that the relatively lower viscosity and low molecular weight of the amides increase the permeability of these components across the sperm plasma membrane and decrease the osmotic damage to the equine sperm. The cryoprotective capacity is even more evident when the amides are used in horses that have shown low motility after freezing their semen with glycerol (Medeiros et al., 2002).

Amino acids protect mammalian cells against cold-shock damages. Koskinen et al. (1989) showed that betaine used at 2.5% increased the number of sperm with progressive motility in equine semen. The incorporation a range of 30–80 mM of glutamine and proline in the freezing medium increased motility parameters (Khlifaoui et al., 2005).

One reason related to the decrease of fertility rates during the cryopreservation process is the peroxidation of the lipids in the cell membrane. Lipid peroxidation is the oxidative degradation by ROS of lipids. Semen contains antioxidants that balance lipid peroxidation and prevents excessive formation of lipids peroxides (Griveau et al., 1995). High concentrations of superoxide dismutase have been found in equine seminal plasma (Mennella and Jones, 1980) and Kankofer et al. (2005) determined positive interaction between seminal plasma and milk-based extender increasing the antioxidant capacity. This hypothesis is supported by the fact that the addition of certain antioxidants, such as ascorbic acid (Aurich, 2005) or pyruvate (Bruemmer, 2006), improved sperm plasma membrane integrity, motility, and fertility of equine sperm. Detergents (Equex STM) have been added to the seminal freezing extenders. This compound increases the emulsion and dispersion of the egg yolk lipids and interact with the sperm plasma membrane. The presence of seminal plasma increases sperm resistance to cold-shock and the ability to survive during the freezing and thawing process. The individual differences of the seminal plasma composition could explain the variability between stallions for sperm tolerance to cryopreservation (Ramires Neto et al., 2014).

In the last 25 years, two extenders and their modifications have been most utilized in equine reproduction. Martin et al. (1979) together with Cochran et al. (1984) demonstrated that the lactose-egg-glycerol extender supplemented with EDTA and sodium bicarbonate maintained suitable percentages of motility and viability after thawing equine semen, achieving pregnancy rates of 63%. On the other hand, Palmer et al. (1984) proposed

the alternative skimmed milk-egg yolk-glycerol extender with good sperm quality results after freezing. Currently, there is a wide variety of commercial extenders available that could be used for equine semen cryopreservation. Gent® (MinitubeIberica SL, Tarragona, Spain) and Botucrio® (BiotechBotucatu, Sao Paulo, Brazil) are the most common commercial medium utilized (Macedo et al., 2017). INRA 96® (IMV Technologies, L'Aigle, France) is the most used in chilled doses, with the possibility of adding different permeable cryoprotectants agents for freezing (Álvarez et al., 2014).

8.4.2.2 CRYOPRESERVATION PROTOCOL

The first step in the cryopreservation protocol is the centrifugation of the semen sample in order to increase the concentration of semen and reduce the amount of seminal plasma (Hoogewijs et al., 2010). Previously, the ejaculate is usually diluted in semen/media ratio of 1:1 (Pickett et al, 1975; Martin et al. 1979; Cochran et al, 1984).

In relation to sperm concentration, Loomis et al. (1983) recommended that high concentrations are preferable, on the contrary to Heitland et al. (1995). Samper and Morris (1998) suggested that freezing at low sperm concentrations could provide high availability of nutrients and cryoprotectants per sperm. This fact would explain the high percentages of motile spermatozoa immediately after thawing when frozen at a concentration of $40x10^6$ sperm/mL. Standard sperm concentrations ranges between $50/200x10^6$ sperm/mL in equine semen. Several storage devices have been used, including vials or glass ampoules, straws of polypropylene, polyvinyl or plastic (generally with volumes between 0.5 and 1 cm^3), macrotubes, containers of aluminum, pellets and plastic bags (Martin et al., 1979; Cochran et al., 1984). Nowadays, straws of 0.5 cm^3 are the devices most utilized worldwide (Fig. 8.1).

Stallion sperm can be successfully frozen when the temperature drop ranges between 5ºC and 45ºC per min (Moore et al., 2006). In a study carried out by Salazar et al. (2011), he determined that a slow cooling curve prior to freezing improved the results with respect to rapid cooling.

Cryopreserved semen involves storage at extremely low temperatures in liquid nitrogen (–196ºC). If the sperms are able to withstand the freeze-thaw cycles, sperm viability could be maintained indefinitely since the metabolic activity is considered null at this temperature (Brinsko et al., 2011).

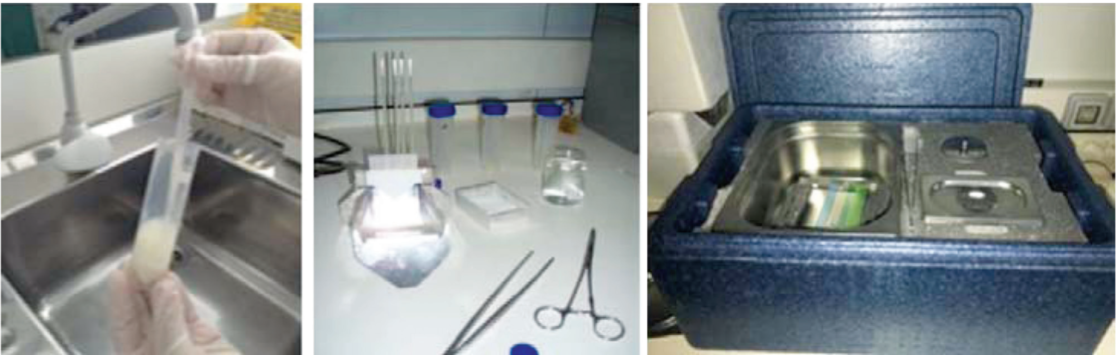


FIGURE 8.1 Steps and schematic design for sperm cryopreservation.

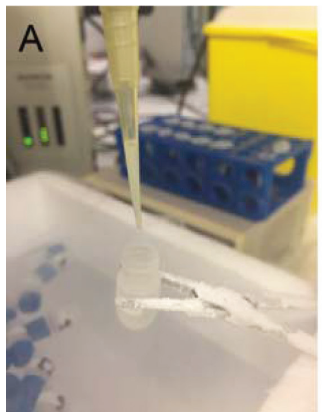
The success of freezing processes is also limited by the thawing process. In general, if the cooling rate is fast, the thawing should also be fast. Alternatively, if the cooling rate is slow, the process must be slow (Amann and Pickett, 1987). There are different thawing protocols, depending on the size of the container. If the sperms are frozen in macrotubes, they are normally thawed in the water at 50°C for 40 s (Martin et al., 1979). There are two protocols available in 0.5 mL straws: 37°C in water bath for 30 s (Cochran et al., 1984; Loomis et al., 1983) or 75°C for 7 s and immediately in another water bath at 37°C for 5 s (Cochran et al., 1984).

8.4.3 SPERM VITRIFICATION

In the last years, long-storage alternatives sperm preservation methods simplest and cost-effective have been proposed in several species. One of them is sperm vitrification. This method preserves the spermatozoa in a hypertonic medium by plunging the cells directly into liquid nitrogen (Rall y Fahy, 1985). The advantages of vitrification are related to the low cost of equipment, simplicity, and low exposure time at low temperatures. However, it produces adverse effects due to the use of hypertonic solutions and high concentrations of cryoprotectants (Lopera et al., 2007).

Vitrification is an ultrafast freezing method (from 37ºC to–196ºC in less than a second), based on the solidification of spermatozoa without the formation of ice crystals (Isachenko et al., 2004; Pradiee et al., 2015). This technique requires high concentrations of permeable cryoprotectants (Isachenko et al., 2008) that increase the viscosity of the medium and prevent the formation of intracellular ice during cooling (Rall and

Fahy, 1985). It is the election method for preservation oocytes, embryos, and other tissues that require rapid cooling rates (Rall and Fahy, 1985). However, the results obtained on sperm preservation are not satisfactory when compared with the results observed in oocytes or embryos applying the same protocols, due to the lack of tolerance of spermatozoa to the high concentration of cryoprotectants (Oldenhof et al., 2017). The kinetic of vitrification has been studied in order to improve vitrification results. It consists of the generation of small volumes of sperm solutions without any permeable cryoprotectant in LN2 (Isachenko et al., 2008), avoiding the formation of intracellular ice (Isachenko et al., 2004). Kinetic vitrification for sperm processing is different compared with the conventional term for vitrification associated with oocytes and embryos, where both the intracellular environment and the extracellular environment must be vitrified (Pradiee et al., 2015) (Fig. 8.2). There is no single protocol established, depending on of sensitivity of the sperm, extenders, cryoprotectants, and devitrification methods (Table 8.3).



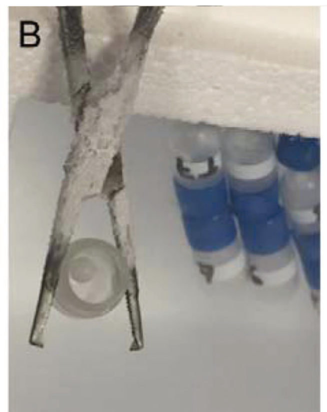


FIGURE 8.2 Stallion sperm vitrification process in cryotubes (A, B) and freeze-dried stallion sperm (C).

TABLE 8.3 Summary of the Principal Vitrification Protocols on Stallion Sperm.

Extender	Volume	Cryoprotectant	Desvitrification	References
Equipro	1.8 mL	8% egg yolk+DMF	37°C 5 min	Restrepo et al. (2012)
INRA 96+BSA	25 µL	Sacarose and trehalose	65°C 5 s and 37°3 min	Pérez-Marín et al. (2018)
INRA 96+BSA	30 µL	Sucrose	42°C 5 s	Hidalgo et al. (2018)
INRA 96+BSA	0.25 mL	Sucrose	43°C 5 s	Consuegra et al. (2018)

Perez-Marín et al. (2018) obtained better results in total and progressive motility with fresh and frozen ejaculate semen than vitrificated sperm with trehalose and sucrose (0.15 M). Another study conducted by Restrepo et al. (2012) found superior results of progressive motility with fresh semen and frozen semen than vitrificated semen. However, other studies determined better motility results when included sucrose and BSA in the vitrification medium with an equilibration period than the traditional freezing process (Consuegra et al., 2018; Hidalgo et al., 2018).

8.4.4 SPERM LYOPHILIZATION

Lyophilization is a preservation method in which frozen material is dried by sublimation of ice, thereby, involving a direct transition from a solid (ice) to a vapor (gas) phase. The main objective of the process is to remove water from the cells avoiding any chemical or biological reaction. It consists of three phases: sample freezing, sublimation (primary drying), and desorption (secondary drying) (Jennings, 2002).

The first attempts to lyophilize spermatozoa were made in rooster semen in 1949 (Polge et al., 1949) and then in human and bovine semen (Sherman, 1954; Bialy and Smith, 1957). Any of these studies did not demonstrate the fertility of freeze-dried sperm. In 1998, Wakayama and Yanagimachi reported the first offspring obtained from freeze-dried mouse sperm. This study demonstrated that although lyophilized sperm were dead, the DNA did not lose their ability to activate the oocyte with the subsequent embryonic development (Kusakabe et al., 2001).

One of the main advantages of this method is that nitrogen is no longer required for the storage and shipment, which can be stored at room temperature or 4°C. The storage and shipping costs are reduced. However, freeze-dried sperm lose their motility, even after rehydration (Kusakabe et al., 2001), therefore sperm are unable to fertilize oocytes by themselves in vivo as by in vitro (Fig. 1.2). It is necessary to use intracytoplasmic sperm injection (ICSI) to perform successful fertilization.

Several factors influence the lyophilization process and produce different sperm damage. One of them is the drying conditions during the lyophilization process (Hara et al., 2014). The interaction between temperature, vacuum pressure, and drying period regulates the kinetics and the degree of dehydration, which have a great impact on the sperm (Kawase et al., 2007). Lyophilized sperm preservation for a long period of time is essential

to protect DNA from physical damage caused by the action of endogenous endonucleases during storage (Kaneko and Serikawa, 2012). Chelating agents, such as EDTA, are necessary. 1,2,2-diamino 2,2', 2'', 2''-tetraacetic) or ethylene glycol-bis (2-aminoethyl ether)-N, N, N'N'-tetraacetic acid (EGTA) are incorporated in the lyophilization solution to prevent sperm DNA fragmentation (Kaneko and Nakagata, 2006). Olaciregui et al., (2016) determined that the presence of EGTA in the lyophilization media provided a protective effect on the DNA sperm than the presence of EDTA. Sugars have been studied as components of the lyophilization medium. Trehalose, a nonreducing disaccharide, plays an important role in the prevention of membrane alterations in cellular dehydration. The incorporation of trehalose to the lyophilization medium maintains the integrity of the DNA after the lyophilization process (McGinnis et al., 2005).

The pH of the lyophilization media is a factor to be taken into account. An alkaline pH (pH:8) contributes to the inactivation of DNA damage. Kaneko and Serikawa (2012) showed that lyophilized sperm in a medium with an alkaline pH maintained the DNA integrity as well as their capacity for embryonic development. Another factor that may influence sperm preservation is the conditions of conservation. Currently, it is considered that 4ºC is the optimum temperature to preserve lyophilized semen samples for long periods of time (Kaneko and Serikawa, 2012).

The rehydration process restores the lyophilized cell to the original formulation. It is a critical step in the process since when reintroducing water into the cells, all the chemical and biological reactions are reactive. The lyophilized sperm are normally rehydrated by adding ultra-pure water, in an equal volume as the original (Gil et al., 2014). Finally, another important factor is the technique of ICSI. After the lyophilization process, the sperm lose motility, so the ICSI technique is necessary to fertilize the oocytes. The development of the ICSI is the key to success in obtaining offspring from lyophilized semen, as published for the first time by Wakayama and Yanagimachi (1998) and Choi et al. (2011) because they obtained the first foal born alive.

In small and farm animal species, satisfactory results have not been obtained, except in the equine species (Choi et al., 2011). In this case, lyophilized stallion semen and sperm extract suspension were utilized from different males. Five pregnancies were obtained an only two foals born. One of them was originated from lyophilized sperm and the other was from the stallion that provides the sperm extract. The lyophilized sperm

and sperm processed by multiple unprotected freezing and thawing cycles (such as sperm extract) can originate viable foals. To our knowledge, this is the first report on the production of live offspring by ICSI with lyophilized sperm in a nonlaboratory animal species.

Lyophilization is a promising semen preservation technique that requires further study to be applied routinely during long-term conservation periods. This technique would be a suitable alternative biobanking option for high genetic values animals.

KEYWORDS

- **stallion**
- **semen preservation**
- **cryopreservation**
- **vitrification**
- **lyophilization**

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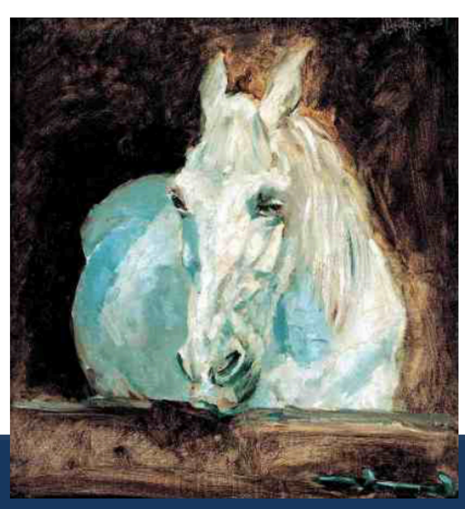


Caballo Blanco (Toulouse-Lautrec)

3. Objetivos

3. Objetivos

En el presente trabajo, los objetivos que nos planteamos se encuentran estrictamente relacionados con la investigación de nuevos componentes en los diluyentes de preservación o con el empleo de nuevas técnicas que permitan mejorar la calidad seminal en la especie equina tras su almacenamiento en N_2L y con ello aumentar los índices de preñez.

Los objetivos son los siguientes:

- Valorar el efecto protector de la adición de calostro de yegua en los diluyentes para la congelación de semen equino (*Equus ferus caballus)* **(Articulo 1)**.
- Valorar el efecto protector de la adición de calostro de burra en los diluyentes para la congelación de semen asnal *(Equus asinus*) **(Artículo 2)**.
- Comparar el efecto protector del calostro de yegua y de burra en la preservación espermática en la especie asnal **(Artículo 3)**.
- Evaluar del uso de crioprotectores permeables, lactosa y trealosa, en la vitrificación de espermatozoides procedentes tanto de origen epididimario como de eyaculado **(Artículo 4)**.

Carrera de caballos (Taylor)

4. Material y métodos

4.1. ANIMALES

Todos los procedimientos experimentales de esta Tesis Doctoral se realizaron de acuerdo con lo establecido en el RD 1201/05 sobre la Ley de Protección Animal, que cumple con la Directiva de la Unión Europea 2010/63/EU de protección de animales utilizados con fines experimentales y otros fines científicos (*ver informe de la comisión ética asesora)*.

4.2. RECOGIDA DE CALOSTRO

El calostro se recogió de 4 yeguas y 4 burras de distintas razas y edades comprendidas entre los 5 y 12 años, procedentes del Centro Militar de Cría Caballar de Zaragoza (CMCCZ), antes de las 8h posparto, mediante ordeño manual y posterior filtrado para eliminar impurezas (Cash, 1999). Previa a la extracción, se limpió la ubre con agua tibia para retirar restos de suciedad e impurezas que pudiesen afectar posteriormente a la calidad.

Se evitó la recogida de yeguas que pudiesen producir calostro de calidad subóptima y/o con posibles alteraciones (yeguas/burras mayores, con enfermedades recurrentes o goteo continuo de calostro o leche antes del parto) (LeBlanc y cols., 1992). Además, a las hembras seleccionadas se les hizo un estudio ecográfico para descartar posibles patologías (mastitis, hematomas, neoplasias, abscesos, lesiones mucosas, inflamaciones o malformaciones congénitas, etc.) (Abshenas y cols., 2014; McKinnon y cols., 2011).

Para el estudio ecográfico, se utilizó un ecógrafo modelo Mindray DP-3300 VET® (*Figura 2*) con sonda lineal y frecuencia de 7,5 MHz. La ubre se limpió de restos de suciedad con agua tibia y se aplicó alcohol en la piel con el objeto de obtener una excelente calidad de imagen (*Figura 3*).

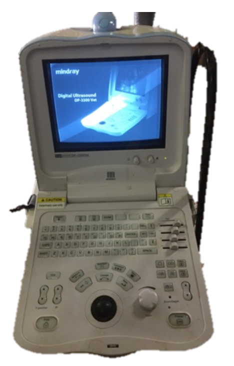


Figura 2. Ecógrafo Mindray DP-3300 VET®

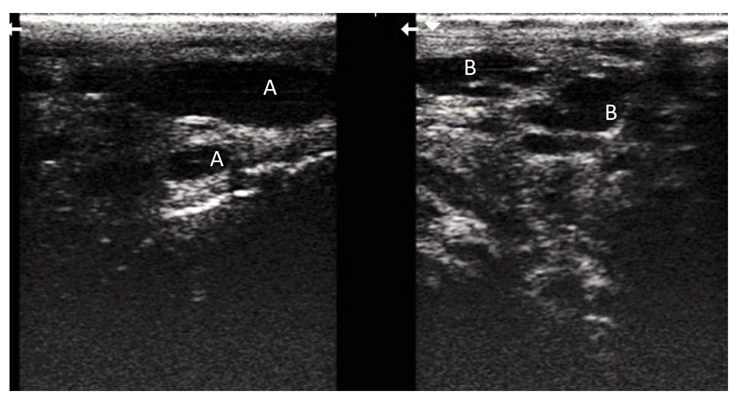


Figura 3. Patrón ecográfico de glándula mamaria normal. A) Cisterna del pezón. B) Conductos lactíferos.

