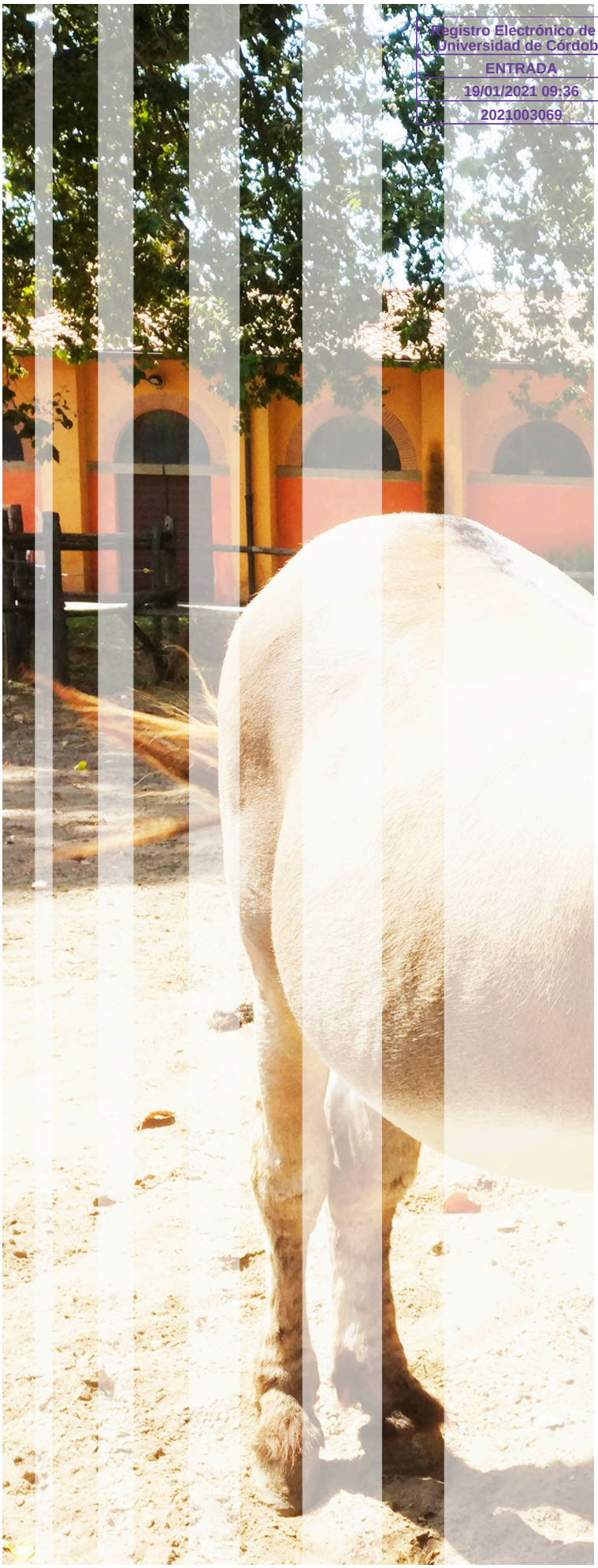
TESIS DOCTORAL

Optimización de la congelación y vitrificación de esperma de asno

Optimization of donkey sperm freezing and vitrification

MARÍA ÁNGELES DÍAZ JIMÉNEZ

2020



UNIVERSIDAD Ð CÓRDOBA

UNIVERSITÀ DI PISA

TITULO: *Optimization of donkey sperm freezing and vitrification*

AUTOR: *María Ángeles Díaz Jiménez*

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Facultad de Veterinaria

UNIVERSITÁ DI PISA

Corso di Dottorato in Scienze Veterinarie

Tesis Doctoral en Cotutela con Mención Internacional

Programa de Doctorado

Biociencias y Ciencias Agroalimentarias

Optimización de la congelación y vitrificación de esperma de asno

"Optimization of donkey sperm freezing and vitrification"

Doctoranda:

María Ángeles Díaz Jiménez

Directores:

Manuel Hidalgo Prieto

Jesús M. Dorado Martín

Alessandra Rota

Córdoba, noviembre de 2020

FINANCIACIÓN

- La Doctoranda ha sido subvencionada para la realización del presente trabajo de Tesis Doctoral por el Programa de ayudas para la Formación de Profesorado Universitario (FPU 15/02472), concedido por el Ministerio de Educación, Cultura y Deporte.

- La estancia realizada durante tres meses en el Departamento de Obstetricia y Ginecología de la Universidad de Colonia (Alemania), para la obtención de la Mención Internacional de la Tesis Doctoral, fue parcialmente financiada por las Ayudas Erasmus+ de movilidad internacional de estudiantes de posgrado para prácticas.

- La estancia realizada en el Departamento de Ciencias Veterinarias de la Universidad de Pisa (Italia) durante seis meses para la realización de la Tesis Doctoral en régimen de cotutela, fue financiada por las Ayudas complementarias de movilidad, para realizar traslados temporales y destinadas a beneficiarios del programa FPU, pertenecientes al Ministerio de Educación y Formación Profesional.

- El Proyecto AGL-2013-42726-R titulado "Optimización de la técnica de vitrificación de gametos y embriones equinos" fue concedido por la Dirección General de Investigación Científica y Técnica del Ministerio de Economía y Competitividad y ha financiado la fase experimental del presente trabajo de Tesis Doctoral

TÍTULO DE LA TESIS:

Optimización de la congelación y vitrificación de esperma de asno

"Optimization of donkey sperm freezing and vitrification"

DOCTORANDA: María Ángeles Díaz Jiménez

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

D. Manuel Hidalgo Prieto y D. Jesús M. Dorado Martín, profesores del Departamento de Medicina y Cirugía Animal de la Facultad de Veterinaria de la Universidad de Córdoba, y Dña. Alessandra Rota, profesora del Departamento de Ciencias Veterinarias de la Universidad de Pisa,

Informan:

Que la Tesis Doctoral titulada **"**Optimización de la congelación y vitrificación de esperma de asno", ha sido realizada por la doctoranda D.ª María Ángeles Díaz Jiménez y dio comienzo en el curso académico 2015/2016. El objetivo principal de la misma fue optimizar la técnica actualmente empleada para la criopreservación del semen de asno, así como desarrollar y optimizar la técnica de vitrificación de esperma.

Parte de los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral han sido publicados como nueve artículos científicos en cinco revistas diferentes indexadas en el JCR, siendo ésta elaborada por compendio de publicaciones.

Tesis por compendio de publicaciones:

1. **M. Diaz-Jimenez**, J. Dorado, I. Ortiz, C. Consuegra, B. Pereira, C.A. Gonzalez-De Cara, R. Aguilera, G. Mari, B. Mislei, C.C. Love, M. Hidalgo. *Cryopreservation of donkey sperm using non-permeable cryoprotectants*. Animal Reproduction Science (2018). Vol 189: 103-109.
2. **M. Diaz-Jimenez**, J. Dorado, B. Pereira, C. Consuegra, I. Ortiz, M. Hidalgo. *Is sperm cryopreservation in absence of permeable cryoprotectants suitable for subfertile donkeys?* Reproduction in Domestic Animals (2019). Vol 54 (4): 102–105.
3. M. Hidalgo**, M. Diaz-Jimenez**, C. Consuegra, B. Pereira, J. Dorado. *Vitrification of donkey sperm: Is it better using permeable cryoprotectants?* Animals (2020). Vol 10 (9): 1-11.
4. **M. Diaz-Jimenez**, B. Pereira, I. Ortiz, C. Consuegra, A. Partyka, J. Dorado, M. Hidalgo. *Effect of different extenders for donkey sperm vitrification in straws.* Reproduction in Domestic Animals (2017). Vol 52 (4): 1-3.
5. **M. Diaz-Jimenez**, J. Dorado, C. Consuegra, I. Ortiz, B. Pereira, J.J. Carrasco, V. Gomez-Arrones, A. Domingo, M Hidalgo. *Optimization of donkey sperm vitrification: effect of sucrose, sperm concentration, volume and package (0.25 and 0.5 mL straws)*. Animal Reproduction Science (2019). Vol 204: 31-38.
6. **M. Diaz-Jimenez**, J. Dorado, B. Pereira, I. Ortiz, C. Consuegra, M. Bottrel, E. Ortiz, M. Hidalgo. *Vitrification in straws conserves motility features better than spheres in donkey sperm*. Reproduction in Domestic Animals (2018). Vol 53 (2): 56-58.
7. **M. Diaz-Jimenez**, J. Dorado, C. Consuegra, B. Pereira, I. Ortiz, C. Vazquez, E. Isachenko, V. Isachenko, M. Hidalgo. *Effect of warming temperatures on donkey sperm vitrification in 0.5mL straws in comparison to conventional freezing.* Spanish Journal of Agricultural Research (2019). Vol 17 (3): 1-7.
8. **M. Diaz-Jimenez,** J. Dorado, C. Consuegra, B. Pereira, M. Hidalgo. *Vitrification of donkey sperm using straws as an alternative to conventional slow freezing.* Reproduction in Domestic Animals (2020). Vol 00: 1-4.
9. **M. Diaz-Jimenez**, A. Rota, J. Dorado, C. Consuegra, B. Pereira, F. Camillo, D. Panzani, D. Fanelli, M. Tesi, D. Monaco, M. Hidalgo. *First pregnancies in jennies with vitrified donkey semen using a new warming method*. Animal Journal (2020). *In Press*

En referencia al Plan de Formación para el Doctorado en Biociencias y Ciencias Agroalimentarias, la doctoranda ha realizado satisfactoriamente las dos actividades obligatorias propuestas, así como varias actividades optativas por curso, como se recoge en el documento de actividades que acompaña esta Tesis.

Con objeto de ampliar su formación y profundizar en el estudio de la vitrificación de esperma, así como para optar a la Mención Internacional, la doctoranda realizó una estancia de tres meses en el Departamento de Obstetricia y Ginecología de la Universidad de Colonia (Alemania).

Asimismo, para realizar la Tesis en cotutela con la Universidad de Pisa (Italia), la doctoranda completó un período de seis meses en el Departamento de Ciencias Veterinarias de dicha Universidad.

La presente Tesis Doctoral ha sido revisada, reuniendo a nuestro juicio todos los requisitos necesarios para su lectura y defensa por compendio de publicaciones, en régimen de cotutela con la Universidad de Pisa (Italia), así como para obtener la Mención internacional.

Y para que conste, en cumplimiento de las disposiciones vigentes, se autoriza la lectura y defensa de la Tesis doctoral.

Córdoba, 3 octubre de 2020

Firma de los directores

Fdo.: D. Manuel Hidalgo Prieto

Fdo.: D. Jesús M. Dorado Martín

Fdo.: Dña. Alessandra Rota

INTERNATIONAL DOCTORS REPORT
DOCTORAL THESIS

REFEREE REPORT ON THE PhD TESIS PRESENTED IN THE UNIVERSITY OF CÓRDOBA (SPAIN) BY **MARÍA DE LOS ÁNGELES DÍAZ JIMÉNEZ**.

TITLE OF THE THESIS:	Optimization of donkey sperm freezing and vitrification

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This thesis meets the requirements for presentation as an oral dissertation: (X) YES ( ) NO

RATING

Originality:	(X) Outstanding	( ) Excellent	( ) Very Good	( ) Good	( ) Sound	( ) Defficient
Scientific/ technical merit:	(X) Outstanding	( ) Excellent	( ) Very Good	( ) Good	( ) Sound	( ) Defficient
Planning/ methodology:	(X) Outstanding	( ) Excellent	( ) Very Good	( ) Good	( ) Sound	( ) Defficient

COMMENTS (Please use additional sheets, if necessary):

The manuscript hereby presented by the candidate María AÁgeles Díaz Jiménez to opt for the grade of Philosophiae doctor by the Universities of Córdoba and Pisa is a clear example of how basic and applied science could be developed at the same time.

During their Ph.D. , Ms. Díaz Jimenez developed, from the very beginning, a new integral technology which allowed, for the first time, to obtain live offspring from vitrified sperm in donkeys. This fact in itself deserves great recognition. But also, during all the chapters of the thesis, the candidate demonstrated the effect of different kind of cryoprotectants, energy sources, freezing technologies, and warming procedures in the viability of the frozen sperm in donkeys, which is a species well know by the lack of reliability and efficiency in sperm freezing

Universidad de Córdoba

techniques. In the opinion of this reviewer, all the research made on endangered and "non-commercial" species is a plus, since the start point is always way more behind that in livestock species.
In terms of quality and productivity, the excellency of this study is demonstrated, beyond any doubt, by the fact that 9 JCR publications were derived from the experimental procedures, most of them in highly prestigious journals in the animal reproduction area.

But also by the fact that the experimental procedures included international collaborations with scientists from Germany and Italy.

Finally, the overall presentation of the thesis is nice, and it easy to read and follow. This is always something to thank.

Therefore, for all the abovementioned reasons I strongly believe that the manuscript hereby presented by the candidate María Ángeles Díaz Jiménez fulfill all the academic requirements to obtain the degree of Philosophiae doctor.

DATE: 15/10/2020

SIGNATURE: Sebastián Demyda Peyrás Firmado digitalmente por Sebastián Demyda Peyrás Fecha: 2020.10.16 10:18:17 +02'00'

INTERNATIONAL DOCTORS REPORT
DOCTORAL THESIS

REFEREE REPORT ON THE PhD TESIS PRESENTED IN THE UNIVERSITY OF CÓRDOBA (SPAIN) BY **MARÍA DE LOS ÁNGELES DÍAZ JIMÉNEZ**.

TITLE OF THE THESIS: Optimization of donkey sperm freezing and vitrification

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This thesis meets the requirements for presentation as an oral dissertation: ● YES ○ NO

RATING

Originality:	● Outstanding	○ Excellent	○ Very Good	○ Good	○ Sound	○ Defficient
Scientific/ technical merit:	● Outstanding	○ Excellent	○ Very Good	○ Good	○ Sound	○ Defficient
Planning/ methodology:	● Outstanding	○ Excellent	○ Very Good	○ Good	○ Sound	○ Defficient

COMMENTS (Please use additional sheets, if necessary):

The Doctoral Thesis presented by Mrs. María de los Ángeles Díaz Jimenez for admission to the degree of Doctor of Philosophy (PhD) as a "Tesis Doctoral en Cotutela con Mención Internacional, Programa de Doctorado Biociencias y Ciencias Agroalimentarias", is extremely well presented, very well structured, written, and extremely coherent. This PhD Thesis includes highly relevant information regarding the development and optimization of donkey sperm freezing and vitrification techniques. The aim of this Thesis was to develop conventional freezing and vitrification, avoiding the use of permeable cryoprotectant agents, as alternative methods for donkey sperm cryopreservation. The Thesis starts with a very self explanatory abstract, in English, Spanish and Italian. It is followed by a thorough Introduction on the subject,

and by five very well defined and important Objectives. The five subsequent chapters, correspond to the nine scientific manuscripts that have resulted from this Doctoral work developped by Mrs. María Díaz-Jiménez. They were published in extremely well known and highly prestigious Journals in the area of animal reproduction. The studies were performed according to the five different objectives considered in this PhD proposal. The excellence of this work is proven by the outstanding scientific output of the work.

The topic of the PhD thesis presented by Mrs. María Díaz Jiménez is highly innovative in the donkey, and therefore adequate for the development of a PhD project. The proposed aims of those studies were fully reached. The experimental design was extremely updated and adequate for the purpose. To achieve those objectives, the PhD candidate mastered a number of different techniques for donkey sperm freezing and analysis. A large amount of experimental work was carried out, the results are correctly presented and scientifically sound and stimulating discussions are presented along the manuscripts. A general Conclusion finalizes the Thesis, in a very objective manner.

This thesis shows that Mrs. María Díaz-Jiménez has acquired solid theoretical concepts in different and complimentary areas of knowledge and has developed sound field and laboratory experience. Also, the way the results are presented, the rigorous approach, the explanation of results, and the extremely adequate and deep discussions show the scientific maturity of the candidate.

In conclusion, this Doctoral thesis is worth for Mrs. María de los Ángeles Díaz Jiménez to be awarded the degree of European Doctorate.

DATE: 08/10/2020

SIGNATURE:

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European/ International Mention in the Doctorate Degree

Köln, den 07. February 2020

Certificate of stay

I hereby confirm that Ms. **Maria Angeles Díaz Jiménez** has steadily stayed in my **Research Group for Reproductive Medicine** at the **Department of Obstetrics and Gynecology, Cologne University,** from **15 April to 15 July, 2018** and has very successfully performed a cycle of investigations in field of cryoprotectants-free vitrification of mammalian spermatozoa. These investigations are directly correlated with her PhD thesis regarding donkey sperm cryopreservation.

Director of Research Group for Reproductive Medicine

Dr. (SU) Vladimir Isachenko

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Alessandra Rota

Cargo o Categoría del o de la responsable / *Position of the researcher in charge*:

Associate Professor

3. DURACIÓN DE LA ESTANCIA BREVE O TRASLADO TEMPORAL / *DURATION OF THE MOBILITY*

Fecha inicio / *Start date*: 04/03/2019

Fecha fin / *End date*: 03/09/2019

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Signature of the researcher in charge:

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AGRADECIMIENTOS

AGRADECIMIENTOS

Si pudiera verdaderamente agradecer a todas las personas que de un modo u otro me han ayudado durante estos años, me han apoyado, escuchado y a las que estoy sumamente agradecida, creo que esta sección sería bastante más larga que la propia Tesis… Tengo y he tenido la suerte de rodearme de gente que siempre me ha apoyado en mis decisiones y animado en los momentos difíciles de estos años. Así que no me queda otra que hacer un *abstract*:

- Ante todo, muchísimas gracias a mis directores,

A mi director Manuel Hidalgo (mi *boss*). Estoy orgullosa de poder decir que, después de más de cuatro años, hemos llegado a entendernos (aunque a veces lo disimulamos bien). Has dedicado a esta tesis casi tantas horas como yo. Gracias por todo lo que me has enseñado Manolo, no solo profesional sino también personalmente. Me has ayudado a desarrollar una paciencia infinita con los demás, a saber cuándo hablar y cómo, casi me sale ya el *charming*… Siempre te has preocupado de nosotros, siempre nos has apoyado y contado con nosotros para todo, siempre buscando las mil becas para echarnos un cable. Me alegro haber tenido la suerte de tenerte como director de tesis y sobre todo como amigo.