La determinación de la calidad de todas las muestras de calostro para los distintos estudios se realizó mediante un refractómetro de Brix (Bellingham y Stanley, Kent, UK) (*Figura 4*), y sólo las muestras con valores superiores o iguales a 23ºBrix (23 ºBrix >60 g/L IgG), (Cash, 1999) fueron aceptadas y posteriormente identificadas y congeladas en alícuotas de 15 ml (Cash, 1999; Chavatte y cols., 1998; McKinnon y cols., 2011).

La descongelación del calostro se realizó con agua tibia a 38 ºC.

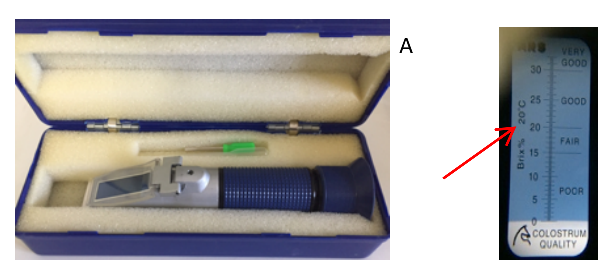


Figura 4. A) Refractómetro de Brix para determinación de la calidad del calostro. B) Escala de valores (en grados Brix).

4.3. RECOGIDA DE MUESTRAS SEMINALES

4.3.1. Recogida de muestras de eyaculado

Para la realización de nuestros estudios, se recogió semen de 12 caballos y 5 burros procedentes del CMCCZ, de distintas edades y en época reproductiva. Sólo se aceptaron eyaculados cuya calidad tenía como mínimo una motilidad progresiva ≥35% y >40% de semen morfológicamente normal ("buenos congeladores") (Alvarenga y cols., 2016; Batellier y cols., 2001; Loomis y Graham, 2008). Para la recolección de semen tanto en caballos como en burros utilizamos la VA modelo Missouri® (*Figura 5*). La presión y temperatura usadas en ella para

cada extracción fueron variables dependiendo del semental. Un manejo frecuente de todos los sementales nos indica cuales son las que mejor se adaptan a cada uno de ellos.

A

B

Figura 5. A) VA modelo Missouri empleada para extracción de semen. Camisa interna desechable de color azul. B) Biberón graduado, con filtro desechable usado como colector, colocado en el extremo final de la VA.

Para la colecta de los eyaculados utilizamos un maniquí regulable en altura y forrado de cuero para evitar que el caballo se pudiese deslizar, el suelo también era antideslizante para favorecer así que el caballo pudiese hacer fuerza con las extremidades posteriores sin resbalarse. Utilizamos para todos los sementales una yegua en celo para estimularles, que se colocó en el potro de contención (*Figura 6*), si bien, algunos sementales están tan entrenados que pueden prescindir de ella. En caso de los burros el manejo fue algo diferente, debido a que la líbido es mucho menor y el tiempo de excitación se alarga bastante. En estos casos, la burra en celo no se introdujo en el potro de contención, sino que fue sujetada por un operario. Además, se utilizó orina de burras en celo, que se fue recogiendo y congelando en diferentes alícuotas ya que es frecuente no disponer de burras activas en el momento de la extracción. Con la burra en celo o con el olor de las feromonas de la orina recogida, los burros se estimularon más rápidamente para la recogida seminal.

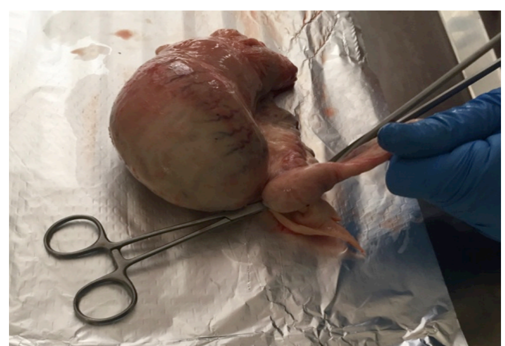
Previamente, todos los sementales se les limpió el pene retrayendo el prepucio para dejar el glande al exterior con agua tibia y posterior secado, con el objeto de evitar suciedad en los eyaculados. Una vez recogido el eyaculado, inmediatamente después se llevó al laboratorio para proceder a su primera evaluación (*in situ*), retirando el filtro del colector (biberón graduado) para eliminar la fracción de gel, y se depositó en un baño María para mantener una temperatura (tª) constante de 37 ºC en todo momento hasta su procesado. Después de evaluarse las muestras, fueron diluidas en INRA 96® (IMV Technologies, L'Aigle, Francia) (dilución 1:1) y se transportaron en envases

individualizados a tª ambiente y protegidos de la luz en cajas de neopor (Minitub Ibérica S.L., Tarragona, España).

Figura 6. Maniquí para extracción de semen y potro de contención de madera para las yeguas. El maniquí es regulable en altura. El pavimento debe ser antideslizante para evitar caídas y lesiones.

4.3.2. Recogida de semen de epidídimo

La técnica utilizada en nuestro trabajo para obtención de espermatozoides fue la de lavado retrógrado empleada por Garde y cols. (1994). Para ello se recogieron 10 testículos en el matadero de Mercazaragoza (MZA) y se transportaron en cajas de poliestireno isotérmicas, en refrigeración (4 ºC) a la FVZ. En el laboratorio, se procedió a diseccionar de cada testículo la cola del epidídimo con su conducto deferente proximal, se canalizó este por la parte seccionada del conducto con una aguja 25G, utilizando unas pinzas mosquito para que la sujeción fuese más sólida. A la aguja, con extremo romo, se le acopló una jeringuilla con 10 ml de INRA 96® a 20ºC como medio de lavado, con la que se ejerció presión para recoger el semen en un vaso de precipitados (*Figura 7*).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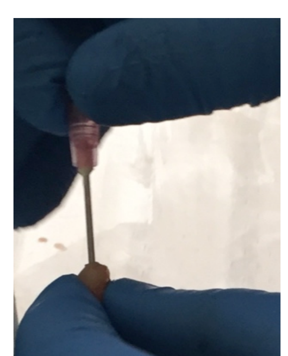
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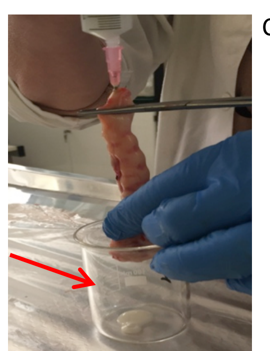
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Figura 7. A) Disección de cola de epidídimo con conducto deferente. B) Canulación. C) Introducción de INRA 96® a presión y recogida de semen en vaso de precipitados.

4.4. PROCESADO DE LAS MUESTRAS DE SEMEN PARA CONGELACION

4.4.1. Centrifugación

Para la centrifugación empleamos INRA 96®. Se hizo una dilución del eyaculado: diluyente de 1:1. Realizamos la centrifugación a 1000 *g* durante 5 min (Alvarenga y cols., 2016; Brinsko y cols., 2011). Tras la centrifugación retiramos la mayor parte del sobrenadante, dejando un 5% del mismo para facilitar la reconstitución del pellet (Lozano y cols., 2011).

4.4.2. Preparación de las muestras seminales

Como medio de dilución para nuestros experimentos se utilizó el Medio Cochran: diluyente a base de lactosa (Cochran y cols., 1984) con 50% (v / v) de lactosa 290 mM, 25% (v / v) de medio de glucosa EDTA (322.20 mM de glucosa, 12.58 mM de citrato de sodio , 9.93 mM de EDTA disódico y 14.28 mM de bicarbonato de sodio).

Al medio Cochran se le añadió el 20% de Calostro (de yegua o de burra), y 5% de un crioprotector permeable (glicerol o DMF) dependiendo del estudio.

Como grupos control para nuestros trabajos, se utilizó el Medio Cochran con 20% de yema de huevo y 5% de glicerol o DFM.

Otro medio de control que se utilizó fue BotuCrio® (NidaCon International AB, Mölndal, Suecia), compuesto por agua ultra-pura, aminoácidos, carbohidratos, glicerol, amidas, gentamicina (0,5 g/L), penicilina (1,0 g/L) y yema de huevo (*Tabla 6)*.

4.4.3. Protocolo de congelación

El protocolo de congelación de las muestras se realizó mediante un descenso lento de la tª con una disminución progresiva durante 2 h hasta alcanzar los 4ºC, evitando con ello el shock térmico. Transcurridas las 2 h, las muestras se introdujeron en pajuelas de congelación de 0,5 ml, con una concentración de 100 x 10^6 espermatozoides/ml (Samper, 2009). Las pajuelas fueron sometidas a vapores de nitrógeno durante 20 min. para, posteriormente pasar

a sumergirse en N_2L (-196ºC). La congelación se realizó en una unidad de congelación de semen (Minitub Ibérica S.L., Tarragona, España).

En el caso de las muestras de BotuCrio®, el protocolo de congelación se realizó según indicaciones de la casa comercial. Las muestras se mantuvieron a 4ºC durante 20 min antes de proceder a someter las muestras a vapores de nitrógeno para su congelación Todas las muestras permanecieron un mínimo de 15 días en los tanques de nitrógeno.

4.4.4. Protocolo de descongelación

Todas las muestras se descongelaron en baño María a 37ºC durante 21 seg, Tras secado de las pajuelas, y apertura de las mismas, las muestras se depositaron en un eppendorf con un volumen de 1.5 ml y se mantuvieron a 37ºC mientras se procedía a las diferentes evaluaciones espermáticas. La primera se realizaba tras la descongelación (5 minutos) y para el Test de Resistencia se realizaba a las 2 horas.

Tabla 6. Composición de los distintos medios de dilución utilizados en nuestros experimentos.

DILUYENTE	Compuestos con efecto protector añadidos al diluyente	CRIOPROTECTOR	TIPO DE CRIOPROTECTOR	METODO
BotuCrio®	--	G + Amidas	Permeables	CONGELACION
Medio Cochran	Calostro de yegua (20%) Calostro de burra (20%) Yema de Huevo (20%)	G (5%)	Permeable	CONGELACION
Medio Cochran	Calostro de yegua (20%) Calostro de burra (20%) Yema de Huevo (20%)	DMF (5%)	Permeable	CONGELACION
INRA 96 ® +BSA (1%)	--	Sacarosa 0,15 M	No permeables	VITRIFICACION
INRA 96® +BSA (1%)	--	Lactosa 0,15 M	No permeable	VITRIFICACION

4.5. PROCESADO DE LAS MUESTRAS DE SEMEN PARA VITRIFICACION

4.5.1. Protocolo de Vitrificación

La vitrificación se llevó a cabo tanto para espermatozoides procedentes de epidídimo como de eyaculados. A su vez, en ambos tipos de espermatozoides, se vitrificó con dos medios diferentes, utilizando dos crioprotectores no permeables: trealosa y lactosa, ambos a una concentración 0,15M. La concentración de estos dos crioprotectores se escogió en base a estudios previos realizados por Perez-Marín y cols. (2018). El diluyente base para vitrificación de semen fue el INRA 96®, al que se le añadió un 1% de albúmina sérica bovina (BSA), según el protocolo modificado de Pérez-Marín y cols., 2018 (*Tabla 6)* y de investigaciones previas realizadas por Hidalgo y cols. (2018). Los grupos experimentales para el estudio fueron:

- Grupos de semen de epidídimo: con trealosa (EPT) y lactosa (EPL).
- Grupos de semen de eyaculado: con trealosa (EYT) y lactosa (EYL).

Las muestras seminales en todos los casos y al igual que en el caso de la congelación, fueron sometidas al mismo tipo de centrifugación, y tras la misma, sobre el pellet se adicionaron los diluyentes de lactosa y trealosa, ajustando la concentración espermática a 50 x 10^6 spmtz/ml.

Para realizar la vitrificación y tras preparación de las muestras, se tomaron 50 µl, usando una micropipeta, de las muestras preparada de cada grupo experimental y se dejaron caer en un crio-tubo que contenía 300 µl de N_2L (en cada crio-tubo se depositaron 3 gotas que, al contacto con el N_2L, se esferificaron) (*Figura 8*). Posteriormente se cerraron y se mantuvieron en tanques de N_2L hasta su análisis.

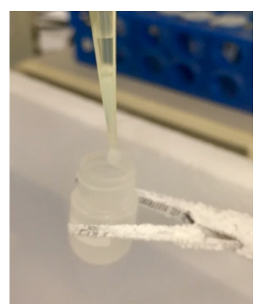
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Figura 8. A) Depósito de 1 gota de 50ml en un crio-tubo con N_2L. B) Esferas de vitrificación contenida en un crio-tubo.

4.5.2. Protocolo de desvitrificación de muestras seminales

Para la desvitrificación: las esferas vitrificadas se sumergieron en 500 µl de medio de desvitrificación (INRA 96® + 1% BSA) a 65ºC durante 5 seg, a continuación, se mantuvieron a 37ºC, 3 min, luego se centrifugaron a 300*g* durante 10 min, y el sedimento final se resuspendió con 50µl de medio de desvitrificación para la evaluación de la calidad seminal (*Figura* 9).

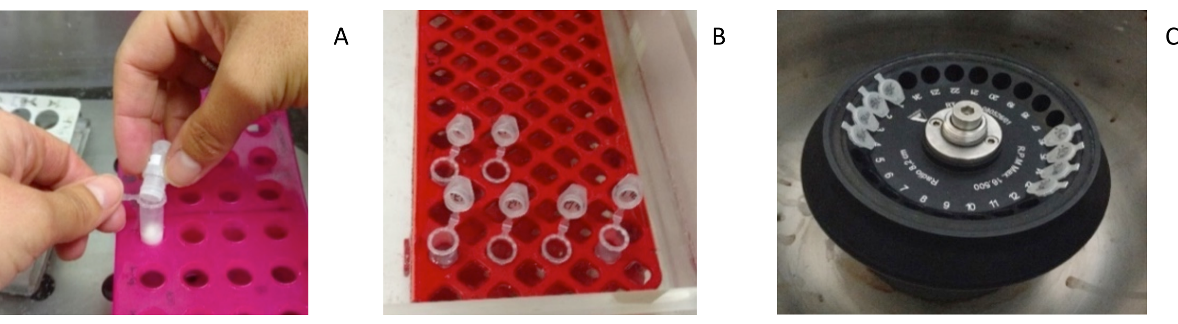


Figura 9. A) Inmersión de la perla a 65ºC. B) Calentamiento en baño a 37ºC. C) Centrifugación.

4.6. EVALUACION DE LA CALIDAD SEMINAL

4.6.1. Evaluación in situ

La primera evaluación se realizó inmediatamente tras la recogida, *in situ*, en el laboratorio del CMCCZ, antes de su traslado a la Unidad de Reproducción de la FVZ. En todas las evaluaciones seminales, los primeros parámetros que se registraron y analizaron fueron: volumen, concentración y motilidad.

Evaluación Macroscópica:

Se evaluaron el volumen y el color. El volumen se determinó por la lectura en el colector graduado. Los valores medios de los volúmenes de eyaculado obtenidos se encontraron entre 30-60 ml. El color, analizado de forma visual, fue blanco lechoso en los sementales y algo más grisáceo en los burros.

Evaluación Microscópica:

A. Concentración: se evaluó mediante un espectrofotómetro denominado Spermacue® (*Figura 10*). Para ello se calibró primero con una micro-cubeta vacía. Una

vez calibrado, se depositó semen con una pipeta en la micro-cubeta, que se absorbió por capilaridad, y se colocó en la rejilla del Spermacue®. Los valores dados por el Spermacue® aparecen directamente como número de partículas contadas por millón en un mililitro de volumen. Los resultados medios para todas las muestras estuvieron comprendidos entre 100-250 x10^6 spmtz/ml.

Figura 10. Spermacue®. Espectrofotómetro utilizado para recuento celular. Resultado = nº x 10^6 spmtz/ml.

B. Motilidad: Se evaluó con un microscopio Olympus C-40 con base termostatizada a 37ºC. Se depositaron 5µl de semen en un portaobjetos previamente calentado en una pletina a 37ºC, se colocó un cubreobjetos atemperado y se hizo una valoración inmediata. La valoración de esta motilidad es subjetiva, si bien, da una idea bastante aproximada de la calidad de la motilidad de la muestra de semen.

Por otro lado, y de forma independiente, también valoramos la líbido del semental, ya que está relacionada con la calidad seminal (Brinsko y cols., 2011). La mayoría de los caballos mostraron una líbido muy buena. Los burros, dentro de las características de su especie, mostraron también buena líbido.

4.6.2. Evaluación de la calidad seminal pre-congelación y tras la descongelación/desvitrificación de muestras

A. *Motilidad:* Todas las muestras procedentes del CMCCZ y de los epidídimos procedentes de los testículos obtenidos en matadero (Mercazaragoza) fueron analizadas mediante el sistema computerizado "Integrates Semen Analysis System-Proiser" (ISAS®) (Valencia, España) antes de ser sometidas a congelación/vitrificación y una vez descongeladas/desvitrificadas (*Figura 11*). Se determinó la motilidad total, motilidad

progresiva, parámetros de velocidad (velocidad curvilínea: VCL; velocidad rectilínea: VSL; velocidad promedio: VAP; índice de linealidad: LIN; índice de rectitud: STR; índice de oscilación: WOB), y la amplitud media de desplazamiento (amplitud lateral de la cabeza: ALH y frecuencia de batida: BCF).

El área de las partículas analizadas se fijó entre 4-75 μm^2, los espermatozoides con VAP <10 µ / seg se consideraron como inmóviles, > 45 µm/seg como normales y > 90 µm/seg como rápidos. El valor mínimo de ALH fue de 10 imágenes.

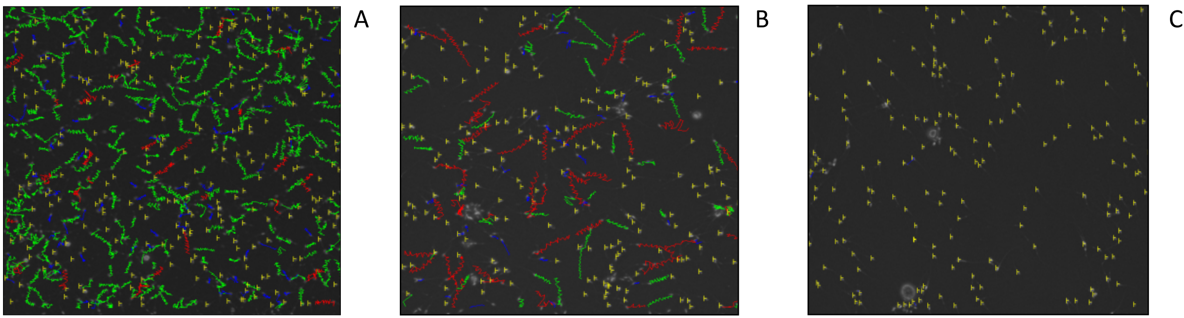


Figura 11. Representación de la motilidad total y progresiva mediante el sistema computerizado ISAS. A) Alto porcentaje de espermatozoides móviles y con buena motilidad progresiva. B) Motilidad buena. C) Espermatozoides inmóviles. Siendo los colores representados: rojo rápidos, verde medios, azul lentos y amarillo estáticos.

B. Vitalidad:

B.1. *Tinción Eosina-nigrosina* (Colembrander y cols., 1992). Para realizar la tinción se colocó en la base de un portaobjetos una gota del colorante (mantenido de forma atemperada en platina térmica, para evitar que el shock térmico pudiese originar datos falsos), se incorporó una gota de semen (5µl) y se mezcló. Con un cubreobjetos colocado con un ángulo de 45º se hizo una extensión y se dejó secar para observarlo posteriormente con el microscopio de campo claro con objetivo 40x. Los espermatozoides dañados aparecieron de color rosáceo al dejar penetrar el colorante, los vivos, al tener la membrana plasmática intacta, no dejaron pasar el colorante, apareciendo sin colorear, pero bordeados en color negro por la nigrosina. Se contaron 100 espermatozoides por muestra (*Figura 12.A*).

B.2. Kit de Viabilidad Espermática LIVE/DEAD® (Molecular Probes Europe, Leiden, Países Bajos). Es un método de tinción a base de SYBR-14 y Ioduro de propidio (PI).

El SYBR-14 es un colorante que penetra en la membrana del espermatozoide, aumentando su tasa de emisión de fluorescencia al acoplarse enérgicamente a los ácidos nucleicos de su ADN. El PI se usa de forma convencional para teñir células muertas. Para su realización se mezcló un volumen de 100 µl de muestra con 150 µl de tampón (Becton Dickinson Inmunochemistry, San José, CA, EE. UU.) para llegar a una concentración final de $20x10^6$ spmtz /ml. A continuación, se agregaron 5 µl de SYBR-14 (concentración final 20 mM) y 5 µl de PI (10 mM) por muestra. Después de incubarse en oscuridad durante 15 min a tª ambiente, se calculó el porcentaje de células espermáticas vivas y muertas. La tinción SYBR-14 penetra tanto las membranas de los espermatozoides intactos como las dañadas, y da fluorescencia verde/amarilla, mientras que el PI solo penetrará si la membrana está dañada. predominando entonces sobre la fluorescencia del SYBR-14 y tiñendo el espermatozoide de color rojo (fluorescencia roja) (Love y cols., 2003; Love, 2018). Las muestras se analizaron usando un microscopio de fluorescencia con contraste de fases (*Figura 12.B*).

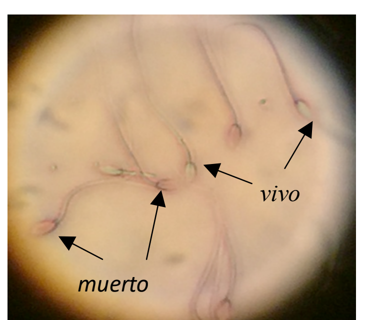


Figura 12. A) Tinción de eosina-nigrosina para determinación de espermatozoides vivos. Los rosas no presentan integridad de membrana (muertos). Absorben colorante. B) Tinción de fluorescencia SYBR-14/PI. Vivos: Fluorescencia verde. Muertos: Fluorescencia roja

C. <u>Integridad de membrana</u>: Test Hipoosmótico (HOST): Para valorar la integridad de membrana de los espermatozoides, se incubaron 100µl de semen durante 30 min a 37ºC en 900 µl de una solución de lactosa 100 mOsm (18 g en 500ml de agua destilada desionizada). Tras la incubación, las muestras se fijaron en una solución buffer al 8% de glutaraldehido. Se evaluaron 100 espermatozoides bajo el microscopio con el objetivo 100x. Los que mostraban colas enrolladas se consideraban como HOST positivos (*Figura 13*) (Lagares y cols., 2000; Neild et al., 1999).

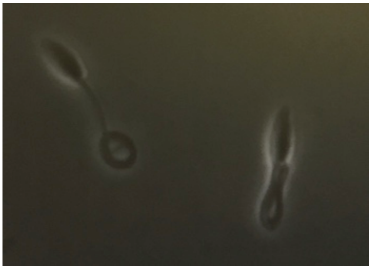


Figura 13. Swellig o hinchamiento. Los dos espermatozoides han reaccionado al HOS test presentando colas enrolladas.

D. <u>Integridad del acrosoma</u>:

D.1. Técnica DIC (Differential Interference Contrast microscopy) (Steinholt y cols., 1991). Para ello en un tubo eppendorf se colocaron 10 µl de semen en 90 µl de glutaraldehido para fijar la muestra. Se homogeneizó con cuidado y se dejó reposar durante 30 min. Para su análisis se depositó una gota de muestra sobre un portaobjetos y se cubrió con un cubreobjetos para poderlo observar directamente al microscopio con el objetivo de inmersión 100x. La porción acrosómica del espermatozoide normal se observa tintada de un color más oscuro que el resto de la célula y en forma de semiluna. Se contaron un total de 100 espermatozoides, valorándose el porcentaje de los acrosomas positivo o íntegros (zona acrosómica oscura) y de los acrosomas negativos (sin zona oscura, o con un halo difuso en la zona apical de la cabeza del espermatozoide) (*Figura 14.A y B*).

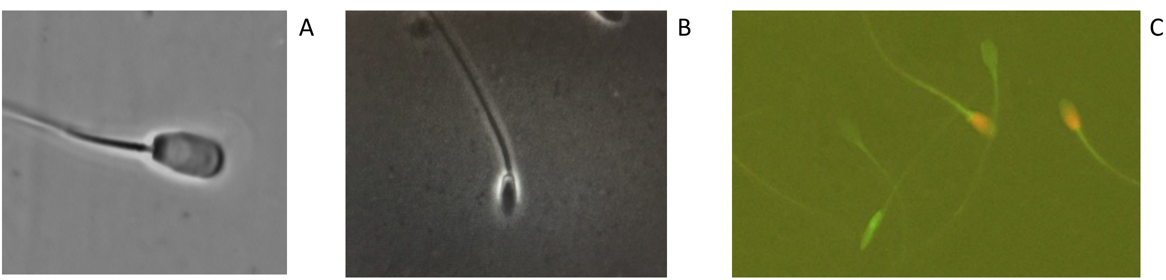


Figura 14. A) Técnica DIC: Acrosoma normal. B) Técnica DIC: Acrosoma perdiéndose. C) Tinción de fluorescencia FICT-PNA/PI. Los espermatozoides con membrana plasmática y acrosomal intacta aparecen en verde PNA-/PI- PNA+/PI+ aparecen rojos con una ligera tinción verde en el borde de la cabeza.

D.2. Tinción FITC-PNA/PI: Fluorocromo de Isotiocianato de Fluoresceína (FICT) conjugado con aglutinina de maní (PNA) y PI). Para su realización añadimos 10 µl de solución FITC-PNA (1 mg/ml en agua bidestilada) y 4 µl de solución PI (500 µg/ml) a 200

µl de la muestra de cada grupo de estudio. Se incubó durante 5 minutos a 38ºC, y finalmente se fijó en paraformaldehído (4% [v/v]) en solución salina. Se examinaron 200 espermatozoides bajo un microscopio de contraste de fase de fluorescencia. Los espermatozoides se clasificaron en cuatro categorías según su integridad plasmática y de membrana acrosómica: plasma y membrana acrosomal intacta (PNA- / PI-), membrana plasmática intacta y daño acrosomal (PNA + / PI-), membrana plasmática dañada y acrosoma intacto (PNA- / PI +) y daños en la membrana plasmática y acrosomal (PNA + / PI +) (Luño y cols., 2014) (*Figura 14.C*).

E. <u>Integridad del Acido Desoxirribonucleico (ADN) espermático</u>: La determinación del grado de fragmentación del ADN fue realizada utilizando tres técnicas diferentes, que por distintos motivos fuimos modificando.

E.1. Test de Dispersión de la Cromatina (SCD), utilizando el kit de diagnóstico rápido de fragmentación de ADN específico para caballos, llamado *Halomax*® (Halotech®, ChromaCell SL, Madrid, España) (*Figura 15.A*). El grado de daño del ADN en cada muestra fue cuantificado utilizando el índice de fragmentación del esperma del ADN (SDFI) (porcentaje de células espermáticas fragmentadas).

E. 2. Azul de Toluidina (AT). Para este procedimiento, las extensiones de semen secadas al aire se fijaron en etanol-acetona al 96% recién preparado (3: 1) durante 1 min, luego se pasaron a etanol a 70% durante 3 min y se hidrolizaron en ácido clorhídrico 0,1 N durante 25 min. Posteriormente, los portaobjetos se enjuagaron con agua destilada y se secaron. Finalmente, se tiñeron con 0,05% de azul de toluidina (en tampón fosfato de citrato de McIlvaine al 50%, pH 3,5 Merck). Se evaluaron al microscopio con el objetivo de 100x, un total de 200 espermatozoides en diferentes áreas de cada portaobjetos. El colorante AT se une al ADN generando reacciones metacromáticas cuando se asocia a la cromatina, permitiendo diferenciar espermatozoides de acuerdo con el grado de condensación de ésta. Las cabezas de células espermáticas con buena integridad de cromatina eran de color azul claro; las que presentaban alteración en la integridad se tiñeron de color azul oscuro/morado, considerándose anormales (*Figura 15.B*).

E.3. Naranja de Acridina (AO). Este método está basado en la susceptibilidad del ADN a la desnaturalización ácida. Para su realización, las muestras de semen fueron secadas al aire en un portaobjetos. Entonces, se fijaron en solución de Carnoy (metanol: ácido acético glacial, 3: 1) durante 3 hrs, y luego se enjuagaron en agua destilada y se secaron. Posteriormente, se cubrieron con una solución de tinción de AO al 0,1%, de acuerdo con el método de Tejada (Tejada y cols, 1984). Después de 5 min se lavaron con agua del grifo y se cubrieron con un cubreobjetos. En la evaluación con microscopio fluorescente a 40x, se contaron un total de 200 espermatozoides. Se determinaron dos tipos diferentes de tinción: espermatozoides con ADN normal (cabezas verdes) y espermatozoides con ADN desnaturalizado de una sola cadena (cabezas con fluorescencia rojo-anaranjado) (*Figura 15.C*).

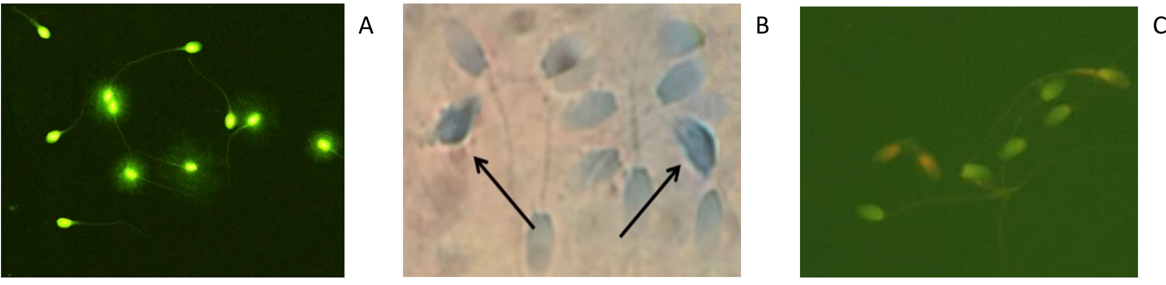


Figura 15.A) Halomax® para determinación de fragmentación de ADN. Los espermatozoides dañados muestran un halo "estallido" alrededor. B) Tinción con Azul de Toluidina. Las flechas negras muestran espermatozoides con ADN alterado. C) Tinción de fluorescencia Naranja de Acridina. Los espermatozoides con ADN desnaturalizado aparecen de color anaranjado.

F. <u>Test de Resistencia</u>: El objetivo de este test fue evaluar la motilidad después de 2 horas (Jiménez, 2010). El daño de la membrana puede no ser completamente expresado justo después de la descongelación, por ello, el semen debe ser valorado pasadas 2 horas y tras ser mantenido en incubación a 37 ºC. Esta evaluación es conocida como prueba de resistencia, de termorresistencia o de incubación. El semen debe tener al menos un 5% de motilidad a las dos horas de descongelado. La motilidad se analizó mediante sistema computerizado ISAS®.

Caballo prehistoria. Cueva Lascaux (Francia)

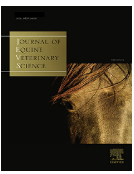
Artículo 1: Effect of Mare Colostrum in Extenders for Freezing Stallion Semen. Journal of Equine Veterinary Science (2019), 77: 23-17

Journal of Equine Veterinary Science 77 (2019) 23–27

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com



Original Research

Effect of Mare Colostrum in Extenders for Freezing Stallion Semen

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ARTICLE INFO

Article history:
Received 7 December 2018
Received in revised form
22 January 2019
Accepted 13 February 2019
Available online 21 February 2019

Keywords:
Mare colostrum
Stallion
Semen
Cryopreservation

ABSTRACT

This study aimed to evaluate the addition of mare colostrum in stallion freezing extenders to improve sperm quality. First, colostrum samples were collected from four mares after the foal's birth and their composition was determined. Ejaculates were collected from nine fertile stallions. Sperm samples were pooled, diluted, and cryopreserved into three experimental extender groups: Lactose-based extender supplemented with mare colostrum (20%), lactose-based extender supplemented with egg yolk (20%), and BotuCrio. The quality of the post-thaw semen samples were evaluated assessing sperm motility by means of computer-assisted analysis, viability by SYBR-14 and propidium iodine (PI) stain, acrosome integrity by fluorescein isothiocyanate and peanut agglutinine (FITC-PNA) and PI stain, plasma membrane functionality by hypo-osmotic swelling (HOS) test, and DNA denaturation by acridine orange (AO) test. There were no significant differences in the percentages of total motility, acrosome integrity, and DNA fragmentation among the extenders after thawing. Kinematics parameters showed significantly higher values in BotuCrio than in lactose extenders ($P < .05$). BotuCrio and lactose colostrum extender yielded significantly better rates for HOS-test, linearity, straightness, and wobble than egg-yolk extender ($P < .05$). However, in relation to sperm viability, lactose egg yolk extender showed significantly better results in comparison to the others seminal experimental media ($P < .05$). In conclusion, the incorporation of mare colostrum into cryopreservation media protected the sperm against cold-shock; therefore, it may be a good cryoprotectant agent alternative in extenders for freezing stallion semen.
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1. Introduction

In equine species, the fertilizing capacity of frozen semen is lower than in other species [1]. There is also a strong individual factor; thus, stallions are qualified as “poor or good freezers” [2,3]. In addition, pregnancy rates in mares inseminated with frozen semen doses are lower in comparison to chilled semen or natural conditions (30%–40%) [4–6].

The cryopreservation medium should ensure the sperm quality after freezing-thawing process due to specific compounds, as cryoprotectants, which decrease cold-shock damage [7]. Egg yolk is currently used as an external cryoprotective agent to freeze equine sperm and other species sperm [8]. It is considered as a good nonpenetrating cryoprotectant due to cholesterol, phospholipids, and low-density lipoprotein (LDL), proteins that have been identified as protective agents against temperature-related injury [9].

Previous research has demonstrated that milk contains some factors and components, such as caseinates, that provide protection to semen during the storage [1,10]. In addition, milk has been shown to have an antioxidant effect responsible for strong plasma membrane protection [1,11]. Colostrum, as the first form of milk produced by the mammary gland, has similar compounds as milk. Furthermore, it has been recognized to protect the foal during its first days of life by providing immunoglobulins (Ig) [12,13]. Notwithstanding, the composition of colostrum differs from milk in the Ig concentrations (IgG > 80%), fat, and lactose levels [13–15]. Likewise, colostrum shows anti-inflammatory and antimicrobial effects because of some active compounds such as lactoferrine, lactoperoxidase, and lysozyme [16].

Mares develop an endometritis in the uterus during 24–36 hours after insemination [6], which is in some cases persistent and incompatible with pregnancy. Some extender components such us

Animal welfare/ethical statement: The authors declare that the care and the use of animals were in accordance with the Spanish Policy for Animal Protection RD 1201/05 and the directive 2010/63/EU for animal experiments.

Conflict of interest statement: The authors declare no conflicts of interest.

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https://doi.org/10.1016/j.jevs.2019.02.010
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glycerol or egg yolk proteins may induce a greater migration of polymorphonuclear neutrophils in the endometrium [17,18]. A recent study investigated the anti-inflammatory effect of exogenous lactoferrine on breeding-induced endometritis [19].

Colostrum may be a viable alternative instead of milk or egg yolk in cryopreservation stallion extender because it could provide protection to spermatozoa against cold-injury and protection in the mare's uterus after insemination [20,21].

Based on the research presented above and taking into account the strong goal to find alternative cryoprotectants, the aim of this study was to test the protective action of mare colostrum in stallion freezing extender.

2. Materials and Methods

2.1. Reagents and Media

All chemicals were obtained from Sigma-Aldrich Química S.A. (Madrid, Spain), unless otherwise indicated. Dimethylformaide (DMF) was from Panreac Química S.L.U. (Barcelona, Spain) and Equex Paste from Minitube Ibérica S.L. (la Selva del Camp, Spain). The medium used for washing semen and centrifugation was INRA 96 (IMV Technologies, L'Aigle, France).

The care and the use of animals were in accordance with the Spanish Policy for Animal Protection RD 1201/05 and the directive 2010/63/EU for animal experiments.

2.2. Mare Colostrum Collection, Evaluation, and Preservation

Colostrum was collected from four mares of different breeds and ages, from the Military Horse Breeding Center (MHBC) in Zaragoza (Spain). The colostrum was collected from the mare immediately after parturition (a total of 120 mL was collected from each mare). The udder region of each mare was meticulously cleaned with warm water before collection, and an ultrasound study (Mindray DP-3300 VET, 7.5 MHz) was performed to rule out any possible pathologies [13,22]. Once colostrum was obtained, it was filtered by cellulose filter (Minitube Ibérica S.L., la Selva del Camp, Spain).

The quality of the colostrum was evaluated using a Brix refractometer [13]. Only samples that showed at least 23° Brix were accepted (23°Brix >60 g/L IgG) [23]. Colostrum samples were frozen at −20°C in 15 mL labeled test tubes [23,24]. Before each experimental study, colostrum was thawed in a water bath at 37°C [13,25], and all the samples were mixed together. The composition of colostrum was analyzed by means of an IR spectroscopy MilkoScan 4,000 Foss Electric (Hilleroed, Denmark).

2.3. Semen Handling and Cryopreservation

Fifteen ejaculates were collected from nine stallions—good freezers—(aged from 7 to 12 years) during the breeding season from the MHBC in Zaragoza (Spain) using an artificial vagina (Missouri-model, Nasco, Ft. Atkinsosn, WI).

Macroscopic and microscopic assessments were performed immediately after their extraction. The initial concentration of spermatozoa was determined using a Spermacue 12300/0500 (Minitube Ibérica S.L., La Selva del Camp, Spain). Motility and kinematic parameters were analyzed using the Integrated Semen Analysis System (ISAS, Projectes I Serveis R + D S.L., Valencia, Spain).

Only semen with at least ≥35% progressive motility was accepted [26] for cryopreservation. Semen samples were diluted in INRA medium and centrifuged at 1,000 g for 5 minutes [2].

Semen samples from every three ejaculates were pooled to avoid individual factors and obtain a more homogeneous group. Every pooled sample was divided into three experimental extender groups, and from each experimental group, 5 samples were frozen.

2.3.1. Experimental Groups

First group, Lactose–mare colostrum extender containing 50% (v/v) of 290 mM L-lactose, 20% (v/v) of mare colostrum, 25% (v/v) of glucose–EDTA medium (322.20 mM glucose, 12.58 mM sodium citrate, 9.93 mM disodium EDTA, 14.28 mM sodium bicarbonate), and 5%(v/v) of DMF [27].

Second group, Lactose–egg-yolk extender containing 50% (v/v) of 290 mM L-lactose, 20% (v/v) of egg yolk, 25% (v/v) of glucose–EDTA medium (322.20 mM glucose, 12.58 mM sodium citrate, 9.93 mM disodium EDTA, 14.28 mM sodium bicarbonate), 0.5% (v/v) of Equex Paste [28], and 5%(v/v) of DMF [27].