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ÍNDICE

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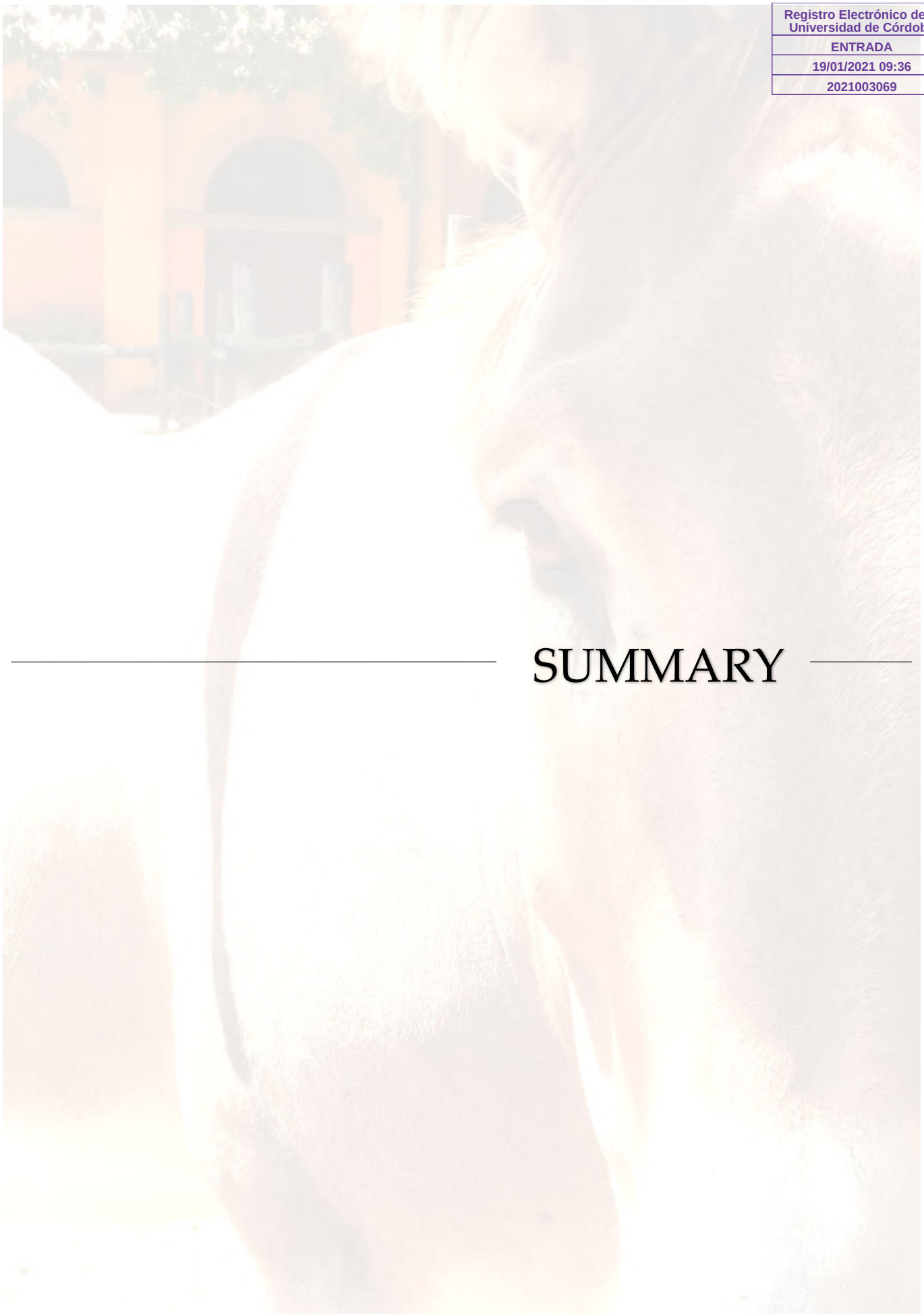


SUMMARY

SUMMARY

Assisted reproductive technologies such as artificial insemination (AI) and semen cryopreservation are important tools to maintain the genetic diversity and preserve endangered species, including most of the European donkey breeds. The aim of this Thesis was to develop conventional freezing and vitrification, avoiding the use of permeable cryoprotectant agents (CPAs), as alternative methods for donkey sperm cryopreservation. In Chapter 1, the sole use of non-permeable CPAs at different concentrations were evaluated for conventional freezing. Sucrose 0.25 molar (M) combined with bovine serum albumin showed similar sperm quality (motility, plasma membrane and DNA integrity) after thawing than conventional freezing with glycerol for fertile donkeys, but is not an option for subfertile ones. In Chapter 2, vitrification in spheres by directly dropping the sperm into the liquid nitrogen was performed for the first time in donkey sperm. Sucrose and bovine serum albumin as non-permeable CPAs resulted in better sperm parameters after warming than the use of extenders containing glycerol. Chapter 3 was designed to develop and optimize a donkey sperm vitrification protocol using straws. It was shown that volumes up to 160 µL could be vitrified at 300 million sperm/mL using 0.25 mL straws with outer covers. Best percentages of total (55.7 ± 16.4 %) and progressive (44.0 ± 11.5 %) sperm motility after vitrification were found using an extender containing egg-yolk and sucrose. Vitrification in straws showed higher motility results when compared to spheres method. In Chapter 4, vitrification of larger volumes of sperm in 0.5 mL straws was tested. Although high warming rates seemed to be more adequate, sperm parameters after vitrification were lower when compared to conventional freezing. In Chapter 5, conventionally frozen and optimized vitrified donkey sperm in straws were compared in terms of sperm quality and fertility. It was found that straws could be directly vitrified and warmed in a water bath at 43 ºC/10 s obtaining similar sperm parameters as conventional

freezing-thawing for total motility (52.7 ± 15.6 % *vs.* 58.2 ± 16.1 %), progressive motility (44.3 ± 15.0 % *vs.* 44.7 ± 18.2 %), plasma membrane integrity (49.2 ± 11.2 % *vs.* 55.4 ± 9.0 %) and acrosome integrity (45.0 ± 11.0 % *vs.* 38.4 ± 19.6 %), respectively. Uterine inflammatory response after AI was similar using vitrified or frozen semen, but uterine reaction solved faster using vitrified semen. Pregnancy rates were greater for vitrified (22 %) than frozen semen (10 %) but not statistically different. These findings confirm the possibility to use vitrified semen as an alternative for AI in jennies.

RESUMEN

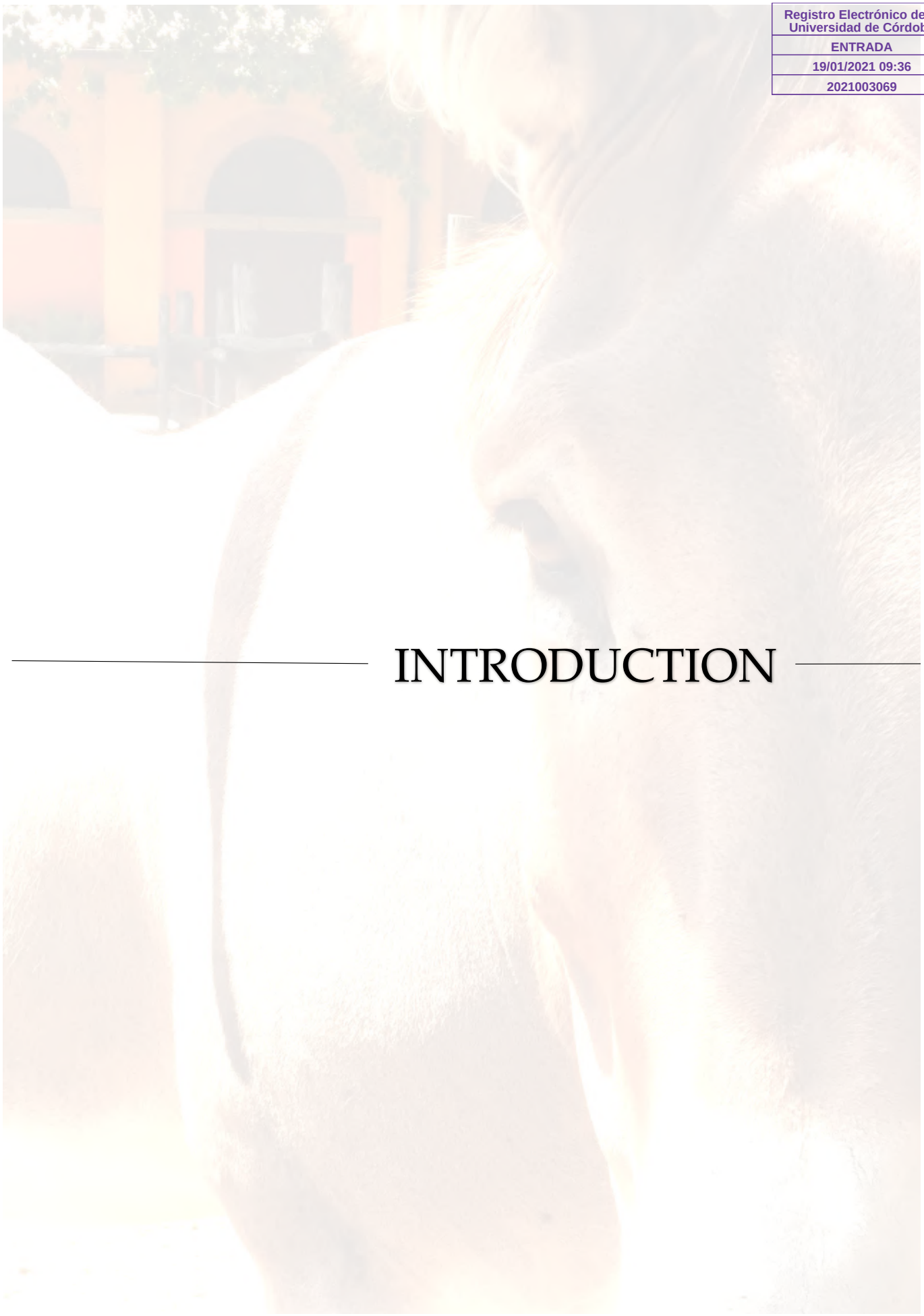
La inseminación artificial (IA) y la crioconservación de esperma son algunas de las técnicas de reproducción asistida más eficaces para el mantenimiento de la diversidad genética, así como para la conservación de especies en peligro de extinción, incluyendo la mayoría de las razas asnales en Europa. El objetivo de la presente Tesis fue desarrollar la congelación convencional y la vitrificación del esperma de asno como métodos de crioconservación alternativos, evitando el uso de agentes crioprotectores (CPAs) permeables. En el Capítulo 1 se estudió la congelación convencional utilizando únicamente CPAs no permeables a distintas concentraciones. Empleando un diluyente con sacarosa a 0,25 molar (M) combinada con albúmina sérica bovina, se obtuvieron parámetros espermáticos (movimiento, integridad de la membrana plasmática y del ADN), similares a los que se obtienen tras la congelación convencional empleando glicerol en asnos fértiles. Sin embargo, en asnos subfértiles ninguna concentración de sacarosa resultó en valores similares a los obtenidos tras la congelación convencional. En el Capítulo 2 se llevó a cabo, por primera vez, la vitrificación de esperma de asno en esferas. Sacarosa y albúmina sérica bovina fueron añadidas al diluyente, como CPAs no permeables, resultando en una mejor calidad espermática tras la descongelación, en comparación con los diluyentes que contienen glicerol. El Capítulo 3 fue diseñado para desarrollar y mejorar la técnica de vitrificación de esperma en esta especie, empleando pajuelas. Se determinó como óptima la vitrificación de volúmenes de hasta 160 µL en pajuelas de 0,25 mL, empleando una concentración de 300 millones de espermatozoides/mL. Los mejores porcentajes de movimiento total (55,7 ± 16,4 %) y progresivo (44,0 ± 11,5 %), tras la vitrificación, se obtuvieron empleando un diluyente suplementado con yema de huevo y sacarosa. El método de vitrificación en pajuelas obtuvo mayores porcentajes de movimiento espermático cuando se comparó con la vitrificación en esferas. En el Capítulo 4 se estudió la vitrificación en volúmenes mayores,

empleando pajuelas de 0,5 mL. Aunque altas velocidades de calentamiento parecen ser más adecuadas, los parámetros espermáticos tras la vitrificación fueron bajos en comparación con la congelación convencional. En el Capítulo 5, la técnica optimizada para la vitrificación en pajuelas se comparó, en cuanto a calidad espermática y fertilidad, con la técnica convencional de congelación, empleando glicerol como CPA. Las pajuelas vitrificadas pueden ser directamente calentadas a 43 ºC/10 s, obteniendo parámetros espermáticos tras la descongelación similares a los que se obtienen con el método convencional para el movimiento total (52,7 ± 15,6 % *vs.* 58,2 ± 16,1 %), movimiento progresivo (44,3 ± 15,0 % *vs.* 44,7 ± 18,2 %), integridad de la membrana plasmática (49,2 ± 11,2 % *vs.* 55,4 ± 9,0 %) e integridad de la membrana acrosómica (45,0 ± 11,0 % *vs.* 38,4 ± 19,6 %), respectivamente. La respuesta inflamatoria uterina tras la IA fue similar empleando dosis sometidas a ambos tratamientos de congelación, pero ésta se resuelve antes empleando esperma vitrificado. Las tasas de gestación fueron mayores empleando esperma vitrificado (22 %) que congelado (10 %), pero las diferencias no fueron significativas. Estos hallazgos confirman la posibilidad de emplear la vitrificación de esperma como alternativa a la congelación convencional para la IA de asnas.

RIASSUNTO

L'inseminazione artificiale (IA) e la crioconservazione del seme sono le tecniche di riproduzione assistita più efficaci per il mantenimento della diversità genetica e la conservazione delle specie e razze in via di estinzione, tra cui la maggior parte delle razze di asini in Europa. Lo scopo di questa tesi è stato quello di sviluppare il congelamento e la vitrificazione convenzionale dello sperma di asino, come metodi alternativi di crioconservazione, evitando l'uso di agenti crioprotettivi permeabili (CPA). Nel Capitolo 1 è stato valutato l'uso esclusivo di CPA non permeabili a diverse concentrazioni per il congelamento convenzionale. Utilizzando un diluente con saccarosio 0,25 molare (M) combinato con albumina di siero bovino i parametri spermatici ottenuti (motilità, integrità della membrana plasmatica e DNA), sono risultati simili a quelli ottenuti dopo il congelamento convenzionale con glicerolo in asini fertili. Tuttavia, negli asini subfertili nessuna concentrazione di saccarosio ha prodotto valori simili a quelli ottenuti dopo il congelamento convenzionale. Nel Capitolo 2 è stata sviluppata la tecnica di vitrificazione in sfere (pellets), ottenuta facendo cadere gocce di seme di asino in azoto liquido. Il saccarosio e l'albumina sierica bovina aggiunti al diluente come CPA non permeabili sono risultati in migliori caratteristiche spermatiche dopo lo scongelamento rispetto all'uso di diluenti con glicerolo. Il Capitolo 3 è stato disegnato per sviluppare e ottimizzare la tecnica di vitrificazione dello sperma degli asini usando paillettes. I risultati migliori sono stati ottenuti quando è stato usato un volume fino a 160 µl, concentrazione di 300 milioni di spermatozoi/mL e paillettes da 0.25 mL. Le migliori percentuali di motilità totale (55.7 ± 16.4%) e progressiva (44.0 ± 11.5%) dopo la vitrificazione sono state ottenute utilizzando un diluente con tuorlo d'uovo e saccarosio. Il metodo di vitrificazione nelle paillette ha ottenuto percentuali più elevate di motilità dello sperma rispetto alla vitrificazione nelle sfere. Nel Capitolo 4 è stata testata la vitrificazione usando volumi di seme maggiori e paillettes da 0.5 mL. I

parametri dopo lo scongelamento sono risultati migliori usando velocità di scongelamento elevate, ma sono risultati peggiori rispetto al congelamento convenzionale. Nel Capitolo 5, la tecnica ottimizzata per la vitrificazione nelle paillette in 0.25 mL è stata confrontata con la tecnica convenzionale di congelamento del seme con glicerolo per quanto riguarda alla qualità dello sperma e la fertilità. Le paillette vitrificate possono essere riscaldate direttamente a 43 ºC/10 s, ottenendo parametri spermatici dopo lo scongelamento simili a quelli ottenuti con il metodo convenzionale per la motilità totale (52.7 ± 15.6 % vs. 58.2 ± 16.1 %), motilità progressiva (44.3 ± 15.0 % vs. 44.7 ± 18.2 %), integrità della membrana plasmatica (49.2 ± 11.2 % vs. 55.4 ± 9.0 %) e integrità della membrana acrosomiale (45.0 ± 11.0 % vs. 38.4 ± 19.6 %), rispettivamente. L'endometrite dopo IA è risultata simile utilizzando entrambi i trattamenti di congelamento, ma la infiammazione uterina si è risolta più velocemente utilizzando sperma vitrificato. I tassi di gravidanza sono stati più alti utilizzando sperma vitrificato (22%) rispetto a quelli congelati (10%), ma le differenze non erano statisticamente significative. Questi risultati confermano la possibilità di utilizzare la vitrificazione del seme di asino come alternativa al congelamento convenzionale.



INTRODUCTION

INTRODUCTION

Donkeys (*Equus africanus asinus*) have been traditionally employed as working animals since they were domesticated millennia ago. They have enabled a wide range of roles over the years, such as transport, pack, riding and draft animals (Davis 2019, Kimura *et al.* 2013). Although these animals are still employed as working animals and economically important in some regions of the world (particularly in less industrialized countries); they have been the most affected livestock breeds by the industrial revolution, depopulation of rural districts and mechanization of agriculture (Camillo *et al.* 2018, Kugler *et al.* 2008). Recently, there is a renewed interest in donkey breeding, but their role is changing: jennies' milk properties have demonstrated to be similar to human milk (Martini *et al.* 2018), being a suitable substitute to cow´s milk in case of allergies, as well as component of skin cosmetics and other beauty products (Kocic *et al.* 2020, Camillo *et al.* 2018). Even so, in Europe this species is in a tough situation: there has been dramatic decrease in donkey population over the last century and breeding areas are scarce and isolated. This has caused a reduced genetic variability as well as increased relatedness within breeds (Kugler *et al.* 2008). According to the Food and Agriculture Organization (FAO), most of the European donkey breeds are currently threatened of extinction (Camillo *et al.* 2018).

Considering the importance of environment and biodiversity preservation of domestic species resources, research should be focused in conservation programs and reproductive technologies that could help to face this situation. In particular, reproductive procedures aiming to preserve all available individuals registered as pure breeds. Most of the animals currently kept in rural areas are crossbreeds and cannot be categorised into specific breed (Kugler *et al.* 2008); besides, inbreeding level is increasing due to crosses between related animals (Aranguren-Méndez *et al.* 2001). Therefore, it would be advisable to create gene banks for all those donkey breeds at risk of extinction, including also those

animals with low fertility, in order to increase the genetic pool and conserve the desirable levels of genetic variability within each species (Chesser *et al.* 1980, Howard *et al.* 2017).

Sperm cells are one of the most commonly preserved material in germplasm banks (O'Brien *et al.* 2019), and the use of frozen semen from selected jacks, different herds and regions, is an important management strategy to cope with their threatened condition. The advantages of using frozen semen in equine industry also includes: minor transportation costs, shipping and availability of semen at any time, less risk of venereal disease transmission and long-term preservation of genetically valuable gametes from males or endangered breeds, among others (Sanchez *et al.* 2009). The use of frozen semen in artificial insemination (AI) represents the main tool for genetic improvement of most domestic animals. One of the most popular sperm preservation technique is conventional freezing; which implies slow cooling rates, long equilibration periods and permeable cryoprotectant agents (CPAs) (O'Brien *et al.* 2019).

Due to the phylogenetic relationship among species, sperm freezing techniques developed for stallions have been directly applied to donkey semen for decades (Vidament *et al.* 2009); however, each species has its own requirements regarding reproductive protocols (Pukazhenthi and Wildt 2004). Conventional slow freezing using glycerol as permeable CPA has been the most employed technique for equine sperm preservation in the last 50 years (Hoffmann *et al.* 2011). Nevertheless, donkey spermatozoa showed a greater sensitivity to glycerol in comparison to stallion, which requires a reduction of its concentration in the freezing extender (Vidament *et al.* 2009, Serres *et al.* 2014). This indicates the obvious differences regarding sperm cryopreservation between species, and that a specific protocol to cryopreserve donkey sperm is required (Canisso *et al.* 2011).

It has aroused a growing interest regarding cryopreservation protocols and strategies to improve post-thaw donkey sperm parameters. Research has mainly focused on procedures pre-freezing, such as: the use of alternative permeable

cryoprotectant agents in the freezing extender (Acha *et al.* 2015, Rota *et al.* 2012), and combinations of them (Oliveira *et al.* 2006, Vidament *et al.* 2009); addition of different non-permeable substances to the freezing extender: Cholesterol-Loaded Cyclodextrins (Oliveira *et al.* 2014), sugars (Dorado *et al.* 2019) and jenny colostrum (Álvarez *et al.* 2019); and the effect of different cooling rates before cryopreservation (Demyda-Peyrás *et al.* 2018). In addition, post-thawing procedures have been also tested: supplementation with seminal plasma (Sabatini *et al.* 2014, Rota *et al.* 2012), with pentoxifylline and/or caffeine (Rota *et al.* 2019) and post-thaw centrifugation for cryoprotectant removal (Ortiz *et al.* 2015b). Despite the exceptional donkey sperm quality after cryopreservation, with sperm motility percentages over 65 % and plasma membrane integrity percentages over 70 % in some studies (Ortiz *et al.* 2015a, Oliveira *et al.* 2016); the ability of jennies to become pregnant after AI with frozen-thawed jack semen is poorer than in mares (Oliveira *et al.* 2006, Rota *et al.* 2012, Vidament *et al.* 2009).

The low fertilising capacity of cryopreserved donkey sperm has been attributed to the impact of permeable CPAs presence in the AI dose (Trimeche *et al.* 1998a); but this hypothesis still remains unclear (Trimeche *et al.* 1998b, Rota *et al.* 2012, Vidament *et al.* 2009). It seems that not only the cryoprotectant toxicity to the sperm cell (Watson 2000), but also the higher post-breeding endometritis that is provoked in jennies in comparison to mares, have a detrimental effect on fertility (Vilés *et al.* 2013, Vidament *et al.* 2009). No differences were found between the use of alternative permeable CPAs (ethylene glycol *vs.* glycerol) by Rota *et al.* (2012); or combinations of them (dimethyl sulphoxide, dimethyl formamide, dimethylacetamide and glycerol) by Oliveira *et al.* (2006), when frozen-thawed semen was employed in AI in jennies; thus the influence of the type of permeable CPAs is not quite clear. However, an improvement on fertility rates was achieved by Serres *et al.* (2014) using fresh semen diluted in freezing extender but removing the cryoprotectant agent from the dose before AI in jennies. Therefore, a negative effect of the CPAs presence in the AI dose seems to be clear in that

study. In order to verify specifically whether the permeable CPAs are the cause of this poor fertility results in donkeys, it seems necessary to develop a freezing protocol avoiding permeable CPAs for donkey sperm cryopreservation.

Sperm cryopreservation using only non-permeable CPAs, has given quite satisfactory results after thawing in sperm motility, plasma membrane and chromatin integrity, and fertility in human (Isachenko *et al.* 2003, Isachenko *et al.* 2004), canine (Sánchez *et al.* 2011), fish (Merino *et al.* 2011), cat (Swanson *et al.* 2017) wild ruminant (Pradieé *et al.* 2018) and stallion (Consuegra *et al.* 2018). Generally, the non-permeable agents added to the extender include carbohydrates (sucrose or trehalose), proteins (human or bovine serum albumin) and other substances (such as lecithin). The concentration of those non-permeable agents seems to be species-specific (Hidalgo *et al.* 2018), so the first step towards developing a cryopreservation protocol for donkey sperm would be the comparison between different concentrations of non-permeable agents for conventional slow freezing.

The identification of a range of substances and concentrations that have a positive effect on post-thaw sperm quality parameters, could eventually lead to its application in other cryopreservation techniques. Sperm vitrification is increasing in popularity in many species, and it is considered one of the two principal methods applied to the cryopreservation of sperm cells in human beings (Tao *et al.* 2020). It consists of an ultra-fast cooling of the sperm suspension in absence of permeable CPAs (Isachenko *et al.* 2008). This method is different from the classic vitrification technique that has been employed in reproductive tissues and cells in which a high proportion of permeable CPAs are required, and both the intracellular and extracellular milieu must become vitrified (Katkov *et al.* 2006). Because of the very low tolerance of spermatozoa to high concentrations of permeable CPAs (Macías García *et al.* 2012), conventional vitrification was inappropriate for the cryopreservation of mammalian spermatozoa. Although there is still some controversy surrounding the use of this nomenclature (Hidalgo

et al. 2018, Pradiee *et al.* 2015), "sperm vitrification" means something different as compared with classic term for vitrification associated with oocytes and embryos (Katkov *et al.* 2006), and it does not imply that the extracellular medium is vitrified (Shaw and Jones, 2003). This methodology does not require special freezing equipment or permeable agents: it is simple, quick, cost-efficient and shows a strong cryoprotective effect, similar to standard frozen-thawed spermatozoa (Isachenko *et al.* 2003). Sperm vitrification is performed by plunging sperm samples directly into liquid nitrogen and has been achieved mostly in two different ways: dropping sperm directly in LN_2, obtaining spheres; or in filled straws and then immersed in LN_2.

Composition of the vitrification extender has been regarded as a key factor for vitrification success (Hidalgo *et al.* 2018). Different sucrose concentrations (between 0.02 to 0.5 M), in combination or not with human or bovine serum albumin (from 0.5 to 2 %), were employed in sperm vitrification in several species: human (Isachenko *et al.* 2008, Riva *et al.* 2018), fish (Merino *et al.* 2011), wild goat (Pradiee *et al.* 2015) and sheep (Pradiee *et al.* 2016), mouflon and fallow deer (Bóveda *et al.* 2018), dog (Sánchez *et al.* 2011), cat (Swanson *et al.* 2017) and stallion (Hidalgo *et al.* 2018). In other studies, a combination of both permeable and non-permeable CPAs were used in research in straws in ruminants (Jiménez-Rabadán et al., 2015; Daramola et al., 2016), and in spheres in fish (Zilli *et al.* 2018, Figueroa *et al.* 2013). In this sense, presence of different non-permeable agents, as well as optimal concentration of them in each vitrification technique (spheres and straws), must be determined in donkey species.

Vitrification process is affected by several factors, not only the extender, as reviewed before, but also sperm concentration, volume and storage device as well as warming procedure. These elements affect the rate of cooling/warming, which would significantly determine the ultimately success or failure of the technique. Reviewing the literature we found differences among studies: human sperm have been successfully vitrified at concentrations ranging from 1 (Sanchez

et al. 2012a) to 15 (Sanchez *et al.* 2012b) millions sperm/mL. This concentration is quite low and inadequate regarding animal sperm freezing. In this regard, inconsistent results were obtained when higher concentrations (up to 200 and 1148 millions sperm/mL) were employed in ram (Jiménez-Rabadán *et al.* 2015) and goat (Daramola *et al.* 2016) sperm vitrification, respectively. On the other hand, different sperm volumes and packages have been employed for sperm vitrification giving satisfactory results in human: from 10 µL in plastic capillaries (Isachenko *et al.* 2012, Isachenko *et al.* 2017) to 500 µL in plastic straws (Isachenko *et al.* 2011); and in animals: from 100 µL using 0.25 into 0.5 mL plastic straws (Jiménez-Rabadán *et al.* 2015) to 200 µL packed directly in 0.5 plastic straws (Daramola *et al.* 2016). Vitrification of large volumes of sperm (up to 0.5 mL) has showed similar (Slabbert *et al.* 2015) and higher (Isachenko *et al.* 2011) sperm motility parameters after warming when compared to conventional freezing in human beings; but almost no motile sperm was recovered after warming when employed in equine species (Restrepo *et al.* 2019, Consuegra *et al.* 2019). Hence, a challenging area in the field of donkey sperm cryopreservation would be a comparison between different sperm concentrations and test different sperm packages, in order to find the most appropriate for this species.

Temperature and time during thawing affect sperm quality after conventional freezing (Athurupana *et al.* 2015), and they are also crucial for sperm vitrification success (Mansilla *et al.* 2016). A review of the literature on this topic found that few studies were performed to compare warming protocols after vitrification, and heterogeneous results were found: Mansilla *et al.* (2016) reported optimum results in human sperm in straws after warming at 42 ºC in comparison to lower temperatures; higher warming temperatures (up to 60 ºC) seemed to be more adequate for wild ruminant vitrification in spheres (Pradiee *et al.* 2015, Pradiee *et al.* 2016); but 37 ºC showed better sperm quality after vitrification in spheres for dog sperm (Caturla-Sánchez *et al.* 2018) in comparison to 65 ºC. The protocol commonly employed after sperm vitrification (spheres and straws) consisted on

the immersion of the vitrified suspension in 1-5 mL of pre-warmed semen extender (37-65 ºC), and then centrifuged. Although this warming protocol is suitable in human reproduction, in which 3 straws with a total of 1.3×10^6 sperm are employed for intrauterine insemination (Sanchez et al., 2012), it is not practical for its use in animal species, especially in equine. Approximately 10 straws would be necessary per an AI dose (500×10^6 sperm), which would imply the use of at least 30 mL of equine extender for each insemination. It is expensive and time consuming, but alternatives warming procedures have not been reviewed yet. Therefore, not only the rate of warming, but a suitable technique for sperm warming after vitrification for donkey sperm is needed.

Considering the high controversy surrounding the above mentioned effect of the CPAs in the exacerbate post-breeding endometritis in jennies, different strategies have been developed: glycerol removal before insemination (Trimeche *et al.* 1998a); glycerol substitution (Vidament *et al.* 2009) or combination with other permeable CPAs (Oliveira *et al.* 2006); performing deep horn AI or increasing the number of inseminations (Oliveira *et al.* 2016) and even AI of jennies using fresh donkey sperm diluted in a freezing extender (Serres *et al.* 2014). Unfortunately, it fails to explain the role of the permeable CPA presence in the post-breeding endometritis, and if it is the cause of the low success of AI with frozen-thawed sperm in jennies. To the best of our knowledge, there are no studies of AI with cryopreserved jack semen with the sole use of non-permeable CPAs. Therefore, the last step towards enhance fertility results in donkeys using cryopreserved sperm would be to perform a fertility trial using vitrified semen without permeable CPAs in comparison to conventionally frozen sperm using glycerol for AI.

OBJECTIVES

OBJECTIVES

The main objective of this Doctoral Thesis was to develop and optimize conventional freezing and vitrification techniques for donkey sperm cryopreservation, in the absence of permeable cryoprotectant agents. To achieve this goal, the following specific objectives were addressed:

Objective 1. To develop conventional donkey sperm freezing in absence of permeable cryoprotectant agents.

This objective has been addressed in **Chapter 1**, which includes:

Chapter 1.1. To evaluate the effect of different concentrations of sucrose-based extenders combined with bovine serum albumin, on post-thaw donkey sperm parameters and to compare the selected sucrose-based extender with a commercial freezing extender containing glycerol.

M. Diaz-Jimenez, J. Dorado, I. Ortiz, C. Consuegra, B. Pereira, C.A. Gonzalez-De Cara, R. Aguilera, G. Mari, B. Mislei, C.C. Love, M. Hidalgo. Cryopreservation of donkey sperm using non-permeable cryoprotectants. Animal Reproduction Science (2018). Vol 189: 103-109.

Chapter 1.2. To determine if the cryopreservation method using non-permeable agents is suitable for subfertile donkeys, comparing sperm variables after thawing with conventional freezing with glycerol.

M. Diaz-Jimenez, J. Dorado, B. Pereira, C. Consuegra, I. Ortiz, M. Hidalgo. Is sperm cryopreservation in absence of permeable cryoprotectants suitable for subfertile donkeys? Reproduction in Domestic Animals (2019). Vol 54: 102–105.

Objective 2. To develop donkey sperm vitrification in spheres.

This objective has been addressed in **Chapter 2**, which has been focused on examination of different concentrations of sucrose and bovine serum albumin for donkey sperm vitrification in spheres, in comparison to a vitrification extender containing glycerol as a permeable agent.

M. Hidalgo, M. Diaz-Jimenez, C. Consuegra, B. Pereira, J. Dorado. Vitrification of donkey sperm: Is it better using permeable cryoprotectants? Animals (2020). Vol 10 (9): 1-11.

Objective 3. To develop and optimize a donkey sperm vitrification protocol using straws.

This objective has been addressed in **Chapter 3**, which includes:

Chapter 3.1. To compare two different extenders supplemented with sucrose and bovine serum albumin for donkey sperm vitrification in 0.25 mL straws.

M. Diaz-Jimenez, B. Pereira, I. Ortiz, C. Consuegra, A. Partyka, J. Dorado, M. Hidalgo. Effect of different extenders for donkey sperm vitrification in straws. Reproduction in Domestic Animals (2017). Vol 52: 55-57.

Chapter 3.2. To assess the effect of sucrose concentration, to evaluate three different concentrations of sperm and to compare different semen volumes using straws.

M. Diaz-Jimenez, J. Dorado, C. Consuegra, I. Ortiz, B. Pereira, J.J. Carrasco, V. Gomez-Arrones, A. Domingo, M Hidalgo. Optimization of donkey sperm vitrification: effect of sucrose, sperm concentration, volume and package (0.25 and 0.5 mL straws). Animal Reproduction Science (2019). Vol 204: 69-77.

Chapter 3.3. To compare sperm motility and plasma membrane integrity after donkey sperm vitrification using straws or spheres methods.

M. Diaz-Jimenez; J. Dorado; B. Pereira; I. Ortiz; C. Consuegra; M. Bottrel; E. Ortiz; M. Hidalgo. Vitrification in straws conserves motility features better than spheres in donkey sperm. Reproduction in Domestic Animals (2018). Vol 53: 56-58.

Objective 4. To develop donkey sperm vitrification in large volumes using 0.5 mL straws.

This objective has been addressed in **Chapter 4**, which has been focused on testing different temperatures and rates for warming in comparison to conventional freezing.

M. Diaz-Jimenez, J. Dorado, C. Consuegra, B. Pereira, I. Ortiz, C. Vazquez, E. Isachenko, V. Isachenko, M. Hidalgo. Effect of warming temperatures on donkey sperm vitrification in 0.5mL straws in comparison to conventional freezing. Spanish Journal of Agricultural Research (2019). Vol 17 (3): 1-7.

Objective 5. To compare conventional freezing and the optimized vitrification protocol in straws in terms of sperm quality and fertility.

This objective has been addressed in **Chapter 5**, which includes:

Chapter 5.1. To compare sperm quality parameters after cryopreservation using conventional freezing and vitrification in 0.25 mL covered straws.

M. Diaz-Jimenez, J. Dorado. C. Consuegra, B. Pereira, M. Hidalgo. Vitrification of donkey sperm using straws as an alternative to conventional slow freezing. Reproduction in Domestic Animals (2020). Vol 00: 1-4.

Chapter 5.2. To evaluate the effect of warming technique and temperature after donkey sperm vitrification in 0.25 mL covered straws and to compare the post breeding uterine inflammatory response and fertility of jennies after artificial insemination with vitrified or frozen jack semen.

M. Diaz-Jimenez, A. Rota, J. Dorado, C. Consuegra, B. Pereira, F. Camillo, D. Panzani, D. Fanelli, M. Tesi, D. Monaco, M. Hidalgo. First pregnancies in jennies with vitrified donkey semen using a new warming method. Animal Journal (2020). In Press

OBJETIVOS

El objetivo general de la presente Tesis Doctoral fue desarrollar y optimizar la congelación convencional y la vitrificación, evitando el uso de crioprotectores permeables. Para la consecución de este objetivo general, se plantearon los siguientes objetivos específicos:

Objetivo 1. Desarrollar la congelación convencional del esperma de asno en ausencia de crioprotectores permeables.

Este objetivo ha sido abordado en el **Capítulo 1**, que incluye:

Capítulo 1.1. Evaluar el efecto de distintas concentraciones de sacarosa, añadida al diluyente en combinación con albúmina sérica bovina, sobre los parámetros espermáticos tras la descongelación; y comparar la concentración elegida con un diluyente comercial con glicerol para congelación del esperma.

M. Diaz-Jimenez, J. Dorado, I. Ortiz, C. Consuegra, B. Pereira, C.A. Gonzalez-De Cara, R. Aguilera, G. Mari, B. Mislei, C.C. Love, M. Hidalgo. Cryopreservation of donkey sperm using non-permeable cryoprotectants. Animal Reproduction Science (2018). Vol 189: 103-109.

Capítulo 1.2. Determinar si el método de congelación convencional, empleando únicamente agentes no permeables, es apropiado para asnos subfértiles, comparando los parámetros espermáticos tras la descongelación con la congelación convencional con glicerol.

M. Diaz-Jimenez, J. Dorado, B. Pereira, C. Consuegra, I. Ortiz, M. Hidalgo. Is sperm cryopreservation in absence of permeable cryoprotectants suitable for subfertile donkeys? Reproduction in Domestic Animals (2019). Vol 54: 102–105.

Objetivo 2. Desarrollar el método vitrificación de esperma de asno en esferas

Este objetivo ha sido abordado en el **Capítulo** 2, y pretendió examinar el efecto de distintas concentraciones de sacarosa y albúmina sérica bovina para la vitrificación de esperma de asno en esferas y comparar con un diluyente de congelación convencional que contiene glicerol como crioprotector permeable.

M. Hidalgo, M. Diaz-Jimenez, C. Consuegra, B. Pereira, J. Dorado. Vitrification of donkey sperm: Is it better using permeable cryoprotectants? Animals (2020). Vol 10 (9): 1-11.

Objetivo 3. Desarrollar y optimizar un protocolo de vitrificación de esperma de asno en pajuelas.

Este objetivo ha sido abordado en el **Capítulo 3**, que incluye:

Capítulo 3.1. Comparar dos diluyentes a los que se añadió sacarosa y albúmina sérica bovina, para la vitrificación de esperma en pajuelas de 0,25 mL.

M. Diaz-Jimenez, B. Pereira, I. Ortiz, C. Consuegra, A. Partyka, J. Dorado, M. Hidalgo. Effect of different extenders for donkey sperm vitrification in straws. Reproduction in Domestic Animals (2017). Vol 52: 55-57.

Capítulo 3.2. Comparar el efecto de la concentración de sacarosa y evaluar el efecto de distintas concentraciones y volúmenes de esperma en la vitrificación del esperma de asno en pajuelas.

M. Diaz-Jimenez, J. Dorado, C. Consuegra, I. Ortiz, B. Pereira, J.J. Carrasco, V. Gomez-Arrones, A. Domingo, M Hidalgo. Optimization of donkey sperm vitrification: effect of sucrose, sperm concentration, volume and package (0.25 and 0.5 mL straws). Animal Reproduction Science (2019). Vol 204: 69-77.

Capítulo 3.3. Comparar los parámetros de movimiento e integridad de la membrana plasmática tras la vitrificación de esperma, empleando el método de pajuelas o esferas.

M. Diaz-Jimenez; J. Dorado; B. Pereira; I. Ortiz; C. Consuegra; M. Bottrel; E. Ortiz; M. Hidalgo. Vitrification in straws conserves motility features better than spheres in donkey sperm. Reproduction in Domestic Animals (2018). Vol 53: 56-58.

Objetivo 4. Desarrollar el método de vitrificación de esperma en grandes volúmenes usando pajuelas de 0,5 mL.

Este objetivo ha sido abordado en el **Capítulo 4,** que se centró en el empleo de distintas temperaturas y velocidades de calentamiento, en comparación con la congelación convencional.

M. Diaz-Jimenez, J. Dorado, C. Consuegra, B. Pereira, I. Ortiz, C. Vazquez, E. Isachenko, V. Isachenko, M. Hidalgo. Effect of warming temperatures on donkey sperm vitrification in 0.5mL straws in comparison to conventional freezing. Spanish Journal of Agricultural Research (2019). Vol 17 (3): 1-7.

Objetivo 5. Comparar el protocolo de congelación convencional y el de vitrificación en pajuelas respecto a la calidad espermática y la fertilidad.