Third group, BotuCrio, standard cryopreservation extender.

The samples were frozen using a stabilization step process with a slow cooling rate of approximately 0.5°C per min. The semen samples were cooled down to 4°C in 2 hours for the lactose extenders, and during 20 minutes for BotuCrio extender samples as instructed in the information leaflet. Then, sperm samples were packed into 0.5 mL polyvinylchloride straws (IVM technologies, L'Aigle, France), which contained 100 × 10^6 spermatozoa/mL [12]. Finally, before submerging the samples, they remained for 20 minutes at 5 cm above liquid nitrogen vapors in a Styrofoam freezer box with neopor insulation block (freezing rate of −60°C) (MinitubIbérica S.L., La Selva del Camp, Spain). The spermatozoa of the five freezing batches from the pooled ejaculates were analyzed.

2.4. Semen Quality Evaluation

Frozen semen samples were thawed in a water bath at 37°C for 21 seconds.

Sperm motility, viability, plasma membrane functionality, acrosome integrity, and DNA fragmentation were assessed. Also, a resistant test—2 hours at 37°C—was performed.

2.4.1. Sperm Motility Analysis

Total motility and velocity parameters were analyzed by means of ISAS (Projectes I Serveis R + D S.L., Valencia, Spain) [29]. The parameters established for the analysis were 25 consecutive digitalized images per sec., and the particles area was 4–75 μ^2. With regard to the setting parameters for the program, sperm, values of average path velocity (VAP) <10 μ/sec were considered as slow and >90 μ/sec were considered as fast. Spermatozoa with 75% of the straightness (STR) were designated as progressive motile. Amplitude of lateral head value ≥ 10 μm (minimum number of images calculated).

After 2 hours at 37°C, a resistant test (RT) was performed to evaluate the motility [30].

2.4.2. Sperm Viability

Sperm viability was evaluated using LIVE/DEAD sperm viability kit (Molecular Probes Europe, Leiden, The Netherlands). The staining was performed following the instructions of the commercial kit. SYBR-14 penetrated the membrane of all the spermatozoa and provided a green fluorescence. Propidium iodine (PI) only penetrated the spermatozoa with altered membrane and showed red fluorescence, which overlapped the green one. A total of 200 cells were counted using a fluorescence phase-contrast microscope.

2.4.3. Membrane and Acrosome Status

Membrane and acrosome status were assessed by fluorescein isothiocyanate conjugated with peanut agglutinin (FITC-PNA) and PI staining [31] (Molecular Probes, Europe, Leiden, The

Table 1
Comparative of colostrum means and results obtained from our colostrum sample after analysis –IR spectroscopy MilkoScan 4,000 Foss Electric (Hilleroed, Denmark).

Composition	Colostrum ranges[a]	Mare Colostrum sample
Fat %	0.92–2.44	1.12
Protein %	8.2–22.22	11
Lactose %	0.64–4.28	3.88
Dry matter %	14.47–24.21	15.28

[a] Peka et al, 2012.

Netherlands). Two hundred microliter of each of the semen samples were supplemented with FITC-PNA solution (1 mg/mL in doubly distilled water) and PI solution (500 μg/mL), kept 5 minutes at 38°C, and finally fixed in paraformaldehyde (4% [v/v]). Two hundred spermatozoa were counted under a fluorescence microscope. Sperm with intact plasma and acrosomal membrane (PNA−/PI−) were determined.

2.4.4. Plasma Membrane Functionality

Plasma membrane functionality was assessed using Hypo-osmotic swelling test (HOST) [32]. This technique consisted of incubating 30 μL of sperm sample with 100 μL of lactose hypo-osmotic solution (100 mOsm/Kg) at 37°C for 30 minutes. The samples were then fixed in 8% glutaraldehyde buffered solution. Spermatozoa with coiled tails were considered as HOST positive.

2.4.5. DNA Status

DNA status was assessed by the acridine orange (AO) test [33]. Metachromatic staining can assess chromatin stability with AO, based on the susceptibility of the sperm DNA to acid-induced denaturation in situ. Air-dried semen samples were fixed in Carnoy solution (3:1methanol:glacialaceticacid) for 3 hours and then rinsed in distilled water and dried. The slides were then covered with 0.1% AO staining solution, according to the method of Tejada [34]. After 5 minutes, the slides were washed with tap water and covered with a coverslip. Under fluorescence microscope evaluation, a total of 200 spermatozoa were counted. Two different partners of staining were determined; spermatozoa carrying a normal DNA appeared as green heads, whereas single-stranded denaturized DNA produced a red-orange fluorescence.

2.5. Statistical Analysis

R-version: 3.1.3 Platform x86_64 Apple-Darwin 13.4.0 (64 bit) was used to analyze the results.

The statistical procedure used for the thawed samples results, began with a visual exploration of data (boxplots) and a summary of centrality estimates (mean and median) and variability (standard deviation). For extenders contrast a nonparametric test (Mann-Whitney-Wilcoxon test) was used. All tests were performed with a significance level of 95. This procedure was executed for all values: total motility, velocity parameters—curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR = VSL/VAP), and wobble (WOB)—viability, HOST, acrosome intact, DNA integrity, and the RT.

3. Results

The colostrum composition determined in this study in relation to fat, protein lactose, and dry matter is shown in Table 1. The results obtained for the four samples after refractometry were 24 ± 1°Brix.

Prefreezing percentages of total sperm motility, acrosome integrity, and viability did not differ among the different extenders. After thawing, semen samples showed values of total motility > 60%; therefore all stallions selected were classified as "good freezers".

The results in this study for total motility, velocity parameters, and the RT for the three groups are shown in Fig. 1. There were no significant differences in the percentage of total motility among the extenders after thawing. BotuCrio showed significantly higher values for kinematics parameters (VCL, VSL, VAP, WOB, LIN, and STR) than the other extenders. In relation to RT, lactose egg yolk semen samples showed higher values for total motility than other extenders, but there were no significant differences ($P > .059$).

The results in this study for viability, HOST positive, acrosome status, and DNA denaturation for the three groups are shown in Fig. 2. Lactose egg yolk extender showed significantly higher values for sperm viability ($P < .02$) and significantly lower percentages for sperm HOST positive ($P < .005$) in comparison to BotuCrio and lactose colostrum extender. DNA fragmentation did not show significant differences in any analyzed sperm samples. Nevertheless, a very low percentage of fragmented DNA was determined in all stained samples. The highest rate of damaged DNA was found in lactose egg yolk samples (3.33%) and the lowest in lactose colostrum samples (2.11%).

4. Discussion

In this research, we demonstrated that freeze-thawed mare colostrum did not lose its properties as described by Cash (1999)

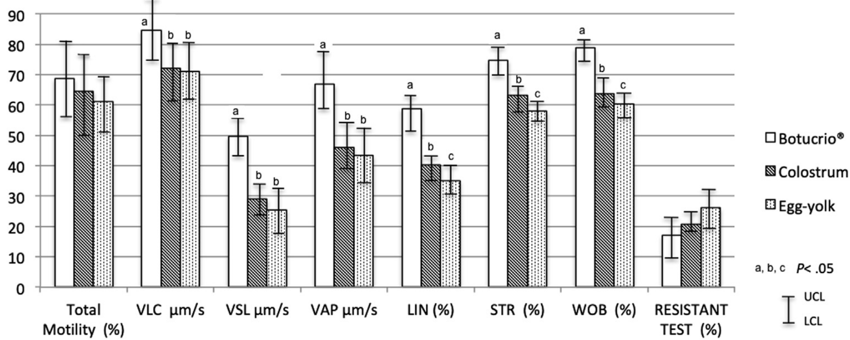

Fig. 1. Three different extenders Mean ± SD (UCL, upper confidence level; LCL, lower confidence level), for motility, kinematic parameters, and resistant test. (n = 15). Superscript letters ([a,b,c]) indicate significant differences ($P < .05$). For BotuCrio samples the results showed higher for all kinetic parameters. For LIN, STR, and WOB, colostrum was significant higher than egg yolk extender. For total motility, there was no significant difference when comparing the three extenders. LIN, linearity; STR, straightness; WOB, wobble.

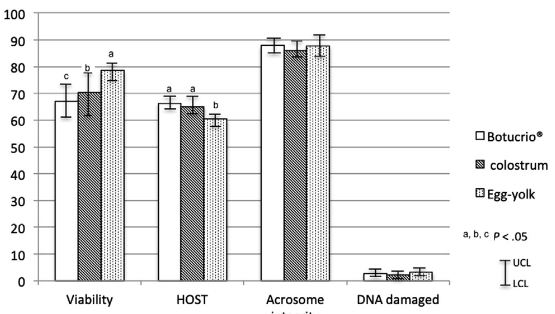

Fig. 2. Three different extenders Mean ± SD (UCL, upper confidence level; LCL, lower confidence level), for vitality, HOST, acrosome integrity, and DNA damaged. (n = 15). Superscript letters ([a,b,c]) indicate significant differences ($P < .05$).

[23]. The determination of IgG quantity and quality was analyzed by Brix refractometer. It is a standard technique to determine the quality of colostrum [24,35]. The colostrum samples remained frozen for a period of 2 months. Once thawed and analyzed, colostrum showed good quality (≥23°Brix, an acceptable IgG levels). The rest of the parameters analyzed, as shown in Table 1, were also within the average ranges for colostrum composition [14]. Mare colostrum quality found to be adequate for our study maintaining the standard quality [14,23].

We obtained positive results of sperm quality after thawing. The threshold for acceptable post-thaw motility is quite different among authors, but in general, results ≥ 35% are considered acceptable [13]. As shown in Fig. 1, post-thawed sperm motility in our study was superior (>60%).

The intracellular cryoprotectant used in our research was DMF. Five percent DMF maintains frozen-thawed stallion sperm quality better than others cryoprotectants including glycerol, improving all seminal parameters evaluated both in ejaculate and epididymis sperm samples [27]. Previous research has documented that DMF reduced the osmotic damage on sperm due to the low viscosity and molecule weight of amides [36].

Egg yolk is one of the most common components in the sperm freezing extender because it minimizes cold-shock effect and maintains sperm quality and functionality [37,38]. BotuCrio contains DMF and pasteurized egg yolk, which has shown to increase the percentages of total motility and fertility when utilized as stallion sperm frozen extender [39]. However, Olacireguiet al, (2014) [40] observed that it did not improve sperm quality on epididymal stallion sperm. Different substances as soybean lecithin, cholesterol-loaded cyclodextrins, or low-density lipoproteins preserved sperm motility and plasma membrane integrity after the freezing process instead of egg yolk [8,39,41].In our study, we tested the effect of a novel substance, mare colostrum, on stallion cryopreservation extender. We determined that colostrum extender samples showed similar percentages of sperm quality parameters as sperm samples cryopreserved with Botucrio. In addition, colostrum extender significantly increased the percentages of sperm HOS test positive, LIN, STR, and WOB in comparison to lactose egg yolk extender.

Colostrum includes some molecules as caseinates in its composition. It has been shown that caseinates act as membrane protectors in sperm during the cryopreservation process [1,10,11]. Thus, colostrum would perform a protective effect not only in sperm plasma membrane but also in DNA integrity. In addition, several molecules with antibacterial and anti-inflammatory properties have been determined in colostrum composition, which could provide a protection against endometritis postbreeding in the mare [35,36].

Different options have been tested in stallion sperm cryopreservation medium. But, to the authors' best knowledge, no publication has been found in literature that addresses the use of mare colostrum to preserve stallions' semen.

Summing up the results, we concluded that the incorporation of colostrum to semen extender protected stallion spermatozoa during the freezing-thawing process. It may be a new possibility in sperm cryopreservation field and a useful alternative to apply in mares to avoid persistent endometritis processes. Subsequent fertility testing is necessary to verify the beneficial effects on post-thaw sperm characteristics.

Acknowledgments

The authors wish to acknowledge the Goverment of Aragon Research Groups-European Social Found-DGA, and Instituto Agroalimentario de Aragón (IA2), Zaragoza, Spain, for their research support. The authors thank the assistance and support of the Military Horse breeding Center (Zaragoza, Spain), Department of Animal Pathology, Obstetric and Reproduction Area (Faculty of Veterinary Medicine, Zaragoza University, Spain), Pablo Alvarez San Martín (Statistical Analysis), Jo During (English Translation), and Dr Lourdes Sanchez Paniagua (Colostrum Analysis).

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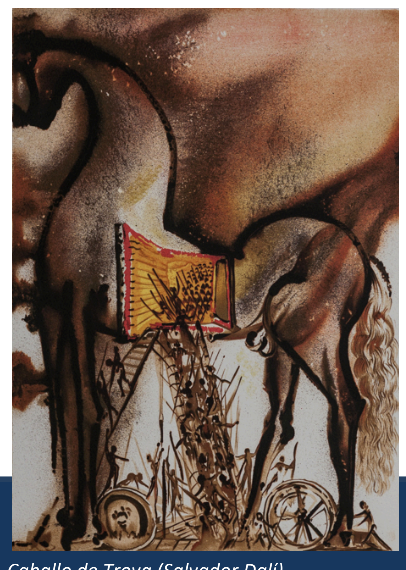


Caballo de Troya (Salvador Dalí)

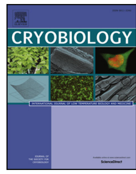
Artículo 2: A preliminary study on the use of jenny colostrum to improve quality in extenders for freezing donkey semen. Cryobiology (2019), 87:110-114

Cryobiology 87 (2019) 110–114

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/cryo



A preliminary study on the use of jenny colostrum to improve quality in extenders for freezing donkey semen

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ARTICLE INFO

Keywords:
Jenny
Colostrum
Donkey
Semen
Cryopreservation

ABSTRACT

Sperm quality in donkeys (*Equus asinus)* after freezing thawing is still considered lower than that from other animals, including horses. The aim of this study was to test a new freezing extender supplemented with jenny colostrum on donkey sperm. After thawing, we evaluated sperm motility by means of computer-assisted analysis, viability by SYBR-14 and propidium iodide (PI), membrane functional integrity by HOS-test and acrosome integrity by isothiocyanate conjugated with peanut agglutinin (FITC-PNA) and PI. Ejaculates were collected from five fertile Donkeys. Sperm samples were pooled, diluted and cryopreserved into three experimental extender groups: BotuCrio®, lactose-extender supplemented with egg yolk (20%) and lactose-extender supplemented with jenny colostrum (20%). The results demonstrated that lactose-jenny colostrum samples displayed significantly higher values in almost all parameters evaluated ($p < 0.05$) compared with the other two extenders after thawing (BotuCrio® and lactose-egg yolk based extender, respectively) –Total Motility, Viability, HOS test, VCL, VSL and VAP. Acrosome status, LIN, STR and WOB despite showing lower values, none of them were statistically significant ($p > 0.05$). In conclusion, the extender containing jenny colostrum can be successfully used for donkey semen crypreservation and could effectively improve donkey sperm qualities after freezing -thawing.

1. Introduction

Global donkey (*Equus asinus*) population is decreasing considerably. Many different breeds of autochthonous donkeys are at high risk of extinction. It is important to make efforts to contribute to the conservation of these animals. The use of artificial insemination (AI) techniques can help in this context [6,42]. Frozen donkey semen, in comparison to horses, shows low conception rates (33%–56% mares vs. 0%–36% jennies). There are only few studies about donkey semen cryopreservation [2,11,45]. Hence, it is necessary to implement different strategies during cryopreservation process in order to improve sperm quality, functionality and fertility [44].

Extenders composition may determinate the sperm quality after thawing. Egg yolk and skim milk have been considered the best non-penetrating cyoprotectants in horse extenders [25].

Previous research has demonstrated that milk contains some factors and substances, as caseinate proteins, that provide semen protection during its storage [7,21]. In addition, milk has been shown to have an antioxidant effect that is responsible for a strong plasma membrane protection [7,8].

With regard to milk properties, it has been demonstrated that donkey milk has similar composition than human milk with analogous antimicrobial and anti-inflammatory properties [27,35,46]. Donkey colostrum, as the first form of milk produced by the mammary glands, has, in general terms, similar composition. However, it differs from milk in several important areas, including higher concentrations of immunoglobulins (Ig) (IgG and IgA) and fat, and lower concentrations of lactose [27,28,34]. It is also composed of a wide range of antimicrobial [35] and immunomodulatory factors, including lactoferrin, lactoperoxidase, lysozyme and oligosaccharides [9].

The uterine reaction of the mares/jennies after insemination is a natural process that aims to eliminate sperm, seminal plasma and other potential pollutants [17]. This inflammation is resolved within 24–36 h [43]. Nevertheless, some mares (and more frequently in jennies), are unable to eliminate inflammation, they consequently develop persistent breeding-induced endometritis, which is incompatible with pregnancy. Some extender components such as glycerol or egg yolk proteins play a major role in this reaction by inducing a greater migration of polymorphonuclear neutrophils (PMNN) in the endometrium [33,41].

We hypothesized that the addition jenny colostrum in an extender for freezing donkey semen could have a protective effect after thawing. Furthermore, if the results obtained are relevant, it may be beneficial in

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https://doi.org/10.1016/j.cryobiol.2019.01.009
Received 17 November 2018; Received in revised form 20 January 2019; Accepted 21 January 2019
Available online 24 January 2019
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the jenny uterus after insemination. The information obtained might help to preserve these species and increase the population of donkeys.

2. Materials and methods

2.1. Reagents and media

All chemicals were obtained from Sigma-Aldrich Química S.A. (Madrid, Spain), unless otherwise indicated. Dimethylformamide (DMF) was from Panreac Química S.L.U. (Barcelona, Spain) and Equex Paste from Minitub Ibérica S.L. (La Selva del Camp, Spain). The medium used for washing the semen and centrifugation was INRA 96® (IMV Technologies, L'Aigle, France).

The care and the use of animals were in accordance with the Spanish Policy for Animal Protection RD 1201/05 and the directive 2010/63/EU for animal experiments.

2.2. Jenny colostrum collection, evaluation and preservation

Colostrum was collected from four jennies of different ages, from the Military Horse Breeding Centre (MHBC) in Zaragoza, Spain.

The colostrum was collected and filtered (cellulose filter, Minitub Ibérica S.L., La Selva del Camp, Spain) from the jennies just after the foal's birth (100 ml of each jenny). Before the collection, an ultrasound study (Mindray DP-3300 VET®, 7.5 MHz) was performed to rule out any possible pathologies [1,28].

The quality of the colostrum was evaluated using a Brix refractometer, after collection. There is a good correlation between the refractometer reading and colostrum IgG (r^2 = 0.74), where r^2 is the coefficient of determination which ranges from 0 to 1 [28]. In our study, only samples that had at least 23° Brix were accepted (23°Brix > 60 g/L IgG), Cash (1999) [12] considered values ≥ 20 °Brix as good quality for colostrum samples. Then the samples were frozen at −20 °C in 15 ml labelled test tubes [12,13].

Once the samples were thawed and evaluated, they were mixed together and added to the extender for study.

2.3. Semen handling and cryopreservation and thawing

Ejaculates were collected from 5 donkeys from the HBMC of Zaragoza, Spain, ages 5–10 years, during the breeding season, using an artificial vagina (Missouri-model, Nasco, Ft. Atkinsosn, WI, USA). Two ejaculates per donkey (n = 10) were used in this experiment.

Macroscopic and microscopic assessments were performed immediately after their extraction. The initial concentration of spermatozoa was determined using Spermacue® 12300/0500 (Minitub Ibérica S.L., La Selva del Camp, Spain). Motility was analysed by means of the Integrated Semen Analysis System (ISAS®, Projectes I Serveis R + D S.L., Valencia, Spain).