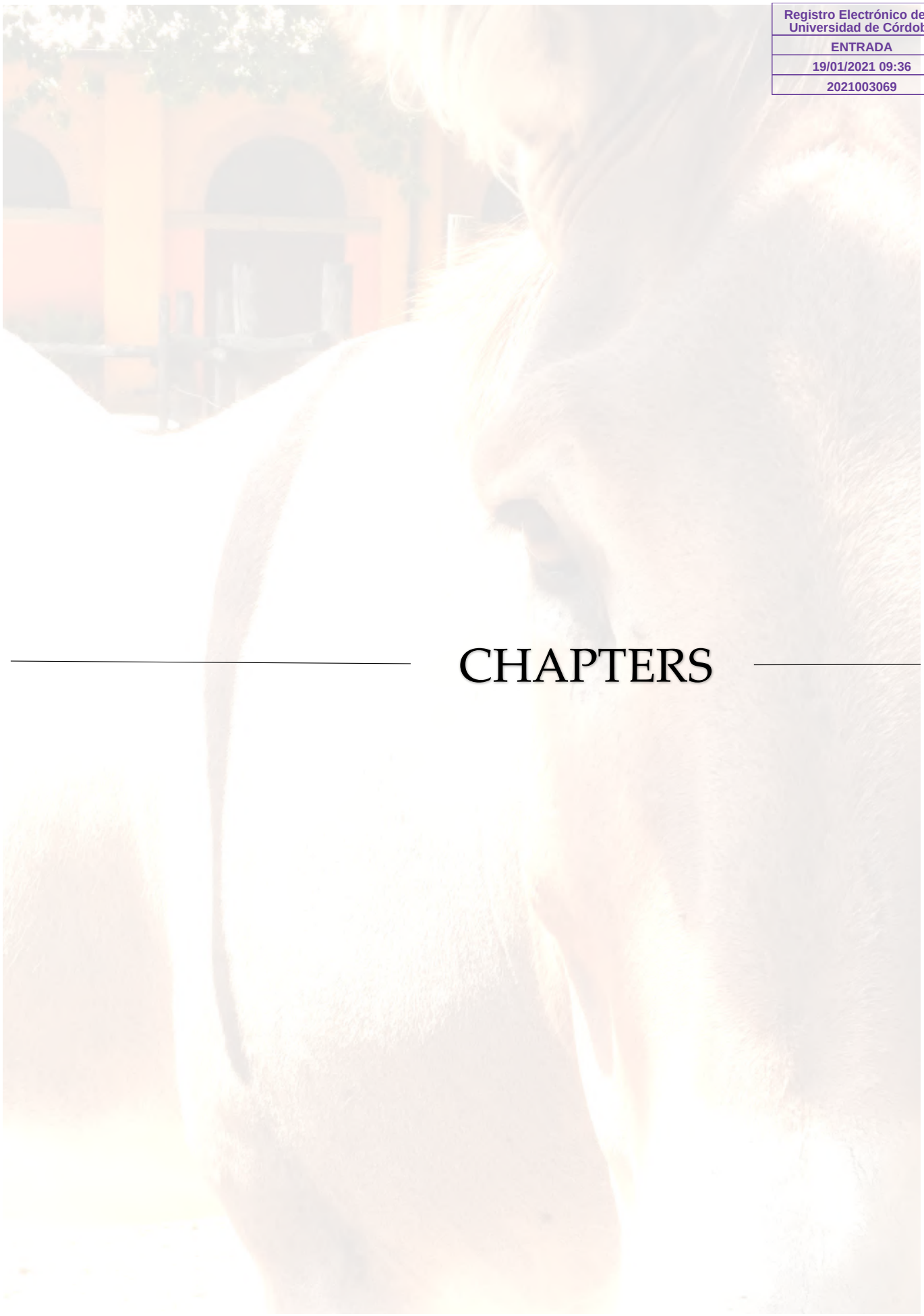
Este objetivo ha sido abordado en el **Capítulo 5**, que incluye:

Capítulo 5.1. Comparar la calidad espermática tras la descongelación, empleando la congelación convencional y la vitrificación en pajuelas cubiertas de 0,25 mL.

M. Diaz-Jimenez, J. Dorado. C. Consuegra, B. Pereira, M. Hidalgo. Vitrification of donkey sperm using straws as an alternative to conventional slow freezing. Reproduction in Domestic Animals (2020). Vol 00: 1-4

Capítulo 5.2. Evaluar el efecto de distintas técnicas de calentamiento y temperatura tras la vitrificación de esperma en pajuelas cubiertas de 0,25 mL y comparar la endometritis post-cubrición de asnas y la fertilidad tras la inseminación artificial empleando esperma congelado y vitrificado de asno.

M. Diaz-Jimenez, A. Rota, J. Dorado, C. Consuegra, B. Pereira, F. Camillo, D. Panzani, D. Fanelli, M. Tesi, D. Monaco, M. Hidalgo. First pregnancies in jennies with vitrified donkey semen using a new warming method. Animal Journal (2020). In Press



CHAPTERS

CHAPTER 1

CHAPTER 1.1

Cryopreservation of donkey sperm using non-permeable cryoprotectants

Diaz-Jimenez et al., 2018. Animal Reproduction Science

CHAPTER 1.2

Is sperm cryopreservation in absence of permeable cryoprotectants suitable for subfertile donkeys?

Diaz-Jimenez et al., 2019. Reproduction in Domestic Animals

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Cryopreservation of donkey sperm using non-permeable cryoprotectants

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ABSTRACT

The aim of this study was to evaluate the effect of different concentrations of sucrose combined with bovine serum albumin (BSA), as non-permeable cryoprotectants, on donkey sperm parameters after cryopreservation, in comparison to a control extender containing glycerol. Semen from five Andalusian donkeys (n = 12) were centrifuged and resuspended with a commercial extender for equine sperm (Gent A, Minitube) adding 1% BSA and different concentrations (M, mol/l) of water-diluted sucrose: 0.05, 0.1, 0.25, 0.35 and 0.45. Thereafter, semen (n = 24) were diluted in the same base extender containing 0.25 M sucrose (S25) or glycerol (GLY, Gent B). Sperm were slowly cooled, filled in 0.5 ml straws and frozen in nitrogen vapours. Post-thaw samples were assessed for sperm motility, plasma membrane and DNA integrity and results were compared by ANOVA. In Experiment 1, sperm motility was significantly higher ($P < 0.001$) for S25 than the remaining treatments, and no differences were found for plasma membrane or DNA integrity. In Experiment 2, no differences were found between S25 or GLY for sperm motility and DNA integrity but plasma membrane integrity was significantly higher ($P < 0.05$) for S25. In conclusion, the extender with sucrose 0.25 M combined with BSA can be considered as an alternative to conventional extenders with glycerol for donkey sperm cryopreservation.

1. Introduction

Cryopreservation of donkey semen is an important tool to maintain the genetic diversity and preserve endangered species (Watson, 2000). Based on Food and Agriculture Organization of the United Nations (FAO) criteria, and according to the Spanish regulations (RD 698/2013), Andalusian donkey breed is in danger of extinction.

It is well known that freezing and thawing lead to cell death or sublethal (apoptotic) cryoinjury, mostly caused by osmotic stress and the toxicity due to unequal distribution of permeable cryoprotective agents (CPAs) on the sperm cell (Morris et al., 2007; Pena et al., 2011; Macías García et al., 2012; Wu et al., 2015). In this regard, donkey sperm seems to be sensitive to glycerol (Vidament et al., 2009), the most used permeable cryoprotectant for equine sperm preservation in the last 50 years (Hoffmann et al., 2011). Besides, its toxic effect has been shown to start as early as the pre-freezing process begins (Vidament et al., 2005; Oliveira et al., 2006; Vidament et al., 2009; Rota et al., 2012; Serres et al., 2014). A negative effect of the permeable CPAs in the uterus of the jenny has

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been also hypothesized (Vidament et al., 2009; Serres et al., 2014; Oliveira et al., 2016), as the post-insemination reaction is more dramatic in jennies than in mares when permeable CPAs are present (Vidament et al., 2009). Moreover, fertility using cryopreserved donkey sperm is also lower in jennies than in mares (Vidament et al., 2009).

Considering the sensitivity of donkey sperm to permeable CPAs (Vidament et al., 2009; Acha et al., 2015), and neither substitution nor dilution avoids completely their toxicity (Oliveira et al., 2006; Vidament et al., 2009; Serres et al., 2014), non-permeable agents such as sucrose-based extenders combined with bovine serum albumin (BSA) could be an alternative for donkey sperm cryopreservation. This alternative has obtained quite satisfactory results in sperm motility, plasma membrane, chromatin integrity and fertility in human (Isachenko et al., 2003; Isachenko et al., 2004), canine (Sánchez et al., 2011), fish (Merino et al., 2011) and wild ruminant (Pradiee et al., 2015) after sperm cryopreservation.

Therefore, the aims of this study were to: 1) evaluate the effect of different concentrations of sucrose-based extenders combined with BSA on post-thaw donkey sperm parameters: motility, plasma membrane and DNA integrity; 2) compare the selected sucrose-based extender with a commercial freezing extender containing glycerol.

2. Material and methods

2.1. Semen collection and evaluation

A total of five healthy Andalusian donkeys, aged from 3 to 19 years were used as semen donors. The jackasses were housed in paddocks at “Centro Rural Malpica” (Palma del Rio, Cordoba, Spain) and the feeding consisted of water “ad libitum”, teff hay and oats. Semen was collected during the breeding season once or twice a week using a Missouri artificial vagina in the presence of a jenny in estrus. Ejaculates were assessed for sperm volume, concentration, morphology and motility before freezing as described by Dorado et al. (2013). All the experiments were approved by the Ethical Committee for Animal Experimentation of the University of Cordoba, in compliance with the Regional Government of Andalusia (no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (RD 53/2013).

2.2. Freezing and thawing

Immediately after collection, semen was extended 1:1 (v:v) with INRA-96 (IMV Technologies, L’Aigle, France) and divided into aliquots. Then, each aliquot was centrifuged 7 min at 400 × *g* (22 °C) in a corning-adapted centrifuge (Eppendorf, model 5702 RH, Eppendorf AG, Hamburg, Germany). Thereafter, the supernatant was removed and the sperm pellet from each aliquot was re-suspended with its corresponding freezing extender (as described in 2.4 Experimental design) to reach a final sperm concentration of 200×10^6 spermatozoa/ml. Samples were equilibrated 10 min at room temperature (22 °C) and then slowly cooled to 5 °C into an Equitainer (Hamilton Research, Inc. Ipswich, Massachusetts, USA) for 2 h. After that, sperm was loaded in 0.5-ml straws and frozen in liquid nitrogen (LN_2) vapours, 2.5 cm above the surface for 5 min, as described by Ortiz et al. (2015). Finally, each straw was plunged into LN_2 and stored for at least 24 h in a tank. A randomly selected straw from each treatment was thawed in a water bath at 37 °C for 30 s.

2.3. Post-thaw sperm evaluation

Thawed sperm were diluted to a final concentration of 25×10^6 sperm/ml with INRA-96 and sperm parameters were analysed as follows:

Total and progressive sperm motility were objectively evaluated using the Sperm Class Analyzer (SCA v.5.4.0.0; Microptic S.L., Barcelona, Spain) system. The SCA settings were previously described by Ortiz et al. (2015). Three drops, and two microscopic fields per drop, were analysed in each semen sample obtaining kinetic parameters: total and progressive motility (TM and PM, %), curvilinear velocity (VCL, μm s^{-1}), straight line velocity (VSL, μm s^{-1}); average path velocity (VAP, μm s^{-1}), linearity (LIN, VSL/VCL, %), straightness (STR, VSL/VAP, %), wobble (WOB, VAP/VCL, %), beat cross frequency (BCF, Hz) and amplitude of lateral head displacement (ALH, μm).

Plasma membrane integrity was assessed using the VitalTest (Halotech DNA SL, Madrid, Spain) as described by Dorado et al. (2014) and sperm with intact plasma membrane were recorded (PMI, %).

Sperm DNA integrity was assessed with the sperm chromatin structure assay (SCSA) using a FACScan Flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Two aliquots of 400 μl and 25×10^6 spermatozoa/ml were saved from each treatment. Those aliquots were kept in Eppendorf tubes and stored at −18 °C right after thawing (T0) and after four hours of incubation at 37 °C (T4). Chromatin integrity was assessed in each sample (T0 and T4) as previously described by Salazar et al. (2011). Around 5000 cells were studied, and the percentage of cells outside the main population, with single-stranded DNA was recorded (COMP-αt, %).

2.4. Experimental design

2.4.1. Experiment 1: comparison of different sucrose-based extenders for donkey semen cryopreservation

Four animals and three ejaculates per donkey (n = 12) were used in this experiment. A commercial extender for equine semen cooling without permeable cryoprotectants, which egg yolk and antibiotics (Gent A, Minitüb, Tiefenbach, Germany) and adding

Table 1
Osmolality (mOsm kg $^{-1}$) values in extenders.

Freezing extender	Osmolality (mOm kg $^{-1}$)
C	353
S05	215
S10	281
S25	438
S35	583
S45	642
GLY	1001

C = Control based extender without sucrose; S05 = Sucrose 0.05 M; S10 = Sucrose 0.10 M; S25 = Sucrose 0.25 M; S35 = Sucrose 0.35 M; S45 = Sucrose 0.45 M; GLY: Control based extender with Glycerol.

bovine serum albumin (BSA, Sigma-Aldrich, Sant Louis, USA) at 1% was used as control base extender (C). Sucrose (Sigma-Aldrich, Sant Louis, USA) was prepared in distilled water as described by Isachenko et al. (2011) and then mixed with the base medium (v/v) to reach a final concentration of 0.05 (S05), 0.10 (S10), 0.25 (S25), 0.35 (S35) and 0.45 (S45) molar (M) in each sucrose-based extender. Osmolality was measured in each extender using a freezing-point digital micro-osmometer Type 6 (Löser Messtechnik, Berlin, Germany) and it is represented in Table 1. Sperm motility, plasma membrane and chromatin integrity were assessed as described above and compared among treatments.

2.4.2. Experiment 2: comparison between the selected sucrose-based extender with the commercial freezing extender containing glycerol

Five donkeys and 24 ejaculates were used in this experiment. Based on the results obtained in Experiment 1, the sucrose-based extender with a concentration of 0.25 M (S25) and BSA 1% was selected and compared with the same commercial base extender formulated for equine sperm cryopreservation, containing egg-yolk, antibiotics but also with glycerol (Gent B, Minitüb, Tiefenbach, Germany) as control.

2.5. Statistical analysis

Statistical analysis of the data was conducted using the Statistical Analysis Software for Windows (SAS v.9.0, SAS Institute Inc., Cary, NC, USA). Comparisons among treatments were assessed using a general lineal model procedure (PROC GLM) followed by the Duncan test for *post hoc* analysis. Treatments, animals and ejaculates were considered as fixed factors. Values were expressed as mean ± standard deviation. Significant differences were considered when $P < 0.05$.

3. Results

Average sperm parameters obtained after fresh semen evaluation were (mean ± SD): gel-free volume 100.4 ± 26.5 ml (range: 55.0–155.0 ml), sperm concentration 247.1 ± 54.4 × 10^6 sperm/ml (range: 173.0–375.0 × 10^6 sperm/ml), total motility 66.4 ± 8.0% (range 51.7–78.8%), progressive motility 55.5 ± 9.5% (range 41.7–71.8%) and normal forms 81.8 ± 10.9% (range 58.0–95.0%).

3.1. Experiment 1: comparison of different sucrose-based extenders for donkey semen cryopreservation

Total motility was significantly higher in S25 ($P < 0.001$) than in the control and the rest of the treatments. Progressive motility and velocity parameters (VCL, VSL and VAP) were significantly ($P < 0.001$) higher in S25, S10 and C extenders. The remaining extenders showed no significant differences for sperm motility except for S45, which obtained the lowest values. PMI showed no differences between treatments except for S45, which again resulted in the lowest value ($P < 0.05$), as shown in Table 2. Mean values of COMP-αt were not significantly different between treatments at T0. However, DNA fragmentation increased significantly ($P < 0.01$) in the sucrose-based extender with the lowest sucrose concentration (S05) at T4 (Fig. 1).

3.2. Experiment 2: comparison between the selected sucrose-based extender with the commercial freezing extender containing glycerol

No significant differences ($P > 0.05$) were found between S25 and GLY for TM (45.8 ± 15.6 vs 40.2 ± 12.7) and PM (31.0 ± 13.2 vs 28.5 ± 9.8) parameters; however, plasma membrane integrity values were significantly higher ($P < 0.05$) for S25 than for GLY (42.7 ± 12.6 vs 37.5 ± 13.6). Chromatin assessment showed no significant differences neither at T0 nor T4 evaluations in both treatments (Fig. 2). GLY obtained higher LIN and WOB and lower ALH in comparison to S25 ($P < 0.05$), but no differences were obtained in the remaining kinetic parameters (Table 3).

Table 2
Comparison between mean values of sperm parameters from frozen-thawed samples (n = 12) using different sucrose-based extenders.

Sperm parameters	C	Sucrose-based extenders					*P*-values
		S05	S10	S25	S35	S45	
TM(%)	22.9 ± 9.8^{b}	16.3 ± 7.1^{c}	23.9 ± 10.8^{b}	31.4 ± 12.9^{a}	11.9 ± 5.8^{c}	5.4 ± 3.6^{d}	< 0.001
PM(%)	15.1 ± 5.8^{a}	8.3 ± 4.7^{b}	15.1 ± 7.4^{a}	19.2 ± 7.6^{a}	4.7 ± 2.3^{bc}	2.4 ± 3.5^{c}	< 0.001
PMI(%)	37.9 ± 15.3^{ab}	41.4 ± 19.6^{a}	42.3 ± 21.3^{a}	39.3 ± 20.6^{a}	33.0 ± 17.5^{ab}	28.9 ± 11.6^{b}	< 0.05
VCL(μm/s)	72.7 ± 7.9^{a}	56.9 ± 13.4^{b}	74.12 ± 13.7^{a}	74.7 ± 22.7^{a}	49.8 ± 15.2^{b}	32.7 ± 16.1^{c}	< 0.001
VSL(μm/s)	61.3 ± 8.6^{a}	45.2 ± 14.2^{b}	62.7 ± 13.3^{a}	60.6 ± 20.4^{a}	36.6 ± 14.8^{b}	22.6 ± 13.2^{c}	< 0.001
VAP(μm/s)	65.8 ± 49.5^{a}	49.5 ± 14.0^{b}	66.9 ± 13.5^{a}	65.6 ± 21.4^{a}	40.5 ± 14.8^{b}	25.1 ± 14.1^{c}	< 0.001
ALH(μm)	1.9 ± 0.3^{a}	1.8 ± 0.4^{a}	1.9 ± 0.3^{a}	2.1 ± 0.7^{a}	2.0 ± 0.9^{a}	1.1 ± 0.7^{b}	< 0.001
LIN(%)	84.0 ± 5.1^{a}	77.9 ± 9.2^{ab}	84.2 ± 4.2^{a}	75.5 ± 21.2^{ab}	69.4 ± 2.0^{bc}	61.3 ± 27.2^{c}	< 0.01
STR(%)	93.1 ± 2.5^{a}	90.4 ± 4.3^{ab}	93.5 ± 2.3^{a}	87.4 ± 17.2^{ab}	85.8 ± 16.2^{ab}	80.8 ± 22.3^{b}	> 0.05
WOB(%)	90.2 ± 3.9^{a}	85.9 ± 6.7^{a}	90.1 ± 2.9^{a}	76.2 ± 18.0^{ab}	78.4 ± 15.3^{ab}	70.0 ± 25.1^{b}	< 0.05
BCF(Hz)	8.8 ± 0.5^{a}	8.5 ± 1.1^{a}	9.2 ± 0.6^{a}	8.5 ± 2.7^{a}	9.1 ± 3.0^{a}	5.2 ± 3.5^{b}	< 0.001

Different superscripts indicate significant differences. C = Control based extender without sucrose; S05 = Sucrose 0.05 M; S10 = Sucrose 0.10 M; S25 = Sucrose 0.25 M; S35 = Sucrose 0.35 M; S45 = Sucrose 0.45 M; GLY: Control based extender with Glycerol. TM = total motility; PM = progressive motility; PMI = plasma membrane integrity; VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; ALH = amplitude of lateral head displacement; LIN = linearity; STR = straightness; WOB = wobble; BCF = beat cross frequency.
Values are expressed as mean ± SD.

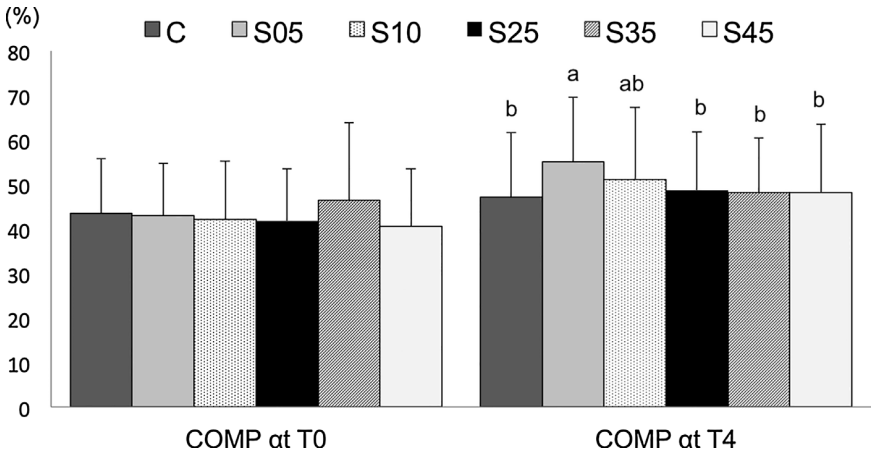

Fig. 1. Comparison of frozen-thawed sperm DNA fragmentation between extenders containing different concentrations of sucrose. Different letters indicate significant differences between treatments ($P < 0.05$). COMP–αt = DNA fragmentation at 0 and 4 h after thawing; S05 = sucrose 0.05 M; S10 = sucrose 0.10 M, S25 = sucrose 0.25 M, S35 = sucrose 0.35 M and S45 = sucrose 0.45 M. Results are expressed as mean ± SD.