The sperm samples were pooled and diluted INRA 96® (IMV Technologies; L'Aigle, France) to avoid individual factors. Then, samples were centrifuged at 1000 g for 5 min and divided into three representative groups:

1st Group, BotuCrio®; cryopreservation commercial extender.

2nd Group, Lactose– egg yolk based extender; containing 50% (v/v) of 290 mM L-lactose, 20% (v/v) of egg yolk, 25% (v/v) of Glucose–EDTA medium (322.20 mM glucose, 12.58 mM sodium citrate, 9.93 mM disodium EDTA, 14.28 mM sodium bicarbonate), 0.5% (v/v) of Equex Paste [18] and 5% (v/v) of DMF [5].

3rd Group, Lactose–jenny colostrum extender; containing 50% (v/v) of 290 mM L-lactose, 20% (v/v) of jenny colostrum, 25% (v/v) of Glucose–EDTA medium (322.20 mM glucose, 12.58 mM sodium citrate, 9.93 mM disodium EDTA, 14.28 mM sodium bicarbonate and 5% DMF.

The samples were frozen following a slow freezing process, whereby the temperature progressively decreased for 2 h until reaching 4 °C. Then, the samples were packed into 0.5 ml polyvinylchloride straws (IVM technologies, L'Aigle, France), at 100 × 10^6 spermatozoa/ml per straw [40], introduced in vapours of liquid nitrogen for 15 min and finally submerged into liquid nitrogen (−196 °C) in a freezer Styrofoam box with neopor insulation block (Minitub Ibérica S.L., La Selva del Camp, Spain). The stabilization step in BotuCrio® samples was performed following the standard protocol for this extender. All samples were thawed in a water bath at 37 °C for 21 s.

2.4. Semen quality assessment

2.4.1. Sperm motility analysis

Total motility, as well as kinetic parameters − curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR = VSL/VAP) and wobble (WOB) −, were analysed by means of ISAS® [19]. The particles area was fixed between 4 and 75 μm², spermatozoa with VAP < 10 μ/sec were considered as immotile, > 45 μm/s as normal and > 90 μm/s as rapid. And ALH minimum value was 10 images.

2.4.2. Sperm viability

Sperm viability was evaluated using LIVE/DEAD® sperm viability kit (Molecular Probes Europe, Leiden, The Netherlands) [24]. SYBR-14 stain penetrates both intact and damaged spermatozoa membranes and fluoresces green, while propidium iodine (PI) only penetrates a damaged membrane, and stains the sperm red [23]. Two hundred spermatozoa were analysed using a fluorescence phase-contrast microscope.

2.4.3. Membrane and acrosome status

Membrane and acrosome status were assessed by FITC-PNA/PI staining. Sperm samples were incubated at 38 °C during 5 min with FITC-PNA solution (1 mg/ml) and PI solution (500 μg/ml) and fixed in paraformaldehyde (4% [v/v]). Two hundred spermatozoa were counted under a fluorescence microscope. Spermatozoa with plasma and acrosomal membrane status (PNA−/PI−) were analysed.

2.4.4. Plasma membrane functionality

Plasma membrane functionality was assessed using HOS test [22]. Semen samples were incubated in lactose hypo-osmotic solution (100 mOsm) for 30 min at 37 °C. The samples were then fixed in 8% glutaraldehyde buffered solution and spermatozoa with coiled tails were considered as HOST-positive.

2.5. Statistical analysis

In order to determine whether the results were statistically valid, a detailed analysis of all the thawed semen samples was performed (R version: 3.1.3 Platform x86_64 Apple-Darwin 13.4.0 (64bit). The statistical procedure began with a visual exploration of data (boxplots), a summary of centrality estimates (mean and median) and variability (standard deviation). Shapiro-Wilk test was used to confirm a normal distribution. For extenders contrast a non-parametric test (Mann-Whitney-Wilcoxon test) was used. All tests were assessed at the conventional significance level of 0.05. Data are expressed as mean ± standard deviation (SD). This procedure was consistently repeated for all values − motility, kinetic parameters, viability, HOST and acrosome − in all extender samples.

3. Results

The values obtained from the four colostrum samples after refractometry analysis were 24.5 ± 1° Brix.

Lactose-based extender with jenny colostrum showed the best results in almost all the compared variables evaluated. Data for comparison of semen quality analysis with different extenders are provided in Fig. 1 and Fig. 2.

Shapiro-Wilk and Mann-Whitney-Wilcoxon tests were performed,

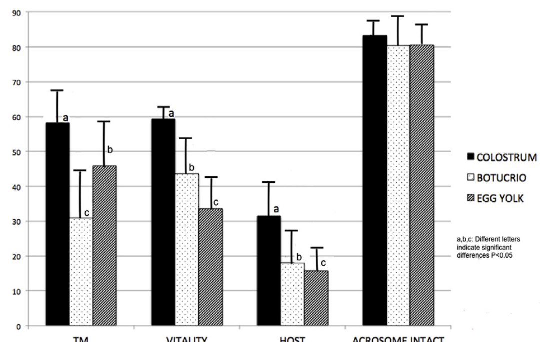

Fig. 1. Post-thawing values for Total Motility, Vitality, HOST and Acrosome intact. Total motility, vitality and HOS test show better results for lactose-Jennie colostrum extenders. Superscripts ([a,b,c]) indicate significant differences ($p < 0,05$).

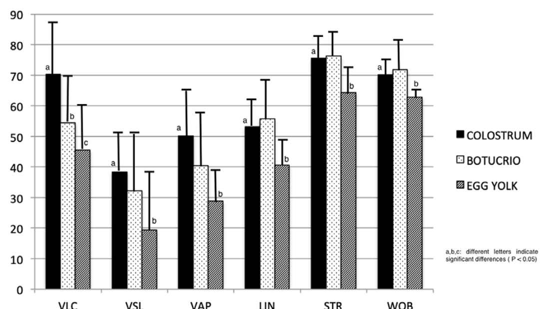

Fig. 2. Post-thawing values for Kinetic parameters. Colostrum was statistically better than the other two extenders for VLC. Superscripts ([a, b, c]) indicate significant differences ($p < 0,05$). For the rest of the parameters analysed, there were only significant differences ($p < 0,05$) between colostrum and egg yolk samples (Superscripts [a, b]). For BotuCrio® samples, in spite of showing higher values for LIN, STR and WOB, no significant differences were shown.

comparing the means of all values (total motility, velocity parameters: VCL, VSL, VAP, LI, STR and WOB, viability, HOST and acrosome integrity).

Cryopreservation affected significantly almost all the sperm parameters assessed ($p < 0.05$) regardless of the extender used. Lactose-jenny colostrum extender was significantly better ($p < 0.05$) for all the samples than Lactose-egg yolk, except for acrosome status, in which there was no significant differences ($p > 0.05$) (Fig. 1).

Different results were showed between jenny-colostrum and BotuCrio® extenders, depending on the evaluated parameter. Lactose-jenny colostrum improved the percentage of sperm total motility, viability, HOST, and VLC ($p < 0.05$). However, the rest of the parameters evaluated did not show significant differences between both extenders.

4. Discussion

In this study, we have demonstrated that jenny colostrum, once thawed and mixed, did not lose its properties as described by Cash (1999) [12]. IgG quantity and quality were analysed by Brix refractometer and we obtained values superior to 23° Brix. This is a standard method to determine reliable results of colostrum quality [15].

Along the years, different studies have proposed several freezing extenders of various compositions in an attempt to improve the quality and the use of frozen stallion semen [10,14,26]. Egg yolk and skim milk are the most common protective agents against cold shock injury utilized on stallion sperm cryopreservation [10,25]. The combination of egg-yolk based medium with glycerol is a standard protocol utilized in commercial extenders as BotuCrio®. However, Álvarez et al. (2014) [5] and Acha et al. (2016) [2], demonstrated that DMF at concentration of 2.5% used as cryoprotectant in donkeys showed better sperm quality − greater values ($p < 0.001$) of total motility, progressive motility and membrane status − than other cryoprotectants after cryopreservation.

DMF reduced the osmotic damage on sperm due the low viscosity and molecule weight of amides [4] and increased the stallion sperm quality after cryopreservation.

BotuCrio® contains pasteurized egg yolk and has shown higher percentage of total motility and fertility when is utilized as stallion sperm frozen extender [32]. However, Olacireguiet al. (2014) [31], observed than pasteurized egg yolk did not improve sperm quality on epididymal stallion sperm. In our study, BotuCrio® showed lower percentages of sperm motility, viability and HOST than Lactose-colostrum extender.

Even though no publication has been found in literature that addresses the use of jenny colostrum to preserve donkey's semen, our research showed that the incorporation of colostrum as a new component in extender for freezing donkey semen increased the percentages of total motility, kinetic parameters and sperm plasma membrane functionality after performing a freezing-thawing procedure.

Our results for sperm motility and kinetic parameters in all extenders were low, but yet in accordance with Rí Zková et al. (2017) [37], with similar mean for all kinetic parameters (VCL 63.23 μm/s, VSL 31.02 μm/s, VAP 47.53 μm/s, STR 65.24%, LIN 48.8%). Results also agreed with Rezagholizadeh et al. (2015) [36] in regards to STR (71.20%) and LIN (45.5%). Kinetic parameters for Lactose-jenny colostrum extender showed better results than for Lactose-egg yolk extender ($p < 0.05$).

Sperm motility is an important parameter to evaluate semen quality in equine species. Donkey semen is less efficiently frozen than that of the stallion, and a significant decrease in total and progressive motility is recorded. Values < 25% post-thaw motility are found to result in a lesser pregnancy rate. In our work, besides the good results of the kinetic parameters, lactose-jenny colostrum also improved total sperm motility (58.3%) after cryopreservation, and it might increase the fertility potential of the sperm samples.

Skim milk-based medium maintains sperm motility, plasma membrane and acrosome integrity in ejaculated and epididymal stallion sperm [30,39]. It has specific proteins as caseinate, which protects sperm even at low concentrations [21]. The colostrum composition, which also contains caseinate, might explain the protecting action on sperm membrane.

We found better percentage on sperm viability on lactose-jenny colostrum extender than on egg yolk-based extenders ($p < 0.05$).

The frozen donkey semen is subjected to conditions that seriously affect sperm functionality and viability, leading to reduce sperm longevity in the jenny genital tract. Moreover, the use of frozen semen induces a stronger inflammatory reaction in the uterus than fresh semen in mares [20,38]. The absence of seminal plasma, certain cryoprotectants [29] such as glycerol, and egg yolk proteins favour this reaction by inducing greater PMNN migration to the endometrium [33,41].

A recent study has demonstrated that the inoculation of colostrum stimulated the uterine immune system and reduced postpartum uterine pathologies in the sows [3]. Another study has demonstrated that Ig, lactoferrin and lactoperoxidase present in colostrum might reduce inflammatory response and oxidative stress of mucous [46].

The use of lactoferrin for its anti-inflammatory and bactericidal properties has also recently been studied by Fedorka et al. (2018) [16] as a treatment on breeding-induced endometritis in susceptible mares.

Consequently, the properties of colostrum shown in those studies could suggest a new field of investigation in which colostrum specific compounds, on the one hand provide good results after thawing and on

the other hand could interact in jenny uterus and decrease reactions due to AI.

It is a preliminary study and subsequent fertility testing is necessary to verify the beneficial effects on post-thaw sperm characteristics.

Summing up the results, we concluded that Jenny-colostrum exerted a protective effective against cold shock, since it improved sperm quality after freezing-thawing process. Therefore, it could be a useful alternative as cryoprotectant on sperm plasma membrane in freezing extenders in donkeys. Furthermore, since extant research on this topic is limited, this paper may create new areas for the study of sperm cryopreservation.

Conflicts of interest

None.

Acknowledgments

The authors would like to acknowledge the assistance and support of the Horse Breeding Military Centre, Zaragoza (Spain); Department of Animal Pathology, Obstetric and Reproduction Area, Faculty of Veterinary Medicine, Universidad de Zaragoza, Spain; Pablo Alvarez San Martín (Statistical Analysis); Lisa Williams (English translation) and Dr. Lourdes Sanchez Paniagua (colostrum analysis).

This work was supported by the Government of Aragon Research Groups- European Social Found, DGA - and IA2.

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Caballo de Azul (Franz Marcí)

Artículo 3: Alternatives in donkey semen preservation: mare vs. Jenny colostrum. Reprod Dom Anim (2019),54 (4), 94-97

Received: 30 April 2019 | Revised: 29 June 2019 | Accepted: 10 July 2019

DOI: 10.1111/rda.13516

SUPPLEMENT ARTICLE

Reproduction in Domestic Animals WILEY

Alternatives in Donkey semen cryopreservation: Mare vs. Jenny Colostrum

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Abstract

The aim of this study was to test and compare two new components in extenders for freezing donkey semen: mare colostrum and jenny colostrum. Colostrum was obtained from four mares and four jennies right after the foal's birth. Ejaculates were collected from five fertile donkeys. Sperm samples were pooled, diluted and cryopreserved in three different experimental extender groups: lactose supplemented with egg yolk extender (20%) as the control group, lactose supplemented with jenny colostrum extender (20%), and lactose supplemented with mare colostrum extender (20%). After thawing, we evaluated the sperm motility by means of computer-assisted analysis, viability by SYBR-14 and propidium iodide (PI), membrane functional by HOS test and acrosome integrity by isothiocyanate conjugated with peanut agglutinin (FITC-PNA) and PI. The results demonstrated that lactose–jenny colostrum extender displayed significantly higher values ($p < .05$) in nearly all parameters evaluated – Total Motility, Viability, HOS test, VCL, VSL, VAP, LIN, STR and WOB –, compared with mare colostrum and egg yolk extenders after thawing. In conclusion, the extender containing jenny colostrum used for donkey semen cryopreservation improved the donkey sperm quality after the freezing–thawing process.

KEYWORDS

Colostrum, Cryopreservation, Donkey, Jenny, Mare, Semen

1 | INTRODUCTION

Frozen donkey semen, in comparison to horses', shows low conception rates after artificial insemination (33%–56% mares vs. 0%–36% jennies). In addition, there are only few studies about donkey semen cryopreservation (Acha et al., 2016).

Colostrum, as the first form of milk produced by the mammary glands, includes higher concentrations of immunoglobulins (Ig) and fat, and lower concentrations of lactose (McKinnon, Squires, & Vaala, 2011; Peka, Dobrzanski, Zachwieja, Szulc, & Czyz, 2012) than milk. It is also composed of antimicrobial and immunomodulatory components – lactoferrin, lactoperoxidase, lysozyme and oligosaccharides. Also, colostrum has anti-inflammatory properties (Massouras, Triantaphyllopoulos, & Theodossiou, 2017; Playford et al. 2000; Yadav, Angolkar, Kaur, & Buttar, 2016). Both the protective and antioxidant effects of the colostrum components added to extenders have been studied separately (Lagares et al., 2012; Martins et al., 2016).

A recent study has demonstrated that jenny colostrum added to an extender for freezing donkey semen yielded high sperm quality after thawing in comparison to egg yolk extenders (Álvarez, Luño, González, & Gil, 2019). We investigated if the addition of mare colostrum and jenny colostrum, which differ in their composition (Brumini, Criscione, Bordonaro, Vegarud, & Marletta, 2016; Marchis et al., 2018; Salimei et al., 2004), could have the same protective effect in donkey sperm cryopreservation.

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TABLE 1 Mean values (±*SEM*) of donkey sperm quality after thawing using different extenders. VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble. Different superscript letters ([a,b,c]) indicate significant differences ($p < .05$)

Extender	Total motility (%)	Vitality (%)	HOST (%)	Acrosome intact (%)	Velocity parameters					
					VLC µm/s	VSL µm/s	VAP µm/s	LIN (%)	STR (%)	WOB (%)
Lactose-based extender + mare colostrum	41.21 ± 8.1^b	41.27 ± 6.1^b	18.27 ± 3.7^b	82.27 ± 1.2	55.02 ± 4.9^b	22.2 ± 6.4^b	32.1 ± 3.5^b	35.3 ± 5.1^b	64.4 ± 4.8^b	59.2 ± 2.8^b
Lactose-based extender + jenny colostrum	58.3 ± 4.8^a	59.4 ± 5.9^a	31.4 ± 3.2^a	83.2 ± 1.2	70.4 ± 5.2^a	38.4 ± 6.8^a	50.2 ± 4.2^a	53.2 ± 3.9^a	75.5 ± 5.0^a	70.1 ± 2.7^a
Lactose-based extender + egg yolk	45.9 ± 7.3^b	33.5 ± 6.9^c	15.7 ± 4.9^b	80.5 ± 1.5	45.4 ± 8.1^c	19.3 ± 7.0^b	28.8 ± 4.6^b	40.6 ± 3.2^b	64.3 ± 4.7^b	62.8 ± 2.6^b

2 | MATERIALS AND METHODS

The care and the use of animals were in accordance with the Spanish Policy for Animal Protection RD 53/2013 and the directive 2010/63/EU for animal experiments. Experimental procedures were approved by the University of Zaragoza Ethics Committee for Animal Experimentation (PI58/18).

2.1 | Jenny and mare colostrum collection, evaluation and preservation

Colostrum was collected from four jennies and four mares of different ages, from the Horse Breeding Military Centre in Zaragoza, Spain (HBMC). About 100 ml of colostrum were collected and filtered (cellulose filter, Minitub-Ibérica S.L., Tarragona, Spain) from each animal right after the foal's birth. The quality of the colostrum was evaluated using a Brix refractometer. There is a good correlation between the refractometer reading and colostrum IgG (r^2 = 0.74) (McKinnon et al., 2011). Values ≥ 20°Brix are considered as good quality. (23°Brix >60 g/L IgG) (Cash, 1999). The samples were frozen at −20°C in 15 ml labelled test tubes (Cash, 1999).

Once the samples were thawed and evaluated, they were pooled and added to the extender for study.

2.2 | Semen handling and cryopreservation and thawing

Two ejaculates were collected from each of the 5 fertile donkeys from the HBMC, using an artificial vagina (Missouri-model, WI, USA).

The sperm samples were pooled and diluted in INRA 96® (IMV Technologies; L'Aigle, France). Then, the samples were centrifuged at 1000 *g* for 5 min and divided into three experimental groups. They were all composed of lactose–based extender; containing 50% (v/v) of 290 mM L-lactose, 25% (v/v) of glucose–EDTA medium (322.20 mM glucose, 12.58 mM sodium citrate, 9.93 mM disodium–EDTA, 14.28 mM sodium bicarbonate) and 5% (v/v) of Glycerol. For the 1st Group – Control – 20% (v/v) of egg yolk and 0.5% (v/v) of Equex Paste (Gil, Galindo-Cardiel, Malo, González, & Álvarez, 2013) was added. For the 2nd Group, 20% (v/v) of jenny colostrum, and for the 3rd Group, 20% (v/v) of mare colostrum.

The samples were frozen following a slow freezing process (4°C/2 hr), packed into 0.5 ml straws (100×10^6 spermatozoa/ml/straw), introduced in vapours of LN2 for 20 min and submerged into LN2 in a freezer Styrofoam box (Minitub-Ibérica S.L., Tarragona, Spain). All samples were thawed in a water bath at 37°C for 21 s.

2.3 | Semen quality assessment

2.3.1 | Sperm motility

Total motility, as well as kinetic parameters, was analysed by means of computer-assisted sperm analyses ISAS® (Valencia, Spain).

2.3.2 | Sperm viability

Sperm viability was evaluated using LIVE/DEAD® sperm viability kit (Molecular Probes, the Netherlands) based on SYBR-14/PI stains.

2.3.3 | Acrosome status

Acrosome status was assessed by FITC-PNA/PI staining. Sperm samples were incubated at 38°C during 5 min with FITC-PNA solution (1 mg/ml) and PI solution (500 μg/ml) and fixed in paraformaldehyde (4% [v/v]).

2.3.4 | Plasma membrane functionality

Plasma membrane functionality was assessed using the HOS test. Semen samples were incubated in lactose hypo-osmotic solution (100 mOsm) for 30 min at 37°C. Afterwards, they were fixed in 8% glutaraldehyde buffered solution.

2.4 | Statistical analysis

The analysis of data was performed using the 'R version: 3.1.3 Platform x86_64 Apple-Darwin 13.4.0'. The boxplots, the Shapiro–Wilk test and the Mann–Whitney–Wilcoxon test were applied. All tests were assessed at the conventional significance level of .05. Data were expressed as mean ± standard deviation.

3 | RESULTS

All colostrum samples showed good quality after being thawed (24.5 ± 1°Brix).

Cryopreservation was significantly affected ($p < .05$) according to the extender used. The Lactose–jenny colostrum extender was significantly better ($p < .05$) than the Lactose–mare colostrum and the Lactose–egg yolk extender, for almost all the quality parameters assessed. However, the percentages of sperm with intact acrosome did not show differences between different extenders. Data for comparison of semen quality analysis with the three different extenders are provided in Table 1. Results also showed that the addition of mare colostrum in the medium increased significantly the sperm viability and VCL parameter in comparison to egg yolk after the cryopreservation process.

4 | DISCUSSION

In this study, we have demonstrated that the incorporation of mare/jenny colostrum as a new component in extender for freezing donkey semen yielded suitable results on sperm quality. Colostrum, once thawed, maintained its properties as described by Cash (1999) and McKinnon et al. (2011).

To date, donkey semen cryopreservation has given disappointed results (Rota, Panzani, Sabatini, & Camillo, 2012; Victor de Oliveira et al., 2016). In our study, jenny and mare colostrum improved total sperm motility (58.3% and 41.2% respectively), as well as kinetic parameters after cryopreservation (Table 1).

According to different studies that have been conducted (Marchis et al., 2018; Salimei et al., 2004), jenny and mare colostrum have different composition. Mare colostrum has higher amount of lactose, which might affect sperm quality. In addition, the casein/whey protein ratio in colostrum is higher in jennies than in mares (Brumini et al., 2016). Colostrum caseinates, which protect sperm even at low concentrations (Lagares et al., 2012), might explain the protecting action on sperm membrane during freezing, and, therefore, why better results were obtained using the Lactose–jenny colostrum extender.

As reported in some studies, there is a stronger inflammatory reaction in the uterus when frozen semen is used to inseminate mares and jennies (Miró & Papas, 2018; Rota et al., 2012). Some compounds including egg yolk, favour this reaction (Miró & Papas, 2018). Recent studies have demonstrated that Ig, lactoferrin and lactoperoxidase present in colostrum show inflammatory and bactericidal properties in the uterus (Fedorka et al., 2018; Yadav et al., 2016). Also, colostrum has been used in sows' uterus to reduce post-partum pathologies (Adres Ramos, 2014).

Subsequent colostrum testing is necessary to verify the beneficial effects of different colostrum types on post-thaw sperm characteristics.

In conclusion, mare and jenny colostrum might be used as alternatives for freezing donkey semen because it provides suitable sperm quality after thawing. In this study, it has been demonstrated that jenny colostrum yielded better sperm quality than mare colostrum and it might be associated with the differences in composition.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the Government of Aragon (Ref. Group A17_17R-RAySA) co-financed with FEDER 2014-2020 'Building Europe from Aragon' for their research support. The authors thank the assistance and support of the Military Horse breeding Center (Zaragoza, Spain), Department of Animal Pathology, Obstetric and Reproduction Area (Faculty of Veterinary Medicine, Zaragoza University, Spain), Pablo Alvarez (Statistical Analysis), Marina Fernández (English language revision).

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

Lydia Gil: designed study and paper corrections. Victoria Luño: contribution to the research, experimental design, results analysis. Felisa Martin: colostrum collection and methods. Noelia Gonzalez: analysis of data and drafted paper.

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How to cite this article: Álvarez C, González N, Luño V, Martínez F, Gil L. Alternatives in Donkey semen cryopreservation: Mare vs. Jenny Colostrum. *Reprod Dom Anim*. 2019;54(Suppl. 4):94–97. https://doi.org/10.1111/rda.13516

Leonardo da Vinci

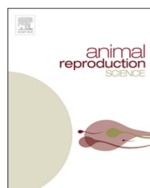
Artículo 4: Ejaculated compared with epididymal stallion sperm vitrification. Anim Reprod Sci (2019), 211 (106205), 1-5

Animal Reproduction Science 211 (2019) 106205

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci



Ejaculated compared with epididymal stallion sperm vitrification

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A R T I C L E I N F O

Keywords:
Cryopreservation
Semen
Stallion
Sucrose
Trehalose
Vitrification

A B S T R A C T

The aim of this study was to evaluate the effect of trehalose and lactose extenders on ejaculated and epididymal stallion sperm vitrification. Ejaculated semen samples were collected from seven fertile stallions, and *cauda* epididymis samples were collected from ten stallion carcasses after slaughter. Both the ejaculated and the epididymis samples were diluted and vitrified using INRA 96® and bovine serum albumin as well as trehalose or lactose. As a control, ejaculated and epididymal samples were collected and frozen using the conventional method. Vitrification was performed by immersing sperm suspensions directly in LN2. After thawing or devitrification, there was assessment of samples for sperm motility using computer-assisted analysis. Viability was assessed using SYBR-14 and propidium iodide (PI) and acrosome integrity by fluorescein using isothiocyanate combined with peanut agglutinin (FITC-PNA) and PI. Epididymal sperm vitrification with trehalose (EPT) or lactose (EPL) resulted in greater progressive sperm motility than sperm of the control sample (EPC). After post-thaw/devitrification of sperm in the EPT group, sperm motility was greater ($P < 0.001$) compared to that using EPL (50.72 ± 5.09% compared with 34.21 ± 3.02%). The results from assessment of ejaculated sperm samples after undergoing the vitrification process indicated cells were less viable ($P < 0.001$) than the control (EJC) sample. In conclusion, vitrification of epididymal stallion sperm using trehalose might be a beneficial alternative for the long-term storage of sperm samples with great economic value. Spermatozoa from vitrified ejaculates of stallions, however, had lesser motility and viability rates than samples subjected to conventional freezing.

1. Introduction

Stallions generally have a marked decrease in spermatozoa fertilization capacity after storage than other sperm of other species (Salazar et al., 2011; Restrepo et al., 2012). There is a large amount of inter-individual variation of values for sperm quality variables after the freezing/thawing processes in which only few stallions produce semen that can be cryopreserved with retention of desirable viability after thawing ("Good freezers"; Hoffmann et al., 2011; Alvarenga et al., 2016). To improve semen quality after thawing, there have been recent studies conducted of new procedures with varying results. One of these procedures is sperm vitrification, which is still a relatively unexplored methodology. The vitrification method has some advantages in practice, such as the reduction of processing time, cost, and equipment needed. It can also be conducted in any andrology laboratory (Rahiminia et al., 2017; Le et al., 2019).

Vitrification consists of the ultra-rapid freezing of a sperm drop immersed directly in liquid nitrogen (LN2), thus avoiding the

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https://doi.org/10.1016/j.anireprosci.2019.106205
Received 15 June 2019; Received in revised form 1 October 2019; Accepted 11 October 2019
Available online 22 October 2019
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detrimental effects of intracellular ice crystallization (Jiménez-Rabadán et al., 2015; Slabbert et al., 2015) and DNA damage that can be caused with use of more prolonged freezing methods (Rahiminia et al., 2017).

In humans, results of different studies indicate vitrification processing of semen led to improvement of values for some sperm variables such as motility, viability, acrosome integrity and DNA integrity compared to use of conventional freezing methods. Inconsistencies in outcomes, however, remain in regard to the most desirable method of cryopreserving human sperm (Le et al., 2019). In animals, there are only few studies on vitrification protocols (Pérez-Marín et al., 2018) and in recent reports there has been little information regarding the vitrification process for stallion semen (Hidalgo et al., 2018; Pérez-Marín et al., 2018).

In stallions and in other animal species, cryoprotectants are essential for these processes to avoid adverse reactions (Pérez-Marín et al., 2018). Disaccharides are used as non-permeable cryoprotectants and the effects have been investigated (Isachenko et al., 2008; Wu et al., 2015; Caturla-Sánchez et al., 2018; Golshahi et al., 2018; Pérez-Marín et al., 2018; Isachenko et al., 2019). With use of disaccharides, there is a recovery rate of motility and protection of the plasma membrane DNA integrity of spermatozoa to the extent sperm viability rates are acceptable for use of these samples for AI (Isachenko et al., 2008).

Hidalgo et al. (2018) analysed the effect of sucrose supplementation, while Pérez-Marín et al. (2018) tested both trehalose and sucrose as cryoprotectants for stallion sperm vitrification. Furthermore, the use of extenders containing trehalose or sucrose as compared to samples without these inclusions, at a range of concentration of between 0.15 and 0.2 M, are associated with a greater protective effect on the quality of sperm. Cryoprotectant-free vitrification approaches to cryopreservation of human sperm compared with conventional freezing results in greater sperm viability after devitrification of the samples (Isachenko et al., 2019). Few studies on vitrification have been published regarding the use of trehalose and lactose as cryoprotectants for stallion semen, and to the best of our knowledge, there are no studies evaluating stallion epididymal sperm vitrification processes. Epididymal collection may be a useful technique that could be used when there is impaired fertility in stallions (e.g., serious traumas or injuries, sudden death, castration or any other incident that makes semen collection impossible).

The purpose of this study was to determine the effect of vitrification on stallion epididymal sperm and to compare the results with ejaculate vitrification after the incorporation of non-permeable cryoprotectant (trehalose or lactose).

2. Materials and methods

The care and treatment of the animals used in this study was conducted in accordance with the Spanish Policy for Animal Protection RD 1201/05 and the directive 2010/63/EU for animal experimentation.

2.1. Reagents and media

All chemicals used in this study were obtained from Sigma-Aldrich Química S.A. (Madrid, Spain), unless otherwise indicated. The medium used for washing the semen and its centrifugation was INRA 96® (IMV Technologies; L'Aigle, France).

Lactose–egg-yolk extender containing 50% (v/v) of 290 mM L-lactose, 20% (v/v) of egg yolk, 25% (v/v) of glucose–EDTA medium (322.20 mM glucose, 12.58 mM sodium citrate, 9.93 mM disodium EDTA, 14.28 mM sodium bicarbonate), 0.5% (v/v) of Equex Paste (Gil et al., 2013) and 5%(v/v) of glycerol, was used as the control freezing extender. Vitrification medium contained INRA 96®, 1% bovine serum albumin (BSA) and 0.15 M trehalose or 0.15 M lactose.

2.2. Experimental design

In Fig. 1 there is a depiction of the schematic of the experimental design.

Sperm samples (n = 7) from the ejaculate and epididymis (n = 10) were treated with identical conservation media.

There were six experimental groups with differences in extenders and sperm origin: EJC (frozen-thawed ejaculate sperm), EJT (trehalose vitrified ejaculate sperm), EJL (lactose vitrified ejaculate sperm), EPC (frozen-thawed epididymal sperm), EPT (trehalose vitrified epididymal sperm) and EPL (lactose vitrified epididymal sperm).

2.3. Semen handling methods

2.3.1. Ejaculate collection, handling and dilution

Ejaculates were collected from seven healthy stallions (age 7–12 years old) for which the semen had adequate viability characteristics after cryopreservation and thawing. Semen collections occurred during the breeding season at the Horse Breeding Military Centre (HBMC) in Zaragoza (Spain) using an artificial vagina (Missouri-model; Nasco, WI, USA).

Macroscopic and microscopic assessments were performed immediately after collection. The initial concentration of spermatozoa was determined using a Spermacue® 12300/0500 device (Minitube Ibérica S.L., Tarragona, Spain). Sperm motility was analysed using the CASA System (ISAS®, Projectes I Serveis R + D S.L., Valencia, Spain). Samples with a minimum sperm concentration of 200×10^6/ml and sperm motility of greater than 50% were selected for the study.

Semen samples were diluted 1:1 in INRA 96® medium and centrifuged at 1000 g for 5 min (Alvarenga et al., 2016) to remove seminal plasma. Samples were divided into three aliquots and re-suspended into different media based on the experimental design (EJC, EJT and EJL). The final semen concentration after dilution was 100 x 10^6 sperm/mL (Samper, 2009).

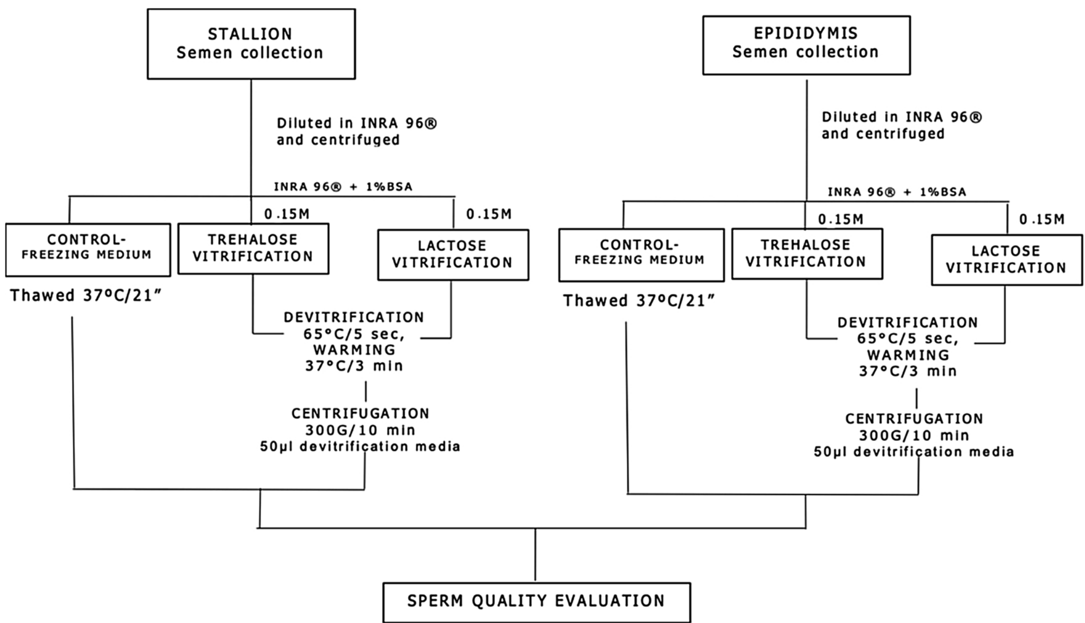

Fig. 1. Schematic of the experimental design – same preservation media was used for sperm samples (n = 7) from ejaculates and epididymides (n = 10); Samples were then placed in two vitrification media, INRA 96® + 1% bovine serum albumin + 0.15 M Trehalose or 0.15 M Lactose. A conventional freezing group was used as a control for both ejaculate and epididymides samples; After thawing/devitrifying, the quality of the sperm samples was evaluated.

2.3.2. Epididymal sperm collection, handling and dilution

Ten epididymides were collected from the carcasses of five stallions at the slaughterhouse (Mercazaragoza, Zaragoza, Spain). Sperm from the epididymis were collected by retrograde flushing of the *cauda* epididymis (Álvarez et al., 2014; Olaciregui et al., 2014).

The *cauda* epididymis was dissected and cannulated with a 25 G needle connected to a 10 ml of INRA 96® syringe at 20 °C. Manual pressure was then applied to the syringe, and spermatozoa were collected in a beaker. Sperm samples were centrifuged at 1,000 *g* for 5 min at room temperature and the supernatant was discarded (Olaciregui et al., 2014). The remaining sperm was extended to achieve a concentration of 100 × 10^6 sperm/mL with the medium (EPC, EPT or EPL).

2.3.3. Cryopreservation

2.3.3.1. Freezing and thawing. The samples were frozen using a stabilization step process at a gradual temperature-cooling rate of approximately 0.5 °C per minute. The samples were cooled to 4 °C in a conventional freezer for 2 h and packaged at 4 °C into 0.5 ml polyvinylchloride straws, (IVM technologies; L´Aigle, France). The samples were then frozen in LN2 vapour, 4 cm above the surface for 20 min. The straws were subsequently plunged directly into LN2 (-196 °C) in a styrofoam freezer box with a neopor insulation block (Minitub Ibérica S.L., Tarragona, Spain). Frozen sperm samples were thawed in circulating water at 37 °C for 21 s.

2.3.3.2. Vitrification and devitrification. Initially, 50 µl of each group were immersed directly in a cryotube containing 300 µl of LN2, which was subsequently sealed. The cryotubes, each containing three spheres, were then stored in LN2 tanks.

The devitrification process consisted of the vitrified sperm spheres being immersed in 500 µl of warming medium (INRA 96® + 1% BSA) at 65 °C for 5 s and maintained at 37 °C for 3 min. The samples were then centrifuged at 300 *g* for 10 min and the final pellet was re-suspended with 50 µl of warming medium for semen quality evaluation.

2.4. Semen quality evaluation

Total motility (TM) and progressive motility (PM) combined with kinematic variables were analysed using a computer-assisted analysis ISAS® (Projectes I Serveis R + D S.L., Spain; Holt et al., 2007). A total of 25 consecutive digital images were obtained with a time lapse of 1 s and the particle area was between 4 and 75 μ^2. With regard to the variables evaluated using the program, spermatozoa with an average path velocity (VAP) < 10 µ/s were considered slow, > 45 µ/s moderate, and > 90 µ/s fast. Spermatozoa with 75% of the straightness (STR) were designated as progressively motile. Spermatozoa were considered hyperactive if curvilinear velocity (VLC) was ≥180 and the amplitude of lateral head displacement (ALH; i.e., twice the maximum displacement of a sperm head from its fitted moving axis in a track segment) was ≥12 (Neuhauser et al., 2018; Rathi et al., 2001).

Sperm viability was evaluated using the LIVE/DEAD® sperm viability kit (Molecular Probes Europe, Leiden, The Netherlands). A

volume of 100 µl of diluted sperm was mixed with 150 µl buffer (Becton Dickinson Immunochemistry, San Jose, CA, USA) with a final concentration of 20 × 10^6 sperm/ml. The SYBR-14 (20 nM) and propidium iodide (PI; 10 nM) were mixed with the different sperm samples. After incubation in the darkness for 15 min at room temperature, the proportion live/dead sperm cells was determined. Samples were analysed using a phase-contrast fluorescence microscope (Leika Microscope, DM2500 LED, l´Hospitalet de Llobregat, Spain). Two hundred spermatozoa were evaluated.

Acrosome status was assessed using fluorescein isothiocyanate combined with peanut agglutinin (FITC-PNA) and a propidium iodide (PI) stain. An aliquot of sperm suspension from each treatment group was supplemented with FITC-PNA solution (1 mg/ml in double distilled water) and PI solution (500 µg/ml), maintained at 38 °C for 5 min, and then fixed in paraformaldehyde (4% [v/v]) in a saline solution. At least 200 spermatozoa were examined using a fluorescence phase-contrast microscope.

2.5. Statistical analysis

All data obtained were analysed using computer software SPSS version 22.0 for Windows (SPSS. PC software, Chicago, Il, USA). The statistical procedure used for the analysis sample began with a visual exploration of data (boxplots) and an outline of centrality (mean and median) and variability (standard deviation) estimates. Kolmogórov-Smirnov tests were used to verify the normality of the data. Comparisons between the values of total motility, progressive motility, viability, acrosome integrity, VCL; VSL, VAP, SRT, BCF from different groups were analysed using a one-way analysis of variance (ANOVA). The LIN, WOB and ALH values were compared using the Kruskal–Wallis test.