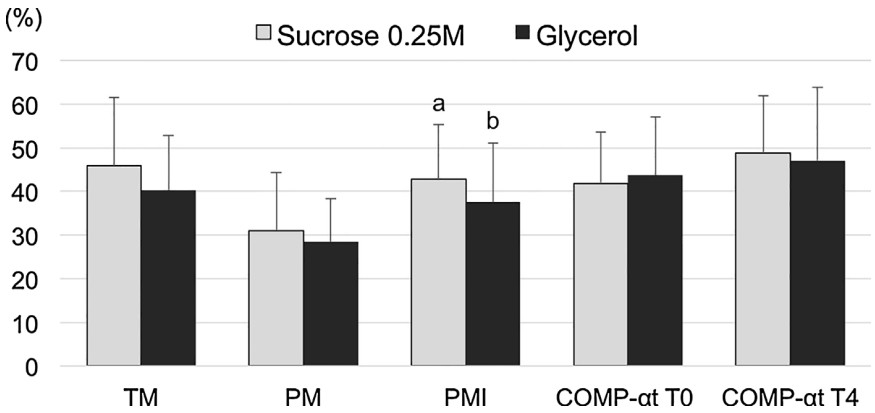

Fig. 2. Comparison of sperm parameters after thawing between extenders containing sucrose 0.25 M or glycerol. Different letters indicate significant differences ($P < 0.05$). TM = total motility; PM = progressive motility; PMI = plasma membrane integrity; COMP–αt = DNA fragmentation at 0 and 4 h after thawing. Results are expressed as mean ± SD.

Table 3
Comparison of mean values of the sperm kinematic parameters from the frozen-thawed samples (n = 24) using two different extenders (S25 or GLY).

Kinetics parameters	Freezing extender	
	S25	GLY
VCL(μm/s)	86.1 ± 20.7[a]	92.8 ± 13.5[a]
VSL(μm/s)	73.3 ± 10.9[a]	78.3 ± 13.1[a]
VAP(μm/s)	79.8 ± 12.1[a]	84.5 ± 13.9[a]
ALH(μm)	2.3 ± 0.3[a]	2.1 ± 0.2[b]
LIN(%)	82.3 ± 3.6[b]	84.1 ± 3.6[a]
STR(%)	91.8 ± 2.5[a]	92.1 ± 2.4[a]
WOB(%)	89.6 ± 2.4[b]	91.2 ± 2.1[a]
BCF(Hz)	12.7 ± 17.1[a]	9.3 ± 0.4[a]

Different superscripts indicate significant differences ($P < 0.05$). S25 = Sucrose 0.25 M; GLY = Control based extender with Glycerol. VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; ALH = amplitude of lateral head displacement; LIN = linearity; STR = straightness; WOB = wobble; BCF = beat cross frequency. Values are expressed as mean ± SD.

4. Discussion

Permeable CPAs have been widely used for sperm freezing in order to prevent cryodamage (Oldenhof et al., 2010; Acha et al., 2015). However, they seem to be harmful for the sperm cell and also increase osmotic stress during freezing and thawing (Glazar et al., 2009), while moving across the plasma membrane.

In the present study, different concentrations of sucrose were added to a base extender for equine semen in order to find an optimal dilution able to protect donkey sperm against cryodamage, without the permeable CPA toxicity. Sucrose is one of the most effective non-permeable CPA (Zhu et al., 2012). Different concentrations have been used in other species always in combination with serum albumin at 1% (Isachenko et al., 2008; Isachenko et al., 2011; Sánchez et al., 2011), giving satisfactory results. Therefore, five sucrose concentrations were compared in this study in presence of BSA 1%, being the first attempt to cryopreserve donkey sperm in absence of permeable CPA.

According to our results, the extender containing sucrose 0.25 M obtained the highest motility percentages and average kinetic parameters in comparison with other extenders with higher and lower sucrose concentrations. Those results are in agreement with the study of Sánchez et al. (2011), in which the highest motility results were obtained with the same sucrose and BSA concentration in dog semen. Lower concentrations seem to be insufficient to preserve motility parameters in both species, and concentrations higher than S25 seem to hamper the motility by increasing the extender viscosity. However, Pradiee et al. (2015) obtained the highest motility values using sucrose 0.1 M in wild Iberian ibex sperm cryopreservation. In this regard, the most suitable sucrose concentration seems to be species-specific.

In our study, a significant decrease in PMI percentage was found only with the highest sucrose concentration, S45. This damage suffered by the equine sperm cell when is exposed to high osmotic stress and then returned to isosmotic conditions was previously described by Oldenhof et al. (2013). The excessive dehydration due to the higher osmolality of this sucrose-based extender (S45 = 642 mOm kg^{-1}) may be the reason for this result. Apparently, hyperosmotic solutions exert a higher damage to the sperm membrane than hypotonic mediums, since there were not significant differences for PMI among the remaining sucrose concentrations. These results are in agreement with Sánchez et al. (2011), where no differences were found in dog sperm for PMI among sucrose concentrations ranging from 0.1 M to 0.4 M.

DNA assessment (COMP-αt) showed no differences between treatments, except for S05 after four hours of incubation. It was shown in previous studies (Nawroth et al., 2002; Gutiérrez-Cepeda et al., 2012) that DNA fragmentation increased after incubation at 37 °C, which could be the reason for the lack of differences between treatments in this parameter at T0. After four hours of incubation, S05 resulted in the highest percentage of chromatin damage. Interestingly, S05 could protect the plasma membrane but not the sperm chromatin. This is in disagreement with the negative correlation found by Sabatini et al. (2014) between damaged chromatin (COMP-αt) and plasma membrane integrity.

Considering that S25 obtained the highest motility parameters and protected the DNA and plasma membrane integrity as good as the control did, it was selected and compared with GLY in Experiment 2. GLY was selected as the permeable cryoprotectant control because it has been traditionally used for sperm cryopreservation, showing also good post-thaw donkey sperm parameters (Ortiz et al., 2015). In general terms, no differences were found in motility percentages between treatments, although motility tends to be even higher for S25 than for GLY. Additionally, PMI results were significantly higher in the treatment with sucrose, which could be due to the different permeability of extenders: the damage suffered by the sperm cell while glycerol is crossing trough the plasma membrane is avoided by the use of non-permeable CPAs. Sugars were previously described to protect plasma membrane and increase PMI percentage in some species including stallions (Rosato and Iaffaldano, 2013; El-Badry et al., 2017). Moreover, no differences were found in DNA fragmentation between extenders. In this sense, S25 seems to be as effective as GLY in terms of both post-thaw sperm motility and chromatin integrity.

Different strategies have been performed to increase pregnancy rates after artificial insemination of jennies with cryopreserved

semen, including the use of permeable extenders with low molecular weight (Vidament et al., 2009; Oliveira et al., 2016), the addition of seminal plasma to frozen-thawed semen (Rota et al., 2012; Sabatini et al., 2014), or removing glycerol by colloid single-layer centrifugation in fresh samples (Serres et al., 2014). To date, none of those alternatives have obtained consistent results in donkey AI with cryopreserved sperm (Vidament et al., 2009; Rota et al., 2012). It could be explained by the damage caused by the glycerol in the sperm cell, whose toxicity has been hypothesized to start as early as the equilibration process (Vidament et al., 2009). However, there are no previous reports of donkey sperm cryopreservation in the absence of permeable CPAs.

In this study, sucrose-based extenders have shown their ability to maintain sperm parameters *in vitro* after thawing. Therefore, replacing glycerol from equine freezing extenders and using instead a combination of BSA and sucrose as non-permeable cryoprotectants could be another strategy to improve pregnancy rates. Unfortunately, no fertility trial was performed in this study, because the main purpose was to evaluate the sperm characteristics of sucrose-based extenders *in vitro*. Further studies including fertility trials would help to clarify this possibility.

In conclusion, the extender containing sucrose 0.25 M combined with 1%-BSA may be considered as an alternative to conventional freezing extenders with glycerol for donkey sperm cryopreservation.

Conflict of interest statement

The authors declare no conflicts of interest.

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SUPPLEMENT ARTICLE

Reproduction in Domestic Animals WILEY

Is sperm cryopreservation in absence of permeable cryoprotectants suitable for subfertile donkeys?

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Abstract

Sperm from fertile donkeys have been successfully frozen in absence of permeable cryoprotectants. The aim of this study was to determine whether this cryopreservation method is suitable for subfertile donkeys in comparison to conventional sperm freezing with glycerol. Ejaculates were collected from four Andalusian Donkeys: three fertile and one subfertile. Semen was frozen with an extender containing glycerol (GLY), or adding instead sucrose 0.25 molar and 1% bovine serum albumin (SUC) as non-permeable cryoprotectants. After thawing, samples were assessed for total (TM, %) and progressive (PM, %) sperm motility by CASA, plasma membrane integrity (PMI, %) by epifluorescence microscopy and DNA integrity (DFI, %) by SCSA. Results (mean ± *SD*) were compared between extenders in fertile and subfertile donkeys using the Student's *t* test. No differences between GLY and SUC treatments were found in the fertile group for the sperm parameters assessed. In subfertile donkey ejaculates, GLY resulted in significantly higher values than SUC for TM (25.5 ± 3.1 vs. 19.6 ± 1.9) and PM (13.3 ± 5.1 vs. 4.0 ± 1.2), respectively. In conclusion, considering all the sperm parameters assessed, sperm freezing in absence of permeable cryoprotectants may not be still an option for cryopreservation of subfertile donkey sperm.

KEYWORDS
cryoprotectant, donkey, fertility, freezing, sperm

1 | INTRODUCTION

Donkey population in Europe has diminished in the last century (Camillo et al., 2018), and Andalusian donkey breed is considered at risk of extinction according to Food and Agriculture Organization of the United Nations (FAO) last update in 2019: breeding population was estimated to be 103 males and 482 females in 2017. Taking into account the importance of environment and biodiversity preservation of domestic species resources, research should be focused in those reproductive procedures aiming to preserve all available individuals registered as pure breeds. In this sense, it would be advisable to create genetic banks for these donkey breeds at risk of extinction, including also those with low fertility in order to conserve the desirable levels of genetic variability within each species (Chesser, Smith, & Brisbin, 1980).

Cryopreservation of semen is an important tool to preserve genetic material and avoid the undesirable effects of inbreeding (Rota, Panzani, Sabatini, & Camillo, 2012), thanks to the possibility of national and international exchange of genetic resources through shipping of frozen semen among others (Kaneko, Kakinuma, Sato, & Jinno-Oue, 2018). However, permeable cryoprotectants used are known to exert a toxic effect in the sperm cell (Fahy, 2010), mostly due to the intrinsic toxicity and osmotic stress (Macías García et al., 2012). Recently though, sperm freezing avoiding permeable cryoprotectants has been successfully developed in fertile stallions (Consuegra et al., 2018) and donkeys (Diaz-Jimenez et al., 2018).

Additionally, the use of non-permeable cryoprotectants has shown to provide protection against cryoinjuries without toxic effects (Swain & Smith, 2010). Sperm banks must include all genetically valuable donkeys, fertile and subfertile, in order to maintain a high degree of genetic polymorphism and increase the breeding population, especially those under endangerment. However, cryopreservation with the sole use of non-permeable agents has not been tried in subfertile donkeys yet. Therefore, the aim of this study was to determine whether this cryopreservation method using non-permeable agents is suitable for subfertile donkeys, comparing sperm variables after thawing with conventional freezing with glycerol.

2 | MATERIALS AND METHODS

Four healthy Andalusian Donkeys, aged from 5 to 9 years, were used as semen donors: three fertile and one subfertile. Jackasses categorization was performed according to the sperm quality and the pregnancy rates per cycle reported by Dorado et al. (2013): fertile >60% and subfertile <40%. Three ejaculates per animal were collected using an artificial vagina. Sperm volume and concentration were recorded, and semen was diluted with INRA-96 (IMV) and centrifuged. Sperm pellets were resuspended to achieve a concentration of 200 × 10^6 sperm/ml with a skimmed milk–egg yolk extender with glycerol (GLY, 1001 mOm/kg, pH 6.8, Minitübe), or same extender without glycerol but adding sucrose 0.25 molar and 1% bovine serum albumin (SUC, 438 mOm/kg, pH 6.8) as non-permeable cryoprotectants (Diaz-Jimenez et al., 2018). Conventional freezing and thawing were performed following a protocol for donkey sperm cryopreservation (Diaz-Jimenez et al., 2018). Briefly, aliquots were slowly cooled for 2 hr at 5°C, filled in 0.5 ml straws and frozen in nitrogen vapours, 2.5 cm above the surface of liquid nitrogen for 5 min. The straws were placed into LN_2 tanks and thawed in a water bath (37°/30 s) after at least 24 hr of storage.

Ejaculates and post-thaw semen samples were evaluated as described by Diaz-Jimenez et al. (2019). Sperm motility was assessed by the Sperm Class Analyzer (SCA v5.4, Microptic S.L.). For each evaluation, two 5 µl drops and three random microscopic fields per drop were analysed including at least 200 spermatozoa. The trajectory of each spermatozoon was determined by the SCA software obtaining total (TM, percentage of spermatozoa with a mean average path velocity >10 µm/s) and progressive (PM, percentage of motile spermatozoa with >75% of the straightness coefficient) sperm motility. Plasma membrane integrity (PMI, %) was evaluated based on the red–green emission of propidium iodide and acridine orange, respectively, from the VitalTest staining (Halotech DNA S.L.) under epifluorescence microscopy. DNA fragmentation index (DFI, %) was evaluated using the Sperm Chromatin Structure Assay (SCSA) with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems) by assessing approximately 5,000 cells and recording the percentage of cells with single-stranded DNA. Sperm parameters assessed were compared between treatments (GLY vs. SUC extenders) and fertile and subfertile donkeys using the Student's *t* test. Results were expressed as mean ± *SD*. Experiments were performed in accordance with the Ethical Animal Experimentation Committee of Cordoba University (project no. 31/08/2017/105) according to the Spanish law for animal welfare and experimentation (Decision 2012/707/UE and RD 53/2013).

3 | RESULTS

Mean average variables of ejaculates used in this study (n = 12) from fertile and subfertile donkeys before cryopreservation are shown in Table 1. No differences between GLY and SUC extenders were found in the fertile group for all the sperm parameters assessed: TM (31.7 ± 12.4 vs. 32.6 ± 14.9), PM (22.0 ± 7.5 vs. 19.6 ± 8.8), PMI (44.0 ± 21.6 vs. 41.7 ± 23.4) and DFI (43.6 ± 13.4 vs. 41.8 ± 11.7) (Figure 1). However, for subfertile donkey (Figure 2), GLY resulted in higher values (p < .05) than SUC for TM (25.5 ± 3.1 vs. 19.6 ± 1.9) and PM (13.3 ± 5.1 vs. 4.0 ± 1.2); but no differences were found for PMI (41.5 ± 18.3 vs. 41.9 ± 19.2) and DFI (32.5 ± 4.6 vs. 30.8 ± 7.5).

4 | DISCUSSION

To the best of the authors' knowledge, this is the first study in which sperm from subfertile donkey has been cryopreserved in absence of permeable cryoprotectants. In agreement to previous studies (Diaz-Jimenez et al., 2018), our results showed cryopreservation of fertile donkey sperm using the extender containing sucrose and bovine serum albumin (BSA) was equally able to protect sperm cells from freezing when compared to conventional cryopreservation with glycerol.

Unexpectedly, regarding the subfertile group, cryopreservation using glycerol resulted in higher values for sperm motility when compared to sucrose and BSA extender. Interestingly though, same combination of non-permeable cryoprotectants was previously employed by Sanchez et al. (2012) in cryopreservation of human sperm from an oligoastenozoospermic patient, and high sperm motility values were found after warming, but vitrification technique was employed.

TABLE 1 Mean values of sperm parameters from ejaculates (n = 12) in fertile and subfertile donkeys

	Animals	
Sperm parameters	**Fertile**	**Subfertile**
Gel-free volume (ml)	106.1 ± 25.8[a]	85.0 ± 32.8[a]
Sperm concentration (×10^6/ml)	227.6 ± 37.8[a]	59.7 ± 37.6[b]
Normal forms (%)	85.0 ± 8.8[a]	62.2 ± 10.2[b]
Total motility (%)	68.8 ± 6.9[a]	47.8 ± 11.0[b]
Progressive motility (%)	58.2 ± 8.9[a]	27.0 ± 9.7[b]
Plasma membrane integrity (%)	63.6 ± 17.8[a]	52.6 ± 15.3[a]

Different letters indicate significant differences (p < .05). Results are expressed as mean ± *SD*.

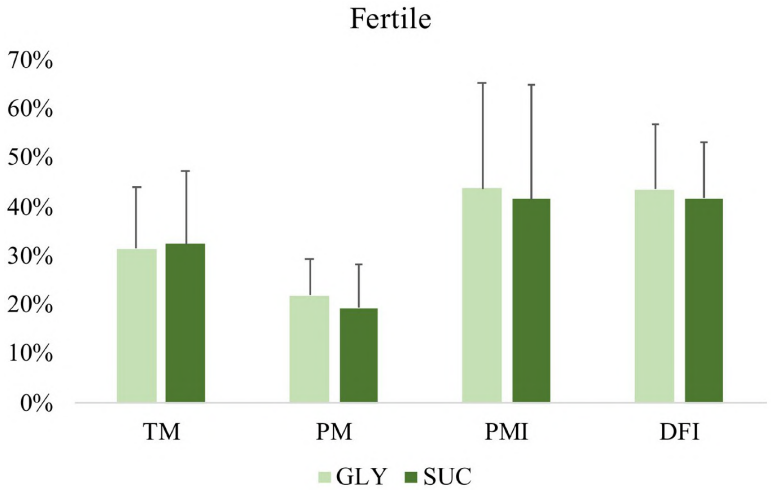

FIGURE 1 Comparison of sperm parameters after thawing between GLY and SUC in fertile donkeys. DFI, DNA fragmentation index; GLY: extender with glycerol; PM, progressive motility; PMI, plasma membrane integrity; SUC: extender with sucrose and bovine serum albumin; TM, total motility. Results are expressed as mean ± *SD*. No significant differences were found between treatments ($p > .05$)

Similarly, a recent study performed in human patients with oligoasthenozoospermia and severe oligoasthenozoospermia showed that sperm vitrification with sucrose as sole cryoprotectant resulted in higher motility results in comparison to conventional freezing with glycerol (Karthikeyan, Arakkal, Mangalaraj, & Kamath, 2019). In this sense, the sole use of non-permeable agents might have different efficiency in subfertile males regarding species. However, sperm vitrification was not performed in this study but conventional freezing, and human sperm has greater cryostability in comparison to other species (Gao, Mazur, & Critser, 1997).

Our results showed the extender containing only non-permeable agents was able to protect plasma membrane and DNA integrity in both groups, as no differences were found in comparison to extender containing glycerol. In the same way, a combination of sucrose and BSA resulted in high plasma membrane and DNA integrity percentages after stallion sperm cryopreservation in previous reports (Consuegra et al., 2018; Hidalgo et al., 2018). Discrepancy in motility results between fertile and subfertile groups regarding the use of glycerol might be explained because the effectiveness of non-permeable cryoprotectants could be subjected to the initial sperm quality of the sample. It has been previously suggested for human sperm that independently from the freezing technique, damage caused after semen cryopreservation is more pronounced in those samples with an initial abnormal quality (Karthikeyan et al., 2019; Verza, Feijo, & Esteves, 2009). In this sense, the concentration of sucrose and BSA here employed is possibly insufficient, and it might be necessary to add other non-permeable substances in those sperm samples which showed reduced motility prior to freezing, as it is in sperm from the subfertile donkeys.

In conclusion, promising results were obtained regarding plasma membrane and DNA integrity after sperm cryopreservation using only non-permeable cryoprotectants in donkeys regardless its fertility. However, considering overall parameters, sperm freezing in absence of permeable cryoprotectants may still not be an option for cryopreservation of subfertile donkey sperm. Further studies are needed including a large number of subfertile animals.