In those cases where significant differences were observed, values were compared using the least significant difference pairwise multiple comparisons *post hoc* test (Tukey HSD test). The quantitative variables were expressed as mean ± standard error. All tests were assessed at the conventional significant level of 0.05.

3. Results

The primary variables for assessing sperm quality were affected by the stallion, sperm origin (ejaculate or epididymis) and process (conventional or vitrification) of sperm preservation ($P < 0.01$).

Results for ejaculate control samples (EJC; conventional freezing) indicated these samples had greater sperm quality ($P < 0.05$) than vitrified samples for the ejaculate when stored with diluents containing trehalose (EJT) or lactose (EJL) for all variables evaluated except progressive motility (Fig. 2). For epididymal sperm samples, the results were different. The EPC group had greater percentages for sperm TM as compared to the EPL group ($P < 0.01$), although there were no differences when there was comparison to the results with these two groups to those of the EPT group. For PM data, with the EPC group there was a greater ($P < 0.05$) PM than with the other experimental groups, except for the EPL group. The vitrification of ejaculate sperm samples resulted in reduced ($P < 0.05$) viability and acrosome integrity percentages as compared to those of epididymal sperm samples and the EJC group.

In addition, when results from sperm origin (ejaculate and epididymis) were compared, the vitrification process with supplementation of diluent with trehalose or lactose resulted in greater ($P < 0.05$) values for epididymal sperm than ejaculate sperm samples in terms of TM, PM, viability and acrosome integrity. There, however, were no differences in ejaculated sperm compared with epididymal sperm when conventional freezing procedures were used.

With regard to velocity variables (Fig. 3), all values were greater ($P < 0.05$) when vitrification methods were used. The ALH data were similar between frozen and vitrified samples regardless of the combination of the disaccharides used (Fig. 4). The BCF after vitrification when there was both trehalose and lactose supplementation, was greater ($P < 0.05$) than with use of conventional freezing procedures (EJC or EPC).

4. Discussion

In the present study, there were similar results to those of previous studies with use of the vitrification technique for ejaculated stallion spermatozoa (Hidalgo et al., 2018; Pérez-Marín et al., 2018). Sperm quality results were different when lactose and/or trehalose were used to supplement the diluents used for sample preservation. Nevertheless, to the best of the authors knowledge, there are no results from previous studies that have addressed vitrification as a method of preserving stallion epididymal spermatozoa, and the results from the present study for the cryopreservation of epididymal sperm varied from that when there was ejaculated sperm vitrification. The results of the present study provide evidence that vitrification of epididymal sperm using trehalose as a diluent supplement results in an enhancement of sperm quality.

Trehalose and sucrose are usually combined with BSA for sperm vitrification (Isachenko et al., 2008; Sánchez et al., 2011; Merino et al., 2012; Slabbert et al., 2015; Diaz-Jimenez et al., 2017; Consuegra et al., 2018). The BSA, among its other properties, reduces oxidative stress (Uysal et al., 2005) and maintains membrane integrity. The BSA used was concentrated to 1% (Hidalgo et al., 2018) because, at a greater concentration, it has negative effects on sperm quality after cryopreservation (Naijian et al., 2013; Nang et al., 2012). The 1% limit in concentration is also consistent with findings from previous studies where there was sperm vitrification in other species (Sánchez et al., 2011; Merino et al., 2012; Pradieé et al., 2018). In the present study, results obtained at a concentration of 1% were also consistent with results from these studies.

In contrast to recent studies, where the use of cryoprotectant free vitrification for humans and fish sperm cryopreservation yielded acceptable results (Merino et al., 2012; Isachenko et al., 2019), the use of non-permeable cryoprotectants was more effective for maintenance of sperm viability of cryopreserved semen of most animals including horses. Trehalose and sucrose are the most studied

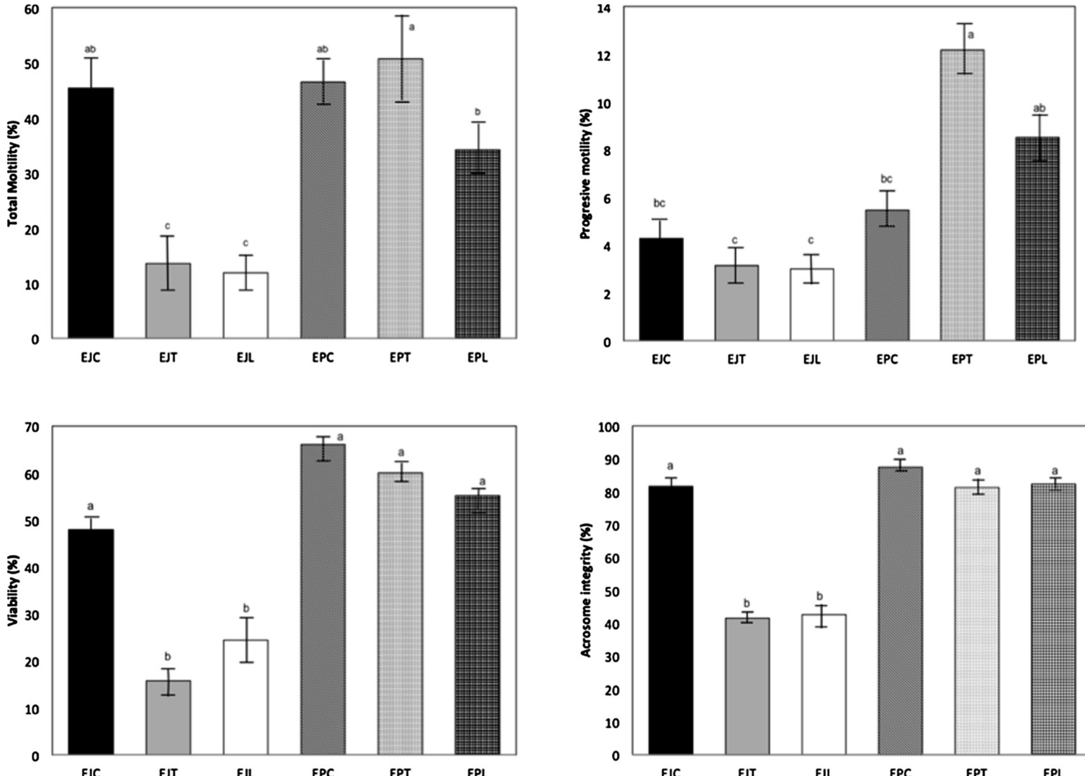

Fig. 2. Effect of sperm origin (ejaculated or epididymal) and extender on total motility, progressive motility, viability and acrosome integrity after preservation; EJC: Ejaculated sperm freeze-thawed; EJT: Ejaculated sperm vitrified with trehalose; EJL: Ejaculated sperm vitrified with lactose; EPC: Epididymal sperm freeze-thawed; EPT: Epididymal sperm vitrified with trehalose; EPL: Epididymal sperm vitrified with lactose (Mean ± SEM, ejaculate samples $n = 7$; epididymis samples $n = 10$); Superscripts ([a,b,c]) indicate differences ($P < 0.05$).

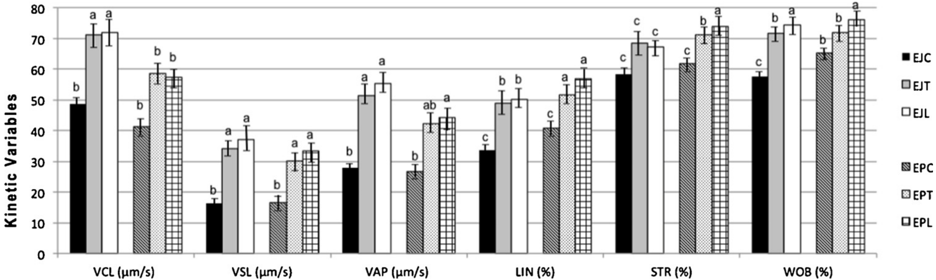

Fig. 3. Effect of sperm origin and extender on kinetic variables (VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness and WOB: wobble) after the conservation process.
EJC: Ejaculated sperm freeze-thawed; EJT: Ejaculated sperm vitrified with trehalose; EJL: Ejaculated sperm vitrified with lactose; EPC: Epididymal sperm freeze-thawed; EPT: Epididymal sperm vitrified with trehalose; EPL: Epididymal sperm vitrified with lactose (Mean ± SEM, ejaculate samples $n = 7$; epididymis samples $n = 10$) Superscripts ([a,b,c]) indicate differences ($P < 0.05$).

diluent supplemental components for spermatozoa preservation with the vitrification process (Caturla-Sánchez et al., 2018; Consuegra et al., 2018; Diaz-Jimenez et al., 2018; Hidalgo et al., 2018; Pérez-Marín et al., 2018). Results of only a few studies indicate trehalose is more effective than sucrose as a non-permeable cryoprotectant for sperm storage (Schulz et al., 2017). Results from the present research indicate that with supplementation of semen diluent with either trehalose or lactose there is no difference in sperm quality outcomes when there is use of these two supplements for cryopreservation of ejaculated vitrified samples. These results were consistent with those from recent studies (Pérez-Marín et al., 2018) in horses, or Caturla-Sánchez et al. (2018) in dogs. Nevertheless, with sperm epididymal samples, the addition of trehalose in the vitrification media resulted in a greater post-thaw motility than with use of lactose.

In results from recent studies (Pérez-Marín et al., 2018; Restrepo et al., 2019), there was a negative effect of the vitrification process on sperm quality as compared with using the conventional freezing process for the preservation of sperm. In addition, in these

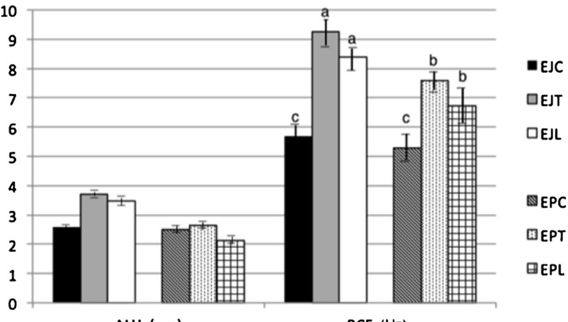

Fig. 4. Effect of sperm origin (ejaculated or epididymal) and extender on ALH (amplitude of lateral head) and BCF (beat cross frequency) variables after the preservation process; EJC: Ejaculated sperm freeze-thawed; EJT: Ejaculated sperm vitrified with trehalose; EJL: Ejaculated sperm vitrified with lactose; EPC: Epididymal sperm freeze-thawed; EPT: Epididymal sperm vitrified with trehalose; EPL: Epididymal sperm vitrified with lactose (Mean ± SEM, ejaculate samples $n = 7$; epididymis samples $n = 10$); Superscripts (^{a,b,c}) indicate differences ($P < 0.05$).

previous studies sperm quality was markedly decreased when the vitrification process was conducted, both with trehalose and lactose in the vitrification medium, with the most desirable results at lesser concentrations (0.15 M). The present study, therefore, was conducted with a 0.15 M concentration and there were similar values for sperm quality when ejaculated sperm samples were evaluated whereas there was a marked decrease in sperm quality when there was use of the vitrification process compared with use of the conventional freezing method for sperm cryopreservation. Nevertheless, post-thaw sperm quality after the imposing of the vitrification process on epididymal samples was similar to that of the control samples. Hidalgo et al. (2018), reported that there were greater sperm quality values for ejaculated samples with the process of vitrification with sucrose plus BSA, but at different concentrations (sucrose at 20, 50, and 100 mM and BSA at 1%, 5%, and 10%).

In the present study, devitrification was performed at 65 °C for 5 s. Caturla-Sánchez et al. (2018) evaluated the optimal temperature during the devitrification process, while Pérez-Marín et al. (2018) reported that in stallions, devitrification at relatively greater temperatures for a short period of time (65 °C for 5 s) resulted in a greater sperm quality as compared with use of other devitrification conditions. Based on the findings from this previous research, in the current study, similar temperatures were applied for both ejaculated semen and epididymal samples. Results with vitrification of the epididymal samples indicated the sperm of these samples were of greater quality than those of ejaculated semen.

From results of the present study, it is concluded that stallion sperm vitrification is affected by sperm origin (i.e., ejaculated compared with epididymal sperm). Thus, vitrification with stallion epididymal spermatozoa resulted in greater sperm quality than that of sperm from stallion ejaculates. There is not exposure of epididymal sperm to seminal plasma which is different from what occurs with ejaculated sperm. Seminal plasma has a greater pH and greater Na^{++} and Cl^{-} content, which have been reported to be detrimental to sperm survival during the conservation process (Kareskoski et al., 2006; Monteiro et al., 2013). In addition, results of different studies indicate there is a deleterious effect of seminal plasma on sperm quality due to biochemical changes that increase membrane permeability (Aurich et al., 1996; Lozano et al., 2011) which cause greater susceptibility to freezing damage on the sperm plasma membrane.

The incorporation of 0.15 M of trehalose in the sperm vitrification medium could be a useful alternative for a long-term storage. Ejaculate-vitrified sperm, however, were of lesser quality for cryopreservation than sperm that are cryopreserved using conventional practices.

The findings of the current study indicate the importance of continued development of reliable protocols regarding the vitrification process for stallion sperm. These protocols could also be extended to other animal species.

Funding

This research was supported by the Government of Aragon (Ref. Group A17_17R-RAySA) co-financed with FEDER 2014-2020 "Building Europe from Aragon”.

Declaration of Competing Interest

None.

Acknowledgments

The authors wish to acknowledge the assistance and support of the Military Horse breeding Centre (Zaragoza, Spain), Department of Animal Pathology, Obstetric and Reproduction Area (Faculty of Veterinary Medicine, Zaragoza University, Spain), Paula Alvarez and Ana Estevez for their contribution on the epididymis vitrification research.

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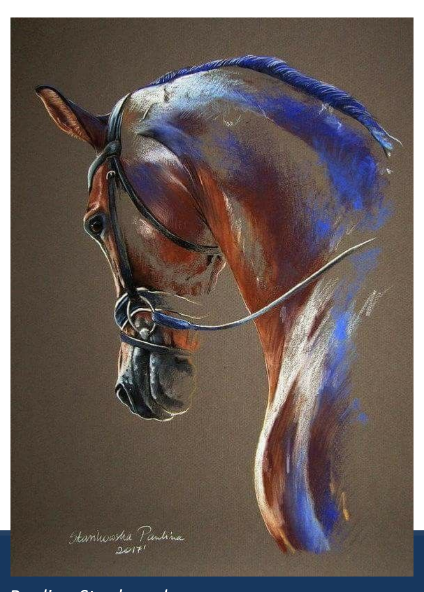
6. Publicaciones previas al Desarrollo de la Tesis Doctoral, iniciación a estudios sobre mejora de la calidad seminal en los equinos



Paulina Stanhowska

Artículo 5: Efecto de la adición de plasma seminal en el semen equino descongelado.

Sanid Mil (2011), 67 (3), 284-290

ACCESIT FIDEL PAGÉS MIRAVÉ 2011

Efecto de la adición de plasma seminal en el semen equino descongelado

Lozano Benito D.[1], Gil Huerta L.[2], Álvarez San Martín C.[3]

Sanid. mil. 2011; 67 (3): 284-290; ISSN: 1887-8571

RESUMEN

Antecedentes y objetivos: El semen criopreservado ofrece beneficios adicionales no presentes en el semen refrigerado. Sin embargo, varios factores afectan al éxito en la inseminación artificial con semen congelado de caballos. El objetivo del trabajo es evaluar si la adición de plasma seminal a diferentes concentraciones, sobre espermatozoides equinos descongelados, afecta a la motilidad espermática, viabilidad y a nivel de membrana. **Material y métodos:** Se utilizaron diferentes razas, cuatro sementales de silla, y dos sementales de tiro. En un primer experimento el semen descongelado se centrifugó, mientras en el segundo no se centrifugó. A continuación, se adicionó el plasma seminal al 10, 20, 30% suspendido en solución tampón fosfato y plasma seminal puro (100%). **Resultados**: En los caballos de silla el plasma seminal no afectó a los parámetros estudiados (p>0,05), pero se apreció un posible efecto tóxico del plasma seminal puro sobre las características espermáticas. En las muestras con plasma seminal de los caballos de tiro, se observaron unos índices mejores en espermatozoides vivos con acrosoma intacto que en las muestras control. Asimismo se obtuvo un porcentaje menor en espermatozoides reaccionados que en las muestras control, encontrando en esta categoría una diferencia significativa (p<0,05). **Conclusiones:** La incubación de los espermatozoides descongelados con plasma seminal puede frenar la reacción acrosómica, reflejado este hecho, en los bajos porcentajes de espermatozoides que han sufrido la verdadera reacción acrosómica.

PALABRAS CLAVE: Plasma seminal, Espermatozoides descongelados, Equino, Reacción acrosómica, Inseminación artificial.

Effect of seminal plasma addition on frozen-thawed equine semen

SUMMARY

Background and objectives: Stallion sperm cryopreservation offers benefits not available in cooled semen. However various factors affect the success of artificial insemination with frozen-thawed equine semen. This study aims to evaluate if adding different concentrations of seminal plasma on frozen-thawed equine spermatozoa affects sperm motility, viability and membrane status. **Material and Methods**: Different breeds were used; four saddle stallions and two draft stallions. In the first experiment thawed semen was centrifuged and in the second one it was not. Subsequent to that, the spermatozoa resuspended with 10, 20, 30% seminal plasma in phosphate buffered saline and pure seminal plasma (100%). **Results:** semen parameters of saddle stallions were not affected (p>0,05), but a possible toxic effect of pure seminal plasma was observed on sperm characteristics. Seminal plasma samples in draft breed got better rates in viable sperm with intact acrosome. A lower percentage was also found on spermatozoa with acrosome reaction than in control samples. This category showed significant differences (p<0,05). **Conclusions:** Post-thawing spermatozoa incubation with seminal plasma can stop acrosome reaction, due to the low percentage of spermatozoa suffering true acrosome reaction.

KEY WORDS: Seminal plasma, Frozen-thawed spermatozoa, Equine, Acrosome reaction, Artificial insemination.