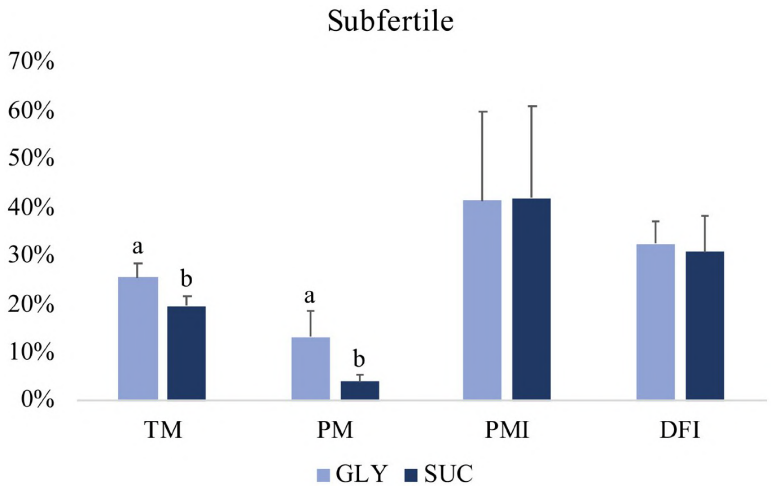

FIGURE 2 Comparison of sperm parameters after thawing between GLY and SUC in subfertile donkeys. DFI, DNA fragmentation index; GLY: extender with glycerol; PM, progressive motility; PMI, plasma membrane integrity; SUC: extender with sucrose and bovine serum albumin; TM, total motility. Results are expressed as mean ± *SD*. Different letters indicate significant differences between treatments ($p < .05$)

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

M Hidalgo and M Diaz-Jimenez contributed to all sections. J Dorado and I Ortiz contributed to the study design, preparation and final approval of the manuscript. C Consuegra and B Pereira performed the experiments. All the authors were involved in revision and approval of the final version of the manuscript.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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CHAPTER 2

Vitrification of donkey sperm: Is it better using permeable cryoprotectants?

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Vitrification of Donkey Sperm: Is It Better Using Permeable Cryoprotectants?

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Simple Summary: Conventional donkey sperm-freezing using permeable cryoprotectants has been successfully performed, and good sperm parameters have been obtained after thawing. Unfortunately, artificial insemination of jennies with cryopreserved semen has given unsatisfactory results. Vitrification by directly dropping the sperm into the liquid nitrogen following the spheres methodology has been developed in human beings as an alternative to conventional freezing. This technique has shown to be a species-specific methodology and the concentration of cryoprotectants should be optimized in donkeys. Additionally, in this study, a permeable cryoprotectant (glycerol) has been tested for the first time for donkey sperm vitrification. According to our findings, vitrification of donkey sperm was effectively carried out using an extender supplemented with sucrose or bovine serum albumin (BSA) as non-permeable agent. When glycerol, a permeable agent, was compared to sucrose 0.1 M and BSA 5%, sperm quality significantly decreased. Therefore, donkey sperm vitrification in the absence of permeable agents obtained better results and gives a new approach to create a pattern for future studies of fertility trials.

Abstract: Vitrification by direct exposure of sperm to liquid nitrogen is increasing in popularity as an alternative to conventional freezing. In this study, the effect of permeable cryoprotectant agents for donkey sperm vitrification was compared to an extender containing non-permeable cryoprotectants. First, three different concentrations of sucrose (0.1, 0.2, and 0.3 molar, M) and bovine serum albumin, BSA (1, 5, and 10%) were compared. Secondly, the concentration of non-permeable agents producing the most desirable results was compared to an extender containing glycerol as permeable agent. Vitrification was performed by dropping 30 µL of sperm suspension directly into LN2 and warming at 42 °C. Sperm motility (total, TM; and progressive, PM) and plasma membrane integrity, PMI (mean ± SEM) were statistically compared between treatments. Sucrose 0.1 M showed a significantly higher percentage of total sperm motility (21.67 ± 9.22%) than sucrose 0.2 M (14.16 ± 4.50%) and 0.3 M (8.58 ± 6.22%); and no differences were found in comparison to the control (19.71 ± 10.16%). Vitrification with sucrose 0.1 M or BSA 5% obtained similar results for TM (21.67 ± 9.22% vs. 19.93 ± 9.93%), PM (13.42 ± 6.85% vs. 12.54 ± 6.37%) and PMI (40.90 ± 13.51% vs. 37.09 ± 14.28); but both showed higher percentages than glycerol (TM = 9.71 ± 4.19%; PM = 5.47 ± 3.17%; PMI = 28.48 ± 15.55%). In conclusion, donkey sperm vitrification in spheres using non-permeable cryoprotectants exhibited better sperm motility and viability parameters after warming than sperm vitrification using extenders containing permeable cryoprotectants.

Keywords: donkey; sperm; vitrification; spheres; sucrose; BSA; glycerol

1. Introduction

According to the Food and Agricultural Organization, European donkey populations have diminished considerably in the last century [1]. According to the Spanish regulations, the Andalusian donkey breed is in danger of extinction, with a breeding population of 100 males and 436 females in the last update in 2020 [2]. In this sense, and considering the importance of environment and biodiversity preservation of domestic species resources, the creation of sperm banks contributes to preserve valuable genetic material from these endangered populations. Unfortunately, artificial insemination (AI) with cryopreserved donkey semen has resulted in poor fertility outcomes [3]. Different strategies have been developed to improve pregnancy rates, including the combination of different cryoprotectant agents (CPAs) [4], addition of seminal plasma to frozen-thawed donkey semen before AI [3], post-thaw centrifugation for cryoprotectant removal [5], study of the jennies endometrial response after AI [6,7], or the influence of different insemination protocols [8]. The low fertilizing capacity of cryopreserved donkey sperm has been attributed to the impact of permeable CPAs, but this hypothesis remains unclear [3,9,10].

In addition, the osmotic stress produced in the sperm cell during conventional freezing and thawing may induce structural and functional damage through the formation of ice crystals, and affects the fertilizing ability of cryopreserved sperm [11]. Vitrification is a cryopreservation method widely used for embryo, oocyte or tissue storage [12,13]. It involves the solidification of a solution, which turns into a glass-like state [14]. During vitrification, viscosity greatly increases, and intracellular or extracellular ice crystals are not formed because water does not precipitate [15]. A high concentration of permeable CPAs has been used to reach the high viscosity needed for oocyte and embryo vitrification [13]. However, this methodology has yet to be applied to the sperm cell due to its higher sensitivity to increasing concentrations of permeable CPAs [16,17]. Nevertheless, it has been demonstrated that the concentration of CPAs required for achieving vitrification is inversely related to the rate of cooling/warming. This means that if the sample is ultra-fast cooled (immersing small volume samples directly into LN_2), high concentration of permeable CPAs are not necessary to achieve a vitrified state, therefore avoiding their toxicity [13,18]. This methodology, combined with non-permeating substances such as proteins and/or carbohydrates, has been called 'kinetic sperm vitrification' or 'permeable cryoprotectant-free sperm vitrification' and has been successfully developed in human [15], dog [19], fish [20,21] wild ungulates [17,22], cats [23] and, recently, in stallions [24] and donkeys [25]. It is a simple, fast, and cost-effective method to cryopreserve sperm, even under field conditions, since a reasonably equipped laboratory is required, making it attractive for the conservation of wild or endangered species and genetically valuable animals distributed in different regions, as it usually happens in endangered donkey breeds. Taking into account that sperm vitrification has led to similar or an increase in sperm quality after warming in comparison to conventional freezing in stallion [24], it could be considered to be another alternative to improve sperm cryopreservation in donkeys.

The optimal concentration of CPAs seems to be species-specific and has been proposed as a key factor for sperm vitrification success, depending on the methodology used [24]. In a preliminary research, donkey sperm vitrified in straws showed significant higher sperm motility percentages when compared to vitrification in spheres [25]. However, only sucrose was tested as cryoprotectant agent, and a fixed concentration was employed for both methods derived from previous studies in other species. Consequently, the optimal concentration of non-permeable CPAs for donkey sperm vitrification using the spheres methodology has not been determined yet. Additionally, as previously stated, sperm vitrification has been developed using non-permeable CPAs [26] and a few attempts for sperm vitrification have been performed using a combination of permeable and non-permeable CPAs [27]. To the best of our knowledge, donkey sperm vitrification in spheres with the sole use of permeable CPAs has not been tested yet.

Therefore, the present study was designed to examine the effectiveness of different concentrations of sucrose and bovine serum albumin for donkey sperm vitrification in spheres, in comparison to a vitrification extender containing permeable CPAs by examining the post-thaw quality in vitro.

2. Materials and Methods

2.1. Animals, Semen Collection, and Processing

All procedures were approved by the Ethical Committee for Animal Experimentation of the University of Cordoba, in compliance with the Regional Government of Andalusia (project no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (RD 53/2013).

The jackasses were housed in individual paddocks at "Centro Rural Malpica" (Palma del Rio, Cordoba, Spain). They were fed with teff hay, oat grains and water "ad libitum". Semen was collected from four adult, healthy and fertile Andalusian donkeys using a Missouri artificial vagina (Minitüb, Tiefenbach, Germany) in the presence of a jenny in estrus. Semen was collected one or twice a week per donkey until a total of 16 ejaculates was completed (four ejaculates per donkey). Immediately after collection, volume (mL) and sperm concentration ($\times 10^6$ spermatozoa/mL) were measured in each gel-free semen sample using a graduated collector and a sperm photometer (Spermacue®, Minitüb, Tiefenbach, Germany) respectively. Sperm motility and viability parameters were evaluated as described below. Thereafter, sperm was extended 1:1 (*v/v*) with INRA96 (IMV Technologies, France) and centrifuged at 400× *g* for 7 min to remove seminal plasma. Sperm pellets were then re-extended in each vitrification media (see experimental design) to reach a final concentration of 200×10^6 spermatozoa/mL. Sperm suspensions were maintained at room temperature for 10 min and slowly cooled for 1 h at 5 °C into a sperm container (Equitainer, Hamilton Research, Inc. Ipswich, Massachusetts, USA) before the vitrification procedure was performed.

2.2. Vitrification and Warming

Sperm vitrification was carried out following the methodology previously described [24,25]. Briefly, a styrofoam box was loaded with LN_2 and five 30 µL drops from each treatment were plunged directly into the LN_2. A micropipette held at an angle of about 45° and a distance of 10 cm from the surface was used (Figure 1a,b). After contact with the LN_2 a sphere is immediately formed (Figure 1c). Spheres were then packaged into 1.8 mL cryotubes and stored LN_2 tanks. The warming procedure was performed by introducing the spheres one by one into two milliliters of extender (INRA-96) previously warmed to 42 °C. A gentle vortexing for a few seconds of each sample was done before centrifugation as described above. Sperm pellets were re-extended with INRA-96 after supernatant removal, to reach final concentration of 25×10^6 spermatozoa/mL for sperm evaluation. Sperm motility was objectively evaluated by using the Sperm Class Analyzer (SCA v.5.4.0, Microptic S.L., Barcelona, Spain) as previously described [4]. The following kinetic parameters were calculated by the system: total (TM, %) and progressive motility (PM, %); curvilinear (VCL, µm/s), straight line (VSL, µm/s) and average path velocities (VAP, µm/s), linearity (LIN, VSL/VCL × 100), straightness rate (STR, VSL/VAP × 100), wobble (WOB, VAP/VCL × 100), lateral head displacement amplitude (ALH, µm) and beat cross frequency (BCF, Hz). Sperm membrane integrity was assessed using Vital-Test commercial kit (Halotech DNA S.L., Madrid, Spain) for sperm staining following the manufacturer´s instructions. In brief, an aliquot of 10 µL of diluted semen was mixed with 1 µL propidium iodide stock solution and 1 µL of acridine orange stock solution and evaluated under epifluorescence microscopy. At least 200 spermatozoa were counted, and sperm with intact plasma membrane was recorded (PMI, %).

2.3. Experimental Design

2.3.1. Experiment 1. Vitrification of Donkey Sperm Using Different Concentrations of Sucrose

Sucrose (Sigma-Aldrich Corp., St. Louis, MI, USA) was added to a control base extender (Control) commonly used for horse sperm. This base extender contains egg yolk and antibiotics (Gent, Minitüb, Tiefenbach, Germany). Powder sucrose was weighted with a precision balance and mixed with the control extender by vortexing. Three concentrations of sucrose (mol/L, M) were compared: 0.1 M,

0.2 M and 0.3 M; each extender was then added to the sperm pellets and vitrified as described before. Post-warming sperm parameters were recorded and compared between treatments.

2.3.2. Experiment 2. Vitrification of Donkey Sperm Using Different Concentrations of Bovine Serum Albumin

Bovine serum albumin (BSA, Sigma-Aldrich, Sant Louis, MI, USA) was weighted and added to the same control base extender as described before at the following concentrations: 1%, 5% and 10%. Each sample was then vitrified, and post-warming analysis was carried out as previously described, comparing the results obtained among treatments.

2.3.3. Experiment 3. Comparison Between Permeable and Non-Permeable CPAs for Donkey Sperm Vitrification

Having identified the best concentration of non-permeable CPAs for donkey sperm vitrification in spheres, a commercial extender for stallion sperm-freezing containing permeable CPAs, in particular glycerol (Gent B, Minitüb, Tiefenbach, Germany), was compared for sperm vitrification. Post-warming sperm parameters were assessed for each treatment as described before.

2.4. Statistical Analysis

Results were analyzed using the Statistical Analysis Systems software (SAS version 9.0; SAS Institute Inc, Cary, NC, USA). All data was first tested for normality of the data distribution and homogeneity of variances using the Kolmogorov–Smirnov and Levene test, respectively. The sperm parameters assessed were compared between treatments using a repeated measured general lineal model (GLM). Animals and ejaculates were considered to be random factors. The Post-Hoc HSD Tukey test was used to compare differences between mean values. Results were expressed as mean ± standard deviation of the mean (SD). The level of significance was set at $p < 0.05$.

3. Results

Mean average parameters of ejaculates used in this study were as follows: gel-free volume of 112 ± 41 mL, sperm concentration of 191 ± 68 × 10^6 spermatozoa/mL, total motility 79.7 ± 12.8%, progressive motility 64.7 ± 15.4% and sperm with intact plasma membrane 58.7 ± 15.7%.

3.1. Experiment 1. Vitrification of Donkey Sperm Using Different Concentrations of Sucrose

Vitrification in spheres using a sucrose concentration of 0.1 M resulted in the greatest values ($p < 0.05$) for all the sperm motility variables assessed in comparison to the other sucrose concentrations (Table 1). There were no significant differences ($p > 0.05$) between control and sucrose treatments for the assessment of plasma membrane integrity. There were no differences between sucrose 0.1 M and the control for total (21.67 ± 9.22 vs. 19.71 ± 10.16) and progressive (13.42 ± 6.85 vs. 12.34 ± 8.13) sperm motility, respectively; however, sucrose 0.1 M showed a tendency to obtain higher values of sperm motility percentages.

Table 1. Vitrification of donkey semen samples ($n = 16$) using different concentrations of sucrose.

Sperm Parameters	Vitrification Extender				*p*-Values
	Control	Sucrose 0.1 M	Sucrose 0.2 M	Sucrose 0.3 M	
TM (%)	19.71 ± 10.16 ^{a,b}	21.67 ± 9.22 ^{a}	14.16 ± 4.50 ^{b}	8.58 ± 6.22 ^{c}	<0.001
PM (%)	12.34 ± 8.13 ^{a}	13.42 ± 6.85 ^{a}	7.69 ± 3.01 ^{b}	3.53 ± 4.42 ^{b}	<0.001
PMI (%)	33.66 ± 14.84	40.90 ± 13.50	39.67 ± 13.12	38.62 ± 10.32	>0.05
VCL (μm/s)	77.04 ± 19.70 ^{a}	82.16 ± 13.31 ^{a}	65.07 ± 13.04 ^{b}	44.69 ± 16.98 ^{c}	<0.001

Table 1. *Cont.*

Sperm Parameters	Vitrification Extender				*p*-Values
	Control	Sucrose 0.1 M	Sucrose 0.2 M	Sucrose 0.3 M	
VSL (μm/s)	62.52 ± 19.12 [a]	66.91 ± 12.48 [a]	50.42 ± 13.80 [b]	32.61 ± 15.43 [c]	<0.001
VAP (μm/s)	67.23 ± 19.51 [a,b]	71.96 ± 12.93 [a]	56.46 ± 11.74 [b]	36.75 ± 16.58 [c]	<0.001
ALH (μm)	2.34 ± 0.29 [a]	2.46 ± 0.43 [a]	2.04 ± 0.56 [b]	1.78 ± 0.67 [b]	<0.001
LIN (%)	80.02 ± 5.69 [a]	81.16 ± 6.19 [a]	76.52 ± 11.01 [a,b]	68.13 ± 17.85 [b]	<0.05
STR (%)	92.74 ± 2.80 [a]	92.84 ± 2.85 [a]	88.08 ± 11.32 [a,b]	84.44 ± 17.97 [b]	<0.05
WOB (%)	86.40 ± 4.32 [a]	87.34 ± 4.64 [a]	86.70 ± 3.79 [a]	78.35 ± 13.85 [b]	<0.05
BCF (Hz)	9.65 ± 0.82 [a]	9.64 ± 0.66 [a]	8.60 ± 1.79 [a,b]	7.68 ± 2.90 [b]	<0.001

Different letters ([a–c]) in the same row indicate significant differences. TM, total motility; PM, progressive motility; PMI, plasma membrane integrity; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; LIN, linearity; STR, straightness; WOB, wobble; BCF, beat cross frequency; Control, control extender without sucrose. Values are expressed as mean ± standard deviation of the mean.

3.2. Experiment 2. Vitrification of Donkey Sperm Using Different Concentrations of Bovine Serum Albumin

The addition of different concentrations of BSA to the vitrification extender resulted in no significant differences ($p > 0.05$) in any of the sperm parameters assessed when compared to control samples (Table 2).

Table 2. Vitrification of donkey semen samples ($n = 16$) using different concentrations of bovine serum albumin (BSA).

Sperm Parameters	Vitrification Extender				*p*-Values
	Control	BSA-1%	BSA-5%	BSA-10%	
TM (%)	19.71 ± 10.16	19.51 ± 9.67	19.93 ± 8.93	15.52 ± 6.37	>0.05
PM (%)	12.34 ± 8.13	12.58 ± 7.65	12.54 ± 6.37	9.24 ± 4.39	>0.05
PMI (%)	33.66 ± 14.84	36.37 ± 11.36	37.09 ± 14.28	35.49 ± 13.28	>0.05
VCL (μm/s)	77.04 ± 19.70	83.74 ± 15.80	83.27 ± 16.31	79.69 ± 16.87	>0.05
VSL (μm/s)	62.52 ± 19.12	68.74 ± 14.78	68.19 ± 15.88	64.57 ± 17.07	>0.05
VAP (μm/s)	67.23 ± 19.51	74.54 ± 15.59	73.86 ± 16.43	69.91 ± 17.06	>0.05
ALH (μm)	2.34 ± 0.29	2.31 ± 0.36	2.46 ± 0.22	2.49 ± 0.31	>0.05
LIN (%)	80.02 ± 5.69	81.72 ± 4.40	81.09 ± 5.06	80.14 ± 5.74	>0.05
STR (%)	92.74 ± 2.77	92.12 ± 2.73	92.08 ± 2.48	91.96 ± 3.16	>0.05
WOB (%)	86.40 ± 4.31	88.67 ± 3.28	88.04 ± 3.95	87.06 ± 4.13	>0.05
BCF (Hz)	9.65 ± 0.82	9.67 ± 0.62	14.82 ± 21.65	15.16 ± 21.58	>0.05

TM, total motility; PM, progressive motility; PMI, plasma membrane integrity; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; LIN, linearity; STR, straightness; WOB, wobble; BCF, beat cross frequency; Control, control extender without BSA; Values are expressed as mean ± standard deviation of the mean.

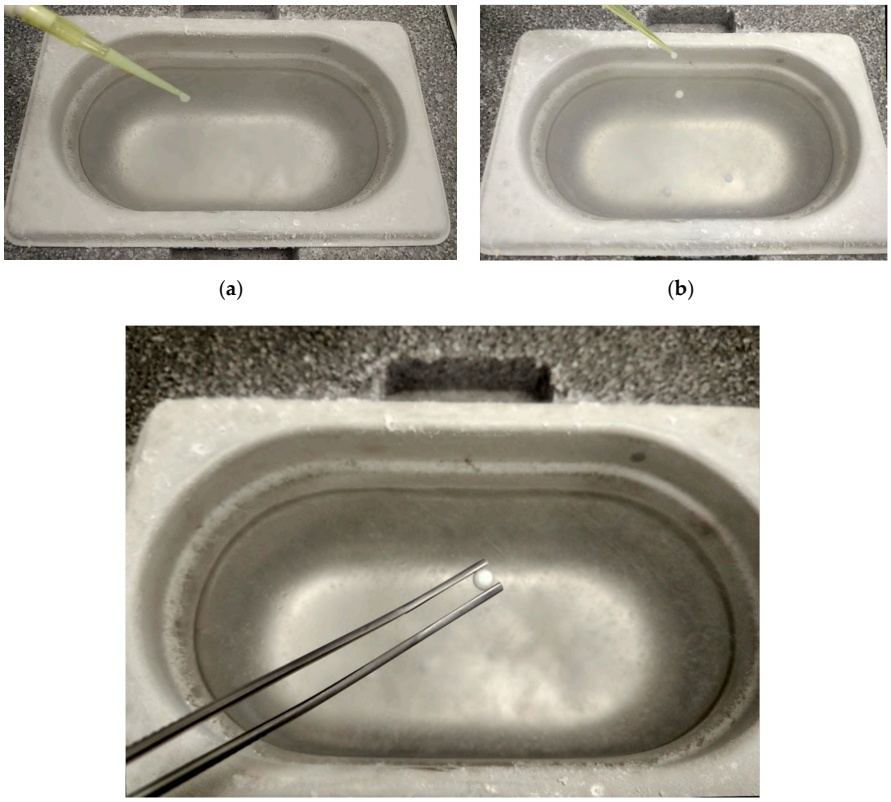
3.3. Experiment 3. Comparison Between Permeable and Non-Permeable CPAs for Donkey Sperm Vitrification

The sole use of glycerol for sperm vitrification in spheres decreased ($p < 0.05$) TM (9.71 ± 4.19%), PM (5.47 ± 3.17%) and PMI (28.48 ± 15.55%) compared to vitrification using sucrose 0.1 M and BSA 5% (Table 3). No significant differences ($p > 0.05$) were found in the remaining sperm parameters assessed.

Table 3. Comparison between permeable (glycerol) and non-permeable (sucrose and BSA) cryoprotectants for donkey sperm vitrification (n = 16).

Sperm Parameters	Vitrification Extender			*p*-Values
	Glycerol	Sucrose 0.1 M	BSA-5%	
TM (%)	9.71 ± 4.19 [b]	21.67 ± 9.22 [a]	19.93 ± 8.93 [a]	<0.001
PM (%)	5.47 ± 3.17 [b]	13.42 ± 6.85 [a]	12.54 ± 6.37 [a]	<0.001
PMI (%)	28.48 ± 15.55 [b]	40.90 ± 13.51 [a]	37.09 ± 14.28 [a]	<0.05
VCL (μm/s)	80.72 ± 17.14	82.16 ± 13.31	83.27 ± 16.31	>0.05
VSL (μm/s)	68.09 ± 18.06	66.91 ± 12.48	68.19 ± 15.88	>0.05
VAP (μm/s)	71.97 ± 17.55	71.96 ± 12.93	13.86 ± 16.43	>0.05
ALH (μm)	2.32 ± 0.54	2.46 ± 0.43	2.46 ± 0.22	>0.05
LIN (%)	83.04 ± 6.19	81.16 ± 6.19	81.09 ± 5.06	>0.05
STR (%)	93.68 ± 3.26	92.84 ± 2.85	92.08 ± 2.48	>0.05
WOB (%)	88.56 ± 4.26	87.34 ± 4.64	88.04 ± 3.95	>0.05
BCF (Hz)	9.47 ± 1.36	9.64 ± 0.66	14.82 ± 21.65	>0.05

Different letters ([a,b]) in the same row indicate significant differences. TM, total motility; PM, progressive motility; PMI, plasma membrane integrity; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; LIN, linearity; STR, straightness; WOB, wobble; BCF, beat cross frequency; Values are expressed as mean ± standard deviation of the mean.



(**a**) (**b**)

(**c**)

Figure 1. Images of the spermatozoa vitrification procedure, (**a**,**b**) 30 μL of spermatozoa suspension dropped into LN_2 using a micropipette, (**c**) sphere formed after sperm vitrification.

4. Discussion

Permeable CPAs have been conventionally employed for the cryopreservation of donkey sperm samples by slow freezing in donkey sperm, and by vitrification in spheres in other species, but they had not been employed for vitrification in donkeys yet. Non-permeable CPAs have been successfully used for sperm vitrification in different animal species [15,19,24,25,28]. It has been pointed out that the optimal concentration of non-permeable cryoprotectants varies between vitrification techniques. In this sense, studies comparing different concentrations of non-permeable cryoprotectants for sperm vitrification using the spheres technique in donkey sperm are so far lacking in the scientific literature.

In the first experiment of the present study, the addition of sucrose 0.1 M to the extender had a positive effect on sperm motility percentages in comparison to the control without sucrose. Sucrose concentrations were selected taking into consideration other reports of sperm vitrification in spheres in mammals [12,17,19,23–25,29], in which various amounts of sucrose between 0.02 M to 0.5 M were added. According to the results obtained in this study, the lowest sucrose concentration (0.1 M) resulted in the greatest values for sperm motility parameters in comparison to higher concentrations of sucrose (0.2 M and 0.3 M). These results are in agreement with previous reports in which different sucrose concentrations were compared, and higher sucrose concentrations (0.3 M and 0.5 M) resulted in significantly lower values for sperm motility in comparison to 0.1 M in wild goat [17] and sheep [22]. Similarly, sucrose 0.1 M has also been successfully used for sperm vitrification in spheres in 14 different wild endangered species [30] and in mouflon and fallow deer [31]. However, previous research of sperm vitrification using the spheres method have shown sucrose requirements to be slightly higher than 0.1 M in other species. In this regard, concentrations of 0.2 M [32] and 0.25 M [29,33] have been successfully employed in studies performed in human; 0.125 M in fish [20]; 0.2 M in cat [23], and 0.25 M in dog [19]. Interestingly, though, despite the phytogenic proximity between horse and donkey species [24], sucrose requirements for vitrification of stallion sperm showed to be much lower than that of in donkeys. It has been reported the upper limit of sucrose concentration to be 0.02 M in stallions [24], which is far lower than those used in the present study.

Our results once again reaffirm the previously described differences of cryoprotectant requirements between species [22,24]. A reasonable explanation for the diverse responses to vitrification extender is that species have different sperm cryosurvival, as described by other authors [12,27]; it seemed to be species-specific, which in turn may be a consequence of the cryostability and properties of the sperm cell: sperm size, water content, membrane fluidity, osmotic content and/or internal compaction [30,31,34]. In agreement with previous reports in other species [17,28], in the current study no differences were found between the different sucrose concentrations and the control extender regarding plasma membrane integrity.

Serum albumin has been traditionally added to the vitrification extender for sperm cryopreservation in several species. Thus, human serum albumin has been employed at 1% in human [29]; and BSA at 0.5% in rabbit [12]; 1% in equine [35], dog [19], fish [28], donkey [36] and wild goat [17]; and 2% in ram [37]. This molecule has shown to reduce oxidative stress [38] and to protect the sperm membrane against cryodamage, although the exact mechanism is still not clear [39]. Few studies have, however, tested different concentrations for sperm vitrification to determine the most adequate [24]. Moreover, to the best of the authors knowledge, the effect of BSA for sperm vitrification has always been examined in combination with sucrose and other cryoprotectants, but not by itself. Therefore, we aimed to test if the sole use of BSA could increase sperm quality after vitrification. Surprisingly, no differences among BSA concentrations nor with the control extender were found in any of the parameters assessed. However, the concentration of BSA 5% showed a tendency to obtain higher motility results.

In the last experiment, and considering that the use of 0.1 M of sucrose in the extender highly improved motility parameters, and BSA 5% also showed a tendency to improve motility results, both extenders were compared with an extender with a permeable CPA for donkey sperm vitrification. Glycerol was selected as the permeable agent because it has been widely and successfully employed

for donkey sperm cryopreservation in previous studies [3,4,40–42], and could be considered to be a starting point to study the impact of permeable agents in donkey sperm vitrification. Glycerol has been previously included in vitrification extenders following the spheres or straws method, with positive results in sperm quality parameters after warming in several species such as ram [16] and sea bream [43]; dimethyl sulfoxide in salmon [21], and a combination of ethylene glycol and dimethyl sulfoxide in goat [44]. According to our results, the addition of glycerol to the vitrification extender significantly reduced sperm motility and plasma membrane integrity after warming; however, similar results were obtained when sperm vitrification was performed using either sucrose 0.1 M or BSA 5% as non-permeable agents. These results agree with previous reports in wild sheep [22], in which glycerol addition to the vitrification extender decreased sperm motility and plasma membrane integrity compared to the sole use of non-permeable CPAs. Nonetheless, other authors reported no motile or viable boar sperm after vitrification with only sucrose, neither in combination with permeable agents [27]. Sperm vitrification in spheres using only non-permeable agents have also been problematic in other species, obtaining few motile or viable sperm after warming in rabbit [12] and ram [37]. Interestingly, in a previous study performed in donkeys [42] in which authors compared between slow freezing using only non-permeable agents and the same freezing protocol but containing glycerol, no differences were found for sperm motility and DNA integrity. Therefore, it seems that glycerol may protect donkey sperm during slow freezing but no during vitrification. As mentioned before, these differences among studies can be explained in part by the cryopreservation method employed, an inadequate concentration and/or type of non-permeable agent [12], sperm cryosurvival regarding the species [27], as well as the lack of equilibration temperature, which have shown to be essential for sperm vitrification in spheres [24].

5. Conclusions

The present study showed that donkey sperm could not be vitrified in small volumes (spheres) using only glycerol as permeable CPAs. Vitrification using non-permeable CPAs (sucrose 0.1 M and BSA 5%) enhanced sperm motility and viability after warming. Further studies will concentrate on evaluating combinations of permeable and non-permeable CPAs for donkey sperm vitrification, assessing a wider range of sperm parameters after warming.

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CHAPTER 3

CHAPTER 3.1

Effect of different extenders for donkey sperm vitrification in straws

Diaz-Jimenez et al., 2017. Reproduction in Domestic Animals

CHAPTER 3.2

Optimization of donkey sperm vitrification: effect of sucrose, sperm concentration, volume and package (0.25 and 0.5 mL straws)

Diaz-Jimenez et al., 2019. Animal Reproduction Science

CHAPTER 3.3

Vitrification in straws conserves motility features better than spheres in donkey sperm

Diaz-Jimenez et al., 2018. Reproduction in Domestic Animals

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SHORT COMMUNICATION

WILEY Reproduction in Domestic Animals

Effect of different extenders for donkey sperm vitrification in straws

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Contents

Aseptic vitrification of semen samples packed in straws has been successfully developed in human but not in donkeys. The aim of this study was to compare the effect of two extenders for donkey sperm vitrification using straws. Ejaculates from four Andalusian donkeys were collected, and samples were extended in INRA-96 (I) or Gent (G) supplemented with sucrose 0.25 M and 1% bovine serum albumin (BSA). Extended samples were cooled for one hour at 5°C. For vitrification, samples were filled in covered 0.25 ml straws and then plunged directly into liquid nitrogen. For warming, straws were immersed in INRA-96 at 43°C. Results showed no significant differences between I and G treatments for TM (34.2% ± 8.7 vs. 30.7% ± 9.6) and PM (26.8% ± 7.3 vs. 24.6% ± 7.9), respectively. In conclusion, donkey sperm could be vitrified in straws either with INRA-96 or with Gent in combination with sucrose and BSA.

1 | INTRODUCTION

Sperm vitrification has been recently used as an alternative to conventional freezing of human (Isachenko, Isachenko, Sanchez, Katkov, & Kreienberg, 2011), dog (Sánchez et al., 2011), fish (Figueroa et al., 2015; Merino et al., 2011) and wild goat (Pradiee et al., 2015) spermatozoa. This technique consists of plunging small volumes of semen samples directly into liquid nitrogen, thus the sample is cooled at ultra-fast speed rates in the absence of permeable cryoprotectants (Isachenko, Isachenko, et al., 2011).

A new aseptic technique packing sperm in sterile straws has been successfully developed for human (Isachenko, Maettner, et al., 2011; Sanchez et al., 2012; Slabbert, du Plessis, & Huyser, 2015) and goat (Daramola et al., 2016) sperm vitrification. The extenders used for sperm vitrification include carbohydrates, proteins and other non-permeable substances. Considering the higher sensitivity of donkey sperm to permeable cryoprotectants (Vidament, Vincent, Martin, Magistrini, & Blesbois, 2009), this method could be an alternative to conventional freezing. The aim of this study was to compare two different extenders (INRA-96 and Gent) supplemented with sucrose and bovine serum albumin (BSA) for donkey sperm vitrification in straws.

2 | MATERIALS AND METHODS

Four healthy, fertile, Andalusian donkeys aged from 4 to 15 were used. Single representative ejaculates from each animal were collected using a Missouri artificial vagina (Minitübe, Tiefenbach, Germany). After collection, ejaculates were assessed for sperm volume, concentration and motility as described by Dorado et al. (2013). After that, semen was diluted 1:1 (v/v) with INRA-96® (INRA; IMV Technologies, France) and centrifuged at 400 × *g* for 7 min at 22°C. Sperm pellets were extended in two extenders: INRA-96 (I, IMV, L'Aigle, France), a milk base extender that contains purified fractions of milk micellar proteins; or Gent (G, Minitübe, Tiefenbach, Germany), a skimmed milk–egg-yolk based extender, both supplemented with sucrose 0.25 M (Molar) and 1% BSA to reach a final concentration of 200 × 10^6 spermatozoa/ml. Extended samples (I and G) were cooled for one hour at 5°C and then 100 µl of each treatment was packed in 0.25 ml French plastic straws, which were horizontally inserted into 0.5 ml CBS™ straws (Cryo Bio System, Paris, France). Thereafter, both ends of the covering straw were sealed and plunged directly into liquid nitrogen for vitrification (Sanchez et al., 2012). For the warming process, 0.5 ml straws were opened with forceps, and each 0.25 straw with the vitrified sperm was immersed in 3 ml of INRA-96 at 43°C until the vitrified solution

became liquid. Thawed samples were then centrifuged at 400 × *g* for 7 min and re-extended with INRA-96 after supernatant removal to reach final concentration of 25 × 10^6 spermatozoa/mL for sperm evaluation. Total (TM, %) and progressive (PM, %) sperm motility were objectively evaluated by the Sperm Class Analyzer (SCA v.5.4.0.0, Microptic S.L., Barcelona, Spain) and compared between treatments using the Student's *t* test. Results were expressed as mean ± standard error of the mean.

3 | RESULTS

Mean average parameters of ejaculates used in this study were as follows: gel-free volume = 91.3 ± 10 ml, sperm concentration = 359.5 ± 59.5 × 10^6 sperm/ml, TM = 89.8 ± 4.1% and PM = 72.6 ± 3.6%. The results obtained in the sperm analysis performed after vitrification are represented in Figure 1. The data showed no significant differences between extenders for TM: I (34.2% ± 8.7) vs. G (30.7% ± 9.6) and PM: I (26.8% ± 7.3) vs. G (24.6% ± 7.9).

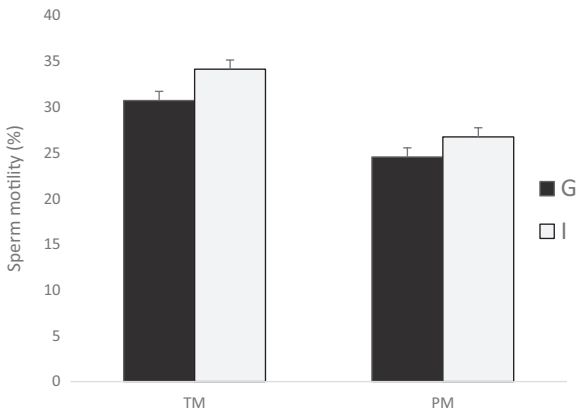

FIGURE 1 Total (TM) and progressive (PM) motility in donkey spermatozoa after vitrification with Gent (G) or INRA (I) extenders supplemented with sucrose 0.25 M and 1% BSA. No statistical differences were found between treatments for both sperm parameters evaluated ($p > .05$)

4 | DISCUSSION

In this study, donkey sperm has been successfully vitrified in straws for the first time. A similar vitrification protocol was described by Sanchez et al. (2012) for human aseptic sperm vitrification, modifying warming temperature as described by Isachenko, Sanchez, Mallmann, Rahimi, and Isachenko (2017) to 43°. These results are in agreement with previous reports in which protein and sucrose addition to a base extender had a cryoprotective effect on motility after vitrification (Isachenko et al., 2008). The same combination of non-permeable cryoprotectants has been successfully used for the sperm vitrification of small volumes (from 1 to 30 µl) in different animal species (Isachenko et al., 2004; Merino et al., 2011; Pradiee et al., 2015; Sánchez et al., 2011).

Our results showed that Gent and INRA-96 extenders supplemented with sucrose 0.25 M and BSA 1% were effective in terms of sperm motility after warming, obtaining similar results to conventional donkey sperm freezing (Rota, Panzani, Sabatini, & Camillo, 2012). However, no comparison between vitrification and conventional cryopreservation has been performed in this study, and only sucrose 0.25 M in combination with BSA was evaluated. In this sense, further studies including a larger number of sperm parameters assessed as well as different sucrose concentrations in comparison with conventional freezing are needed. Previous studies were able to vitrify larger volumes of semen samples in human (Isachenko, Maettner, et al., 2011) and goat sperm (Daramola et al., 2016). Therefore, for a better application of this technique in the equine industry, it would be interesting to vitrify sperm in different packages with higher volume and sperm concentration.

In conclusion, the current study has demonstrated, for the first time, that donkey sperm may be vitrified in straws either with INRA-96 or with Gent in combination with sucrose and BSA, as this procedure provides acceptable total and progressive motility after warming.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

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Optimization of donkey sperm vitrification: Effect of sucrose, sperm concentration, volume and package (0.25 and 0.5 mL straws)

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Livestock

ABSTRACT

The aim of this study was to assess the effect of different factors affecting vitrification success of donkey sperm: extender, sperm concentration, volume and storage vessel type. In Experiment 1, sucrose supplementations at 0.25 and 0.1 M were compared using two base extenders (containing or not egg-yolk); in Experiment 2, three sperm concentrations were assessed: 100, 200 or 300 million sperm/mL; and in Experiment 3, three different sperm volumes (100, 160 and 200 μL) and two different storage vessels (0.25 and 0.5 mL straws) were assessed. Sperm motility variables (CASA), plasma membrane and acrosome (evaluated under fluorescence microscopy) and sperm DNA integrity (flow cytometry) were evaluated after warming with comparisons of protocols. There was a greater total (55.7 ± 16.4%) and progressive (44.0 ± 11.5%) motility using the extender with egg-yolk and 0.1 M sucrose. There were no effects of sperm concentrations on vitrification results ($P > 0.05$). The 0.25 mL covered straw showed higher values than the 0.5 mL straw for total (50.0 ± 17.3% vs 2.0 ± 6.7%) and progressive (40.5 ± 14.9% vs 0.9 ± 1.5%) motility, plasma membrane (43.9 ± 14.4% vs 14.0 ± 16.4%) and acrosome integrity (51.5 ± 13.6% vs 28.0 ± 14.7%), respectively. In conclusion, values for donkey sperm quality variables after vitrification were greater using an extender containing egg-yolk and 0.1 M sucrose, at 300 million sperm/mL in 0.25 mL straws with outer covers.