Quija Board L. (Mc. Mahon)

Artículo 6: *Effect of cholesterol and Equex-STM addition to an egg yolk extender on Pure Spanish Stallion cryopreserved sperm, ISRN Vet. Sci. (2013), 21–23*

Hindawi Publishing Corporation
ISRN Veterinary Science
Volume 2013, Article ID 280143, 6 pages
http://dx.doi.org/10.1155/2013/280143

Research Article

Effect of Cholesterol and Equex-STM Addition to an Egg Yolk Extender on Pure Spanish Stallion Cryopreserved Sperm

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Received 26 September 2013; Accepted 30 October 2013

Academic Editors: Ø. Bergh and M. H. Kogut

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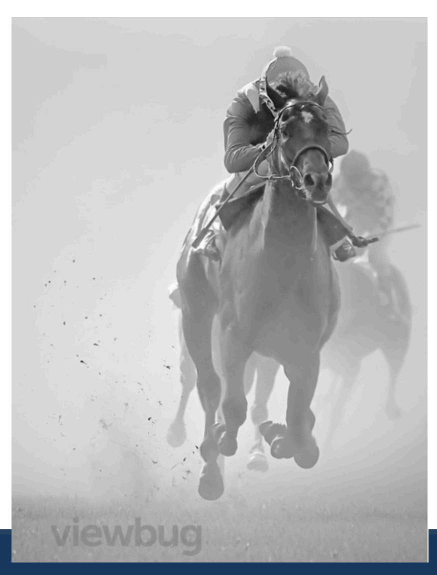
Cholesterol and Equex-STM are frequently added to different commercial and experimental extenders improving postthawing sperm quality. Doses of 125–150 mM of cholesterol from pig liver and 0.5–0.7% of Equex-STM were evaluated in a standard eggyolk extender (Martin et al., 1979). Six ejaculates per stallion from six pure Spanish stallions (6–8 years old) were collected in Martin's extender (B) and different mixtures of 125 mM-0.5% (I), 125 mM-0.7% (II), 150 mM-0.5% (III), and 150 mM-0.7% (IV) were added to original Martin's extender. Samples were frozen in 0.5 mL straws (100×10^6 spermatozoa) and thawed (21 s., 37°C water bath). After thawing the following parameters were evaluated: viability (V), motility (computer assisted sperm analysis, CASA; % nonprogressive NP; % progressive MP), hipoosmotic swelling test (HOST), acrosome integrity (A), fluorescence test (FL), and resistance test (RT). Sperm quality was significantly affected by stallion (in the parameters V, VI, NP, MP, HOST, A, FL, and RT), extraction (VI, NP, MP, HOST, A, and FL), and the different combinations of Equex-STM-cholesterol (FL). We concluded that 0.5% of Equex-STM mixed with 125 mM of cholesterol has obtained better sperm quality results than those of original Martin's extender, showing a simple and economic improvement of this home-made practical seminal extender.



Races at Zagreb Hippodrome (Bojan Bencic)

Artículo 7: *Equine sperm post-thaw evaluation after the addition of different cryoprotectants added to INRA 96® extender.*

Cryobiology (2014), 69, 144–148

Cryobiology 69 (2014) 144–148

Contents lists available at ScienceDirect

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journal homepage: www.elsevier.com/locate/ycryo

Equine sperm post-thaw evaluation after the addition of different cryoprotectants added to INRA 96® extender ☆

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A R T I C L E I N F O

Article history:
Received 30 January 2014
Accepted 25 June 2014
Available online 2 July 2014

Keywords:
Cryopreservation
Equine sperm
Dimethylformamide
Glycerol
Acetal

A B S T R A C T

The rise of assisted reproduction techniques in equine medicine has fostered investigations that seek to optimize methods to increase fertility rates. Since cryopreservation continues to give low values of viability in stallions, the handling and preservation of the sperm is of vital importance. This reduction of fertility makes it essential for farmers to find new options that ensure reliability in the use of these techniques. The main objective of this study was to assess the effect of INRA 96® (manufactured commercial extender for cooling of Equine semen) as an extender for cryopreservation in combination with different cryoprotectants: Acetal (5%), Dimethylformamide (5%) and Glycerol (5%), alone and combined (2.5% each) on ejaculated and epididymal spermatozoa. Ejaculates collected from mature stallion and epididymal sperm samples were cryopreserved in INRA® varying content of cryoprotectant and cryopreserved. Sperm motility, viability, hypoosmotic swelling test (HOST) and acrosome integrity were evaluated post-thawing. We conclude that INRA 96® is suited as extender for freezing when it is used in combination with Dimethylformamide (5%) or Dimethylformamide (2.5%) + Glycerol (2.5%) for samples of ejaculate. The combination of Dimethylformamide (2.5%) + Glycerol (2.5%) showed the best results on epididymal spermatozoa. In conclusion, the combination of Dimethylformamide and Glycerol as cryoprotectants in INRA® medium enhanced equine epididymal and ejaculated spermatozoa quality after cryopreservation.

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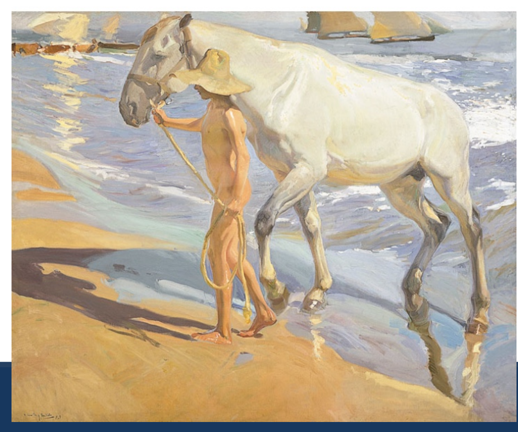

El baño del caballo (Joaquín Sorolla)

7. Conclusiones/ Conclusions

1. El calostro de yeguas utilizado como un componente en el diluyente para congelación de espermatozoides equinos ejerce un efecto protector en ellos y podría ser una interesante alternativa en los sistemas de criopreservación, además, por su composición podría resultar útil en yeguas que presentan cuadros de endometritis persistentes.

2. En la congelación de eyaculados de burro, la calidad seminal post-descongelación es significativamente mejor cuando se utiliza calostro de burra en la composición de los diluyentes de congelación, si bien, la calidad espermática es buena igualmente cuando se adiciona el calostro de yegua. Ambos tipos de calostro se pueden considerar como una alternativa a otros componentes de los diluyentes de criopreservación espermática.

3. La vitrificación de los espermatozoides procedentes de epidídimo o de eyaculados, en la especie equina, puede considerarse una alternativa a las técnicas de criopreservación, siendo los espermatozoides epididimarios los que afrontan mejor el proceso, siempre que el crioprotector utilizado sea la Trealosa a una concentración 0,15M incorporada en el medio de vitrificación.

7. Conclusions

1. Mare Colostrum used as a component in equine sperm freezing extenders exerts a protective effect, and it could be an interesting alternative in cryopreservation methods. In addition, due to its composition, it could be useful in mares which have persistent endometritis processes.

2. Post-thaw seminal quality of donkey semen freezing is significantly better when donkey colostrum is used in freezing extenders composition, although sperm quality is also good when mare colostrum is added. Both types of colostrum can be considered as an alternative to other components for sperm cryopreservation extenders.

3. Vitrification of epididymis or ejaculate spermatozoa, in equine species, can be considered an alternative to cryopreservation techniques. Epididymal sperm is the one that best cope with the process, provided that the cryoprotectant used is Trehalose at a 0.15M concentration incorporated into the vitrification medium.

Francois Bergeron

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9. Apéndice

Factor de Impacto y Área Temática de las revistas

REVISTA	FACTOR DE IMPACTO WoS-JCR	QUARTILE	AREA TEMATICA
CRIOBIOLOGY	2.141	Q2	BIOLOGY
JOURNAL OF EQUINE VETERIARY SCIENCE	0.927	Q3	VETERINARY SCIENCES
REPRODUCTION IN DOMESTIC ANIMALS	1.638	Q2	VETERINARY SCIENCE
ANIMAL REPRODUCTION SCIENCE	1.817	Q1	AGRICULTURE, DIARY & ANIMAL SCIENCE

CONTRIBUCION:

La modalidad elegida para desarrollar esta Tesis Doctoral ha sido la modalidad por compendio de publicaciones, por lo que paso a detallar como he contribuido en los artículos que la componen:

He realizado la revisión bibliográfica de todos ellos, para poder establecer así el diseño experimental de una manera coherente, con el material y métodos adecuados.

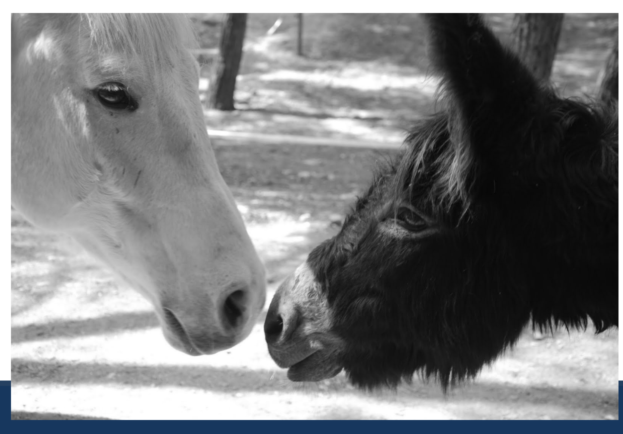
Una vez establecido el material y métodos a seguir, contribuí con la aportación de parte del material biológico con el que se trabajó en cada uno de ellos, obteniendo los eyaculados provenientes de sementales vivos. Las muestras obtenidas a partir de testículos de matadero fueron obtenidas por el equipo que me ha ayudado en el desarrollo de la tesis, puesto que para acceder al matadero eran necesarios permisos especiales. Una vez en el laboratorio, ya

pude colaborar en el procesado de las mismas. El calostro lo obtuve de las yeguas y burras gestantes del CMCCZ justo en el momento del parto y antes de transcurridas 3 horas.

También he desarrollado la mayor parte del trabajo experimental en el laboratorio, procesando las muestras en sus diferentes protocolos de criopreservación y posteriormente de contrastación seminal.

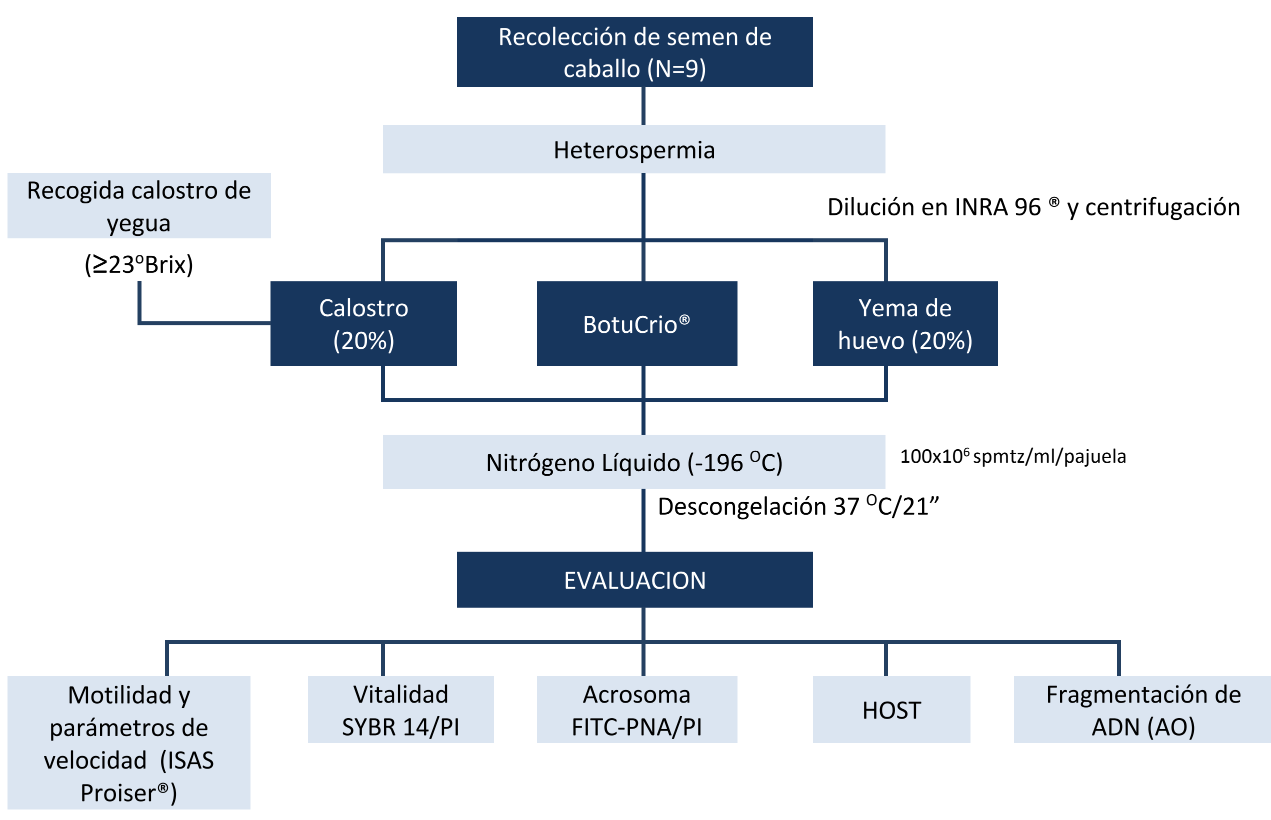
Sobre los resultados obtenidos he aplicado un tratamiento estadístico para poder llegar a las conclusiones en cada uno de los artículos, y con todo ello poder llegar a la redacción de los mismos.

Además, en la redacción del capítulo 8 del libro "*Biotechnologies applied to animal reproduction, current trends and practical applications for reproductive management*", he colaborado en la ampliación y redacción de las nuevas técnicas relacionadas con las mejoras en la calidad seminal en la criopreservación de la especie equina, trabajo en el que se ha centrado todo mi trabajo experimental y que es, además, título de la Tesis que nos ocupa.

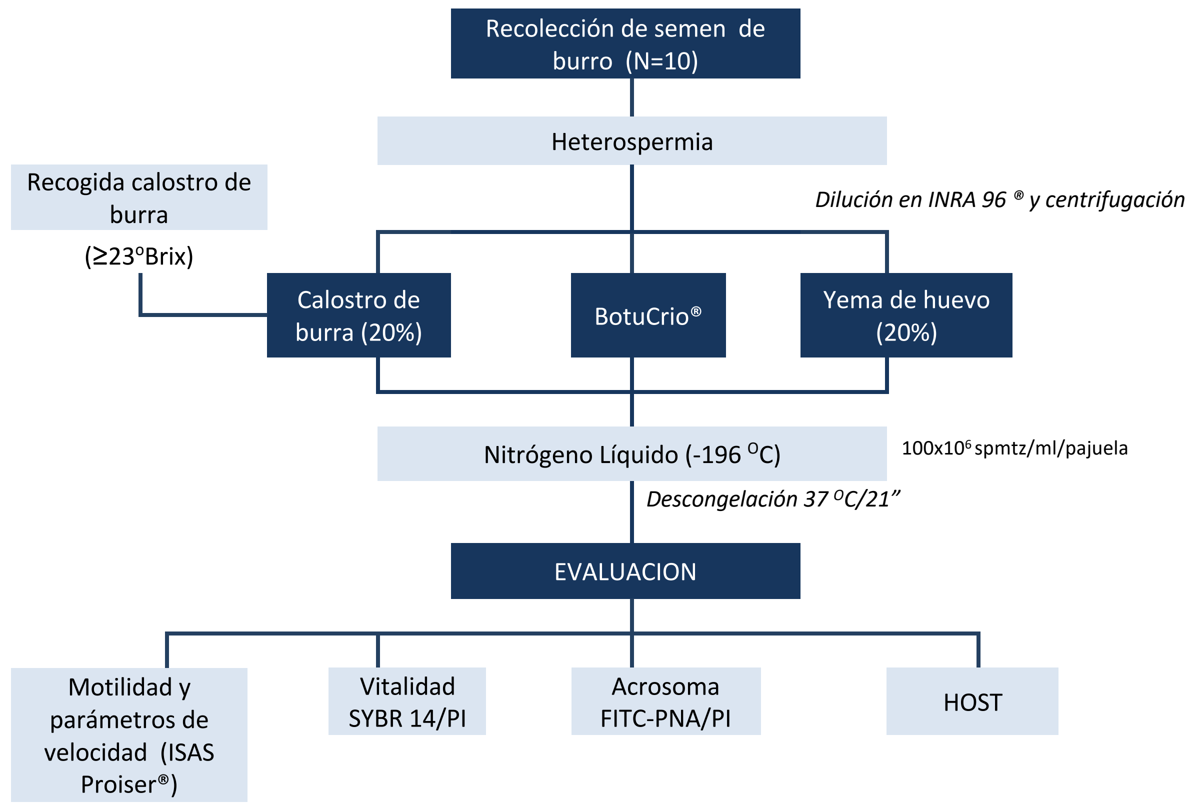


ANEXO I- Diseños experimentales

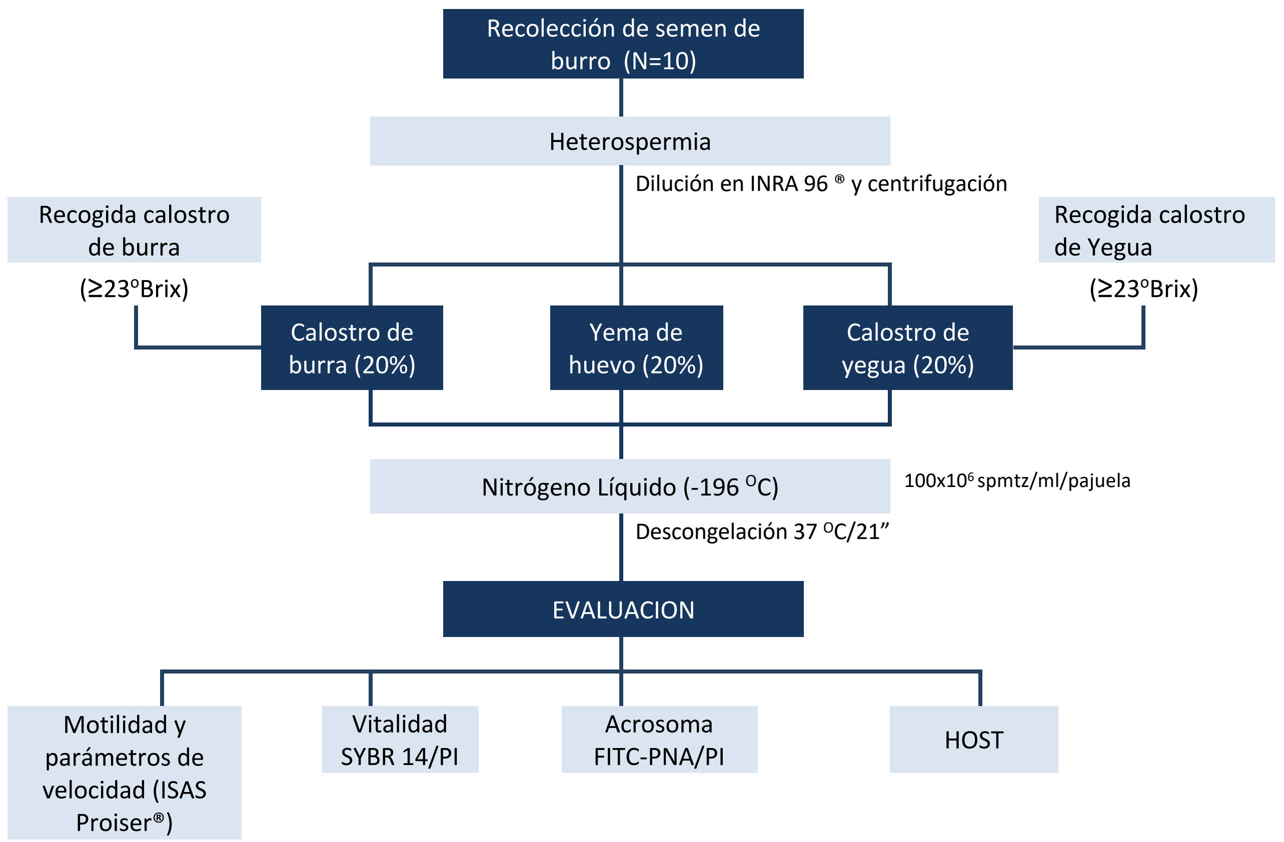
ARTICULO 1. Effect of Mare Colostrum in Extenders for Freezing Stallion Semen.



ARTICULO 2. A preliminary study on the use of jenny colostrum to improve quality in extenders for freezing donkey semen.



ARTICULO 3. Alternatives in Donkey semen cryopreservation: Mare vs. Jenny Colostrum.



ARTICULO 4. Vitrification of ejaculated and epididymal sperm: the quality after vitrification is affected by sperm origin.