1. Introduction

Vitrification of sperm has been developed in different species as a potential alternative to conventional freezing (Isachenko et al., 2011a; Sánchez et al., 2011; Figueroa et al., 2015; Pradiee et al., 2015). Vitrification has been achieved through an abrupt cooling by dropping the semen samples directly into liquid nitrogen (Isachenko et al., 2003, 2008; Pradiee et al., 2015). This non-aseptic technique requires a small volume of the sample, which is suitable for "*in vitro* fertilization" (IVF) or "intracytoplasmic sperm injection" (ICSI), however, is not practical for intrauterine insemination, as previously described (Isachenko et al., 2011b). To solve these limitations, sperm vitrification has been developed using straws (Isachenko et al., 2011b; Slabbert et al., 2015). This recently-developed method has been used primarily for investigations involving human sperm vitrification (Isachenko et al., 2011b; Sanchez

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et al., 2012a; Merino et al., 2015; Slabbert et al., 2015; Mansilla et al., 2016; Schulz et al., 2017) using different combinations of carbohydrates (sucrose or trehalose) and proteins (human serum albumin) instead of permeable cryoprotectants (Sanchez et al., 2012b). There is little information about the use of this technique in animal species and combination of both permeable and non-permeable cryoprotectants were used in previous research (Jiménez-Rabadán et al., 2015; Daramola et al., 2016). Recently, it has been reported that the sperm vitrification technique using 0.25 into 0.5 mL French straws resulted in successful vitrification of donkey semen (Diaz-Jimenez et al., 2017, 2018b); but a fixed concentration of non-permeable agents (sucrose and bovine serum albumin) derived from human studies were used (Sanchez et al., 2012a, b; Slabbert et al., 2015). The concentration of sucrose and proteins to be used for cryopreservation of semen are species-specific (Consuegra et al., 2018a; Diaz-Jimenez et al., 2018a; Hidalgo et al., 2018) and properly designed studies with donkey sperm have not been conducted comparing different concentrations of these compounds. Additionally, results from previous studies indicate usefulness of base extenders containing or not egg-yolk (Sánchez et al., 2011; Pradiee et al., 2015, 2016; Swanson et al., 2017; Bóveda et al., 2018; Consuegra et al., 2018b) for sperm vitrification, but there is little information about comparisons between them (Diaz-Jimenez et al., 2017).

Human sperm have been successfully vitrified at low sperm concentrations, ranging from 1 (Sanchez et al., 2012a) to 15 (Sanchez et al., 2012b) million sperm/mL. This concentration is lower than those usually stored for artificial insemination in animal species, including donkeys, in which sperm are usually frozen in concentrations of 50 (De Oliveira et al., 2017) to 200 million sperm/mL (Acha et al., 2015; Oliveira et al., 2016; Diaz-Jimenez et al., 2018a). Furthermore, the concentration must be increased to compensate for the relatively lesser volumes commonly used for sperm vitrification (30 to 100 µL). But inconsistent results were obtained when there was goat (Daramola et al., 2016) and ram (Jiménez-Rabadán et al., 2015) sperm vitrification at high sperm concentration. This finding indicates that vitrification success in straws could be also related to sperm concentration, and different concentrations for vitrification purposes have not been previously compared in donkeys.

The success in using vitrification procedures for semen cryopreservation seems to be affected by the procedure used for sperm storage (Rosato and Iaffaldano, 2013), which implies different factors including sperm volume, speed of cooling/warming and storage device. Different sperm volumes and packages have been assessed for human sperm vitrification, ranging from 10 µL in plastic capillaries (Isachenko et al., 2012, 2017b) to 500 µL in plastic straws (Isachenko et al., 2011b); and in animals from 100 µL in 0.25 mL covered straws (Jiménez-Rabadán et al., 2015; Consuegra et al., 2018b) to 200 µL placed directly into 0.5 plastic straws (Daramola et al., 2016). To our knowledge, there are no previous reports about donkey sperm vitrification when there is direct placement in 0.5 plastic straws, nor even a comparison between different storage vessel types for donkey sperm vitrification.

The aims of the present study, therefore, focused on the optimization of donkey sperm vitrification by: (1) assessing the effect of sucrose concentration in extenders containing or not containing egg-yolk; (2) evaluating three different concentrations of sperm and (3) comparing different semen volumes using straws.

2. Materials and methods

All the experiments were performed in accordance with the Ethical Animal Experimentation Committee of Cordoba University (Project No. 31/08/2017/105) according to the Spanish law for animal welfare and experimentation (Decision 2012/707/UE and RD 53/2013).

2.1. Animals

A total of five healthy mature Andalusian donkeys, ranging from 4 to 15 years of age were used as semen donors. Animals were managed in paddocks and fed water *"ad libitum"*, teff hay and oats. Semen was collected twice per week, using a Missouri-model artificial vagina with an in-line gel filter (Minitüb GmbH, Tiefenbach, Germany) in the presence of a jenny in estrus. For each experiment, three different ejaculates from each of the five donkeys were obtained.

2.2. Sperm processing

Immediately after collection, the gel-free semen volume was quantified in the graduated collecting tube and sperm concentration was calculated with a sperm photometer (Sperma-Cue, Minitüb GmbH, Tiefenbach, Germany). Thereafter, semen was extended 1:1 (v:v) with INRA-96 (IMV Technologies, L'Aigle, France). Before vitrification, sperm morphology was determined in diff-quick stained smears and sperm motility, plasma membrane and acrosome integrity were assessed as subsequently described. Extended semen was divided into aliquots and centrifuged (7 min/400 x g/22 °C) in a corning-adapted centrifuge (Eppendorf, model 5702 RH, Eppendorf AG, Hamburg, Germany). The supernatant was removed and the sperm pellets were re-suspended with the corresponding vitrification extender (see *2.3. Experimental design*). Samples were maintained 10 min at room temperature (≈ 22 °C) and then slowly cooled in an Equitainer (Hamilton Research, Inc. Ipswich, Massachusetts, USA) for 1 h as previously described (Diaz-Jimenez et al., 2017). Vitrification and warming processes are subsequently described for each experiment.

2.3. Experimental design

2.3.1. Experiment 1: effect of sucrose and egg-yolk for donkey sperm vitrification in 0.25 mL straws

Sucrose and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Sant Louis, USA). The effect of two sucrose concentrations was evaluated using a procedure that has been described for previous studies: (S1) sucrose powder added directly to

the base extender without egg-yolk (INRA-96) to obtain a final concentration of 0.1 M (Hidalgo et al., 2018); or (S2) sucrose prepared in distilled water and diluted 1:1 with the same base extender to obtain a final concentration of 0.25 M, adding BSA at 1% (Diaz-Jimenez et al., 2018a). A second commercial base extender containing egg-yolk (EY, Gent, Minitüb GmbH, Tiefenbach, Germany) was assessed, and both sucrose concentrations were added using the procedures previously described in this manuscript (S1-EY, S2-EY). Osmolality was measured in each extender using a freezing-point digital micro-osmometer Type 6 (Löser Messtechnik, Berlin, Germany).

Semen was divided in four aliquots. After centrifugation, sperm pellets were extended in each vitrification media to reach a final concentration of 200 million sperm/mL (Diaz-Jimenez et al., 2018a). After the cooling process was conducted, 100 µL of each sample was packaged in 0.25 mL French plastic straws, which were horizontally inserted into 0.5 mL straws (CBS™, Cryo Bio System, Paris, France) (Diaz-Jimenez et al., 2017). Thereafter, both ends of the outer straw were sealed and it was directly plunged into liquid nitrogen for vitrification. For the warming process, 0.5 mL straws were opened with forceps, and each 0.25 mL straw with the vitrified sperm was immersed in vertical position into a tube containing 3 mL of INRA-96 at 43 °C until the vitrified solution became liquid (Diaz-Jimenez et al., 2017). Warmed samples were then centrifuged (7 min/400 x *g*/22 °C) and re-extended with INRA-96 to a final concentration of 25 million sperm/mL for sperm evaluation (as described in *2.4. Post-warming sperm evaluation*).

2.3.2. Experiment 2: effect of sperm concentration on vitrification process in 0.25 mL straws

The treatment with the most desirable results in Experiment 1 (S1-EY) was selected for the subsequent experiments of this study. In Experiment 2, sperm were divided in three aliquots and pellets were extended in S1-EY to reach a final concentration of 100, 200 or 300 million sperm/mL. Vitrification and warming processes were performed using 0.25 mL straws using the procedures described in Experiment 1.

2.3.3. Experiment 3: comparison of different sperm volumes and packaging methods (0.25 mL or 0.5 mL straws) for donkey sperm vitrification

The sperm concentration that was determined to be most desirable for vitrification from conducting Experiment 2 (300 million sperm/mL) was used in Experiment 3 using the extender S1-EY. Three sperm volumes and two different packaging processes were compared: 100 and 160 µL of sperm stored in 0.25 mL straws as previously described in this manuscript; and 200 µL of sperm directly loaded in 0.5 mL straws (Isachenko et al., 2011b; Slabbert et al., 2015). Vitrification was performed by plunging straws directly into liquid nitrogen. The 0.25 mL straws were warmed as explained in Experiment 1, and the 0.5 mL straws were immersed in a water bath at 42 °C for 20 s using the procedures described by Slabbert et al. (2015), and then samples were diluted to a final concentration of 25 million sperm/mL with INRA-96 for sperm evaluation.

2.4. Post-warming sperm evaluation

2.4.1. Sperm motility

Sperm motility was objectively evaluated using the Sperm Class Analyzer system (SCA, v.5.4; Microptic S.L., Barcelona, Spain) as previously described by Ortiz et al. (2015). For each sample, two 5 µL drops of diluted sample were evaluated and three microscopic fields were randomly filmed for each drop that was placed on slides. A minimum of 200 spermatozoa were analyzed. The following motility features were recorded: total and progressive motility (TM and PM, %), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, VSL/VCL, %), straightness (STR, VSL/VAP, %), wobble (WOB, VAP/VCL, %), beat cross frequency (BCF, Hz) and amplitude of lateral head displacement (ALH, µm).

2.4.2. Plasma membrane integrity

Plasma membranes were assessed using the VitalTest stain (Halotech DNA SL, Madrid, Spain) according to the manufacturer instructions (Ortiz et al., 2017). In brief, an aliquot of diluted semen from each sample was mixed with 1 µL of propidium iodide stock solution and 1 µL of acridine orange stock solution. At least 200 spermatozoa per sample were evaluated under fluorescence microscopy (Olympus BX40, Tokyo, Japan), using a U-ND25-2 filter (a 460–490 nm excitation filter). Spermatozoa with an intact plasma membrane stained green and those with a damaged membrane stained red. Results were expressed as plasma membrane integrity percentage (PMI, %).

2.4.3. Acrosome membrane integrity

For sperm acrosome evaluation, the double staining (Sigma-Aldrich, Sant Louis, USA) propidium iodide (PI)/peanutagglutinin–fluorescein isothiocyanate (FITC-PNA) procedure was used as described by Dorado et al. (2014). Briefly, a 10 µL aliquot of each diluted sample was spread on a slide and permeabilized with ethanol for 30 s. A mixture of 10 µL PI and 20 µL FITC-PNA was spread over each smear and the slides were incubated in a dark, moist chamber at 4 °C for 30 min. Slides were then evaluated using an epifluorescence microscope and at least 200 spermatozoa were evaluated. Two staining patterns were used to discern: acrosome-intact spermatozoa (acrosomal region of the sperm displayed green fluorescence; PI+/FICT-PNA+) and acrosome-reacted spermatozoa (equatorial segment displayed green fluorescence or no anterior acrosomal staining; PI+/FICT-PNA–). The percentage of acrosome-intact sperm (AIS, %) was recorded.

2.4.4. Sperm DNA analysis

Sperm DNA integrity was assessed with the Sperm Chromatin Structure Assay (SCSA) using a Cytomics FC500 MPL Flow

Cytometer (Beckman Coulter, Miami, FL) with subsequent data analysis using Summit v4.2 software (DakoCytomation, Fort Collins, CO). Two aliquots of 400 µL at 25 million sperm/mL were saved from each treatment, stored in Eppendorf tubes at -80 °C and thawed at room temperature before evaluation as previously described by Salazar Jr et al. (2011). Sperm DNA integrity was assessed in each sample immediately after warming (T0) and after 4 h of incubation at 37 °C (T4). Approximately 10,000 cells/sample were studied, and the percentage of sperm cells with fragmented DNA was recorded as DNA fragmentation index (DFI, %) (Evenson, 2016).

2.5. Statistical analysis

Statistical analyses of the data were conducted using the Statistical Analysis Software (SAS, v.9.0, SAS Institute Inc., Cary, NC, USA). For each variable, normality of the data distribution and homogeneity of variances were assessed using the Kolmogorov–Smirnov and Levene test, respectively. When values were not normally distributed, results were transformed to a logarithmic scale. Comparisons between treatments were assessed using a general lineal model procedure (PROC GLM) followed by the Duncan test for *post hoc* analysis. Animals and ejaculates were considered as random factors. Values were expressed as mean ± standard deviation. Significant differences were considered when $P < 0.05$.

3. Results

Sperm quality variables from the ejaculates used in this study had the following average values: gel-free volume 65.6 ± 22.7 mL (range: 25.0–117.0 mL), sperm concentration 316.5 ± 99.2 million sperm/mL (range: 147.0–557.0 million sperm/mL), total motility 86.6 ± 7.7% (range: 66.2–97.0%), progressive motility 64.4 ± 13.6% (range: 37.0–88.6%), normal forms 87.9 ± 8.1% (range: 61.0–98.0%), plasma membrane integrity 60.5 ± 12.6% (range: 35.0–82.5%) and acrosome-intact sperm 60.8 ± 19.8% (range: 14.7–88.7%). The extenders used in Experiment 1 had the following osmolality values: S1 = 423 mOsm/kg; S2 = 403 mOsm/kg, S1-EY = 442 mOsm/kg and S2-EY = 433 mOsm/kg.

3.1. Experiment 1: effect of sucrose and egg-yolk for donkey sperm vitrification in 0.25 mL straws

Sperm values for TM, PM and velocity features (VCL, VSL and VAP) were greater ($P < 0.001$) with use of the extenders containing egg-yolk (S1-EY and S2-EY) in comparison to the extenders without egg-yolk (S1 and S2). The values for other variables that were assessed indicated there were no differences between sucrose concentrations nor extenders ($P > 0.05$). The S1-EY extender was selected for the subsequent experiments because of the tendency for a greater TM, PM, PMI and AIS than S2-EY. Results from Experiment 1 are provided in Table 1.

3.2. Experiment 2: effect of sperm concentration on vitrification process in 0.25 mL straws

Mean values for TM and PM ($P < 0.05$); VCL, VSL and VAP ($P < 0.01$) were greater with 200 and 300 million sperm/mL than 100 million sperm/mL. There were no differences in values assessed for the other sperm variables ($P > 0.05$). The greater

Table 1
Mean values of sperm variables from vitrified-warmed samples ($n = 15$) using different concentrations of sucrose with extenders containing or not egg-yolk.

Sperm variables	Vitrification media				*P*-values
	S1	S2	S1-EY	S2-EY	
TM (%)	34.3 ± 15.2[b]	28.7 ± 12.3[b]	55.7 ± 16.4[a]	48.4 ± 12.7[a]	< 0.001
PM (%)	24.9 ± 15.3[b]	20.5 ± 9.8[b]	44.0 ± 11.5[a]	39.0 ± 11.6[a]	< 0.001
PMI (%)	37.1 ± 9.7	37.7 ± 9.1	43.2 ± 18.1	34.1 ± 7.2	> 0.05
AIS (%)	46.1 ± 15.4	37.4 ± 17.4	43.8 ± 18.0	38.6 ± 18.6	> 0.05
VCL (µm/s)	75.1 ± 21.7[b]	79.2 ± 11.2[b]	94.1 ± 9.9[a]	91.2 ± 12.2[a]	< 0.001
VSL (µm/s)	65.2 ± 22.4[b]	69.7 ± 11.1[b]	82.2 ± 7.6[a]	81.7 ± 9.9[a]	< 0.001
VAP (µm/s)	68.7 ± 22.7[b]	73.2 ± 11.5[b]	87.2 ± 8.6[a]	85.5 ± 10.8[a]	< 0.001
ALH (µm)	1.7 ± 0.1	1.7 ± 0.3	1.8 ± 0.2	1.7 ± 0.3	> 0.05
LIN (%)	83.9 ± 14.7	87.8 ± 4.2	87.6 ± 6.4	89.8 ± 3.8	> 0.05
STR (%)	93.2 ± 1.6	95.2 ± 1.6	94.3 ± 4.8	95.6 ± 1.0	> 0.05
WOB (%)	89.1 ± 11.9	92.0 ± 6.7	92.9 ± 2.9	94.0 ± 3.7	> 0.05
BCF (Hz)	9.2 ± 1.9	8.9 ± 2.0	9.1 ± 1.7	8.9 ± 2.4	> 0.05
DFI T0 (%)	5.8 ± 6.9	5.9 ± 7.2	5.2 ± 6.3	4.6 ± 5.9	> 0.05
DFI T4 (%)	5.0 ± 5.4	5.0 ± 5.4	5.5 ± 6.7	6.0 ± 6.8	> 0.05

Different letters within the same rows indicate significant differences among treatments; TM = total motility; PM = progressive motility; PMI = plasma membrane integrity; AIS = acrosome intact sperm; VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; ALH = amplitude of lateral head displacement; LIN = linearity; STR = straightness; WOB = wobble; BCF = beat cross frequency; DFI T0 = fragmentation index measured at 0 h; DFI T4 = fragmentation index measured after 4 h of incubation; S1 = sucrose 0.1 M; S2 = sucrose 0.25 M + 1% BSA. EY = egg-yolk; Values are expressed as mean ± SD.

Table 2
Effect of sperm concentration on mean values of sperm variables from vitrified-warmed samples (n = 15).

Sperm variables	Sperm concentration in vitrified 0.25 mL straws [million sperm/mL]			*P*-values
	[100]	[200]	[300]	
TM (%)	39.2 ± 18.4[b]	42.3 ± 16.0[ab]	51.3 ± 14.3[a]	< 0.05
PM (%)	27.8 ± 13.5[b]	33.6 ± 14.1[ab]	39.8 ± 15.8[a]	< 0.05
PMI (%)	32.9 ± 11.4	38.7 ± 15.1	44.2 ± 14.9	> 0.05
AIS (%)	54.1 ± 21.6	55.2 ± 15.4	55.5 ± 12.9	> 0.05
VCL (μm/s)	65.3 ± 27.1[b]	82.6 ± 17.2[a]	89.3 ± 19.9[a]	< 0.01
VSL (μm/s)	57.0 ± 25.3[b]	73.7 ± 17.3[a]	78.7 ± 17.3[a]	< 0.01
VAP (μm/s)	59.9 ± 25.9[b]	76.9 ± 17.7[a]	82.8 ± 18.2[a]	< 0.01
ALH (μm)	1.7 ± 0.5	1.8 ± 0.3	1.9 ± 0.3	> 0.05
LIN (%)	80.6 ± 22.9	88.5 ± 4.7	88.1 ± 4.1	> 0.05
STR (%)	88.3 ± 24.6	95.7 ± 1.0	95.0 ± 2.2	> 0.05
WOB (%)	85.1 ± 23.8	92.4 ± 4.5	92.8 ± 2.9	> 0.05
BCF (Hz)	8.2 ± 2.4	8.9 ± 1.3	9.3 ± 1.7	> 0.05
DFI T0 (%)	3.5 ± 2.8	3.6 ± 2.4	3.0 ± 2.4	> 0.05
DFI T4 (%)	3.7 ± 3.0	3.6 ± 3.0	3.0 ± 2.4	> 0.05

Different letters within the same rows indicate significant differences among treatments; TM = total motility; PM = progressive motility; PMI = plasma membrane integrity; AIS = acrosome intact sperm; VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; ALH = amplitude of lateral head displacement; LIN = linearity; STR = straightness; WOB = wobble; BCF = beat cross frequency; DFI T0 = fragmentation index measured at 0 h; DFI T4 = fragmentation index measured after 4 h of incubation; S1 = sucrose 0.1 M; S2 = sucrose 0.25 M + 1% BSA; EY = egg-yolk; Values are expressed as mean ± SD.

concentration (300 million sperm/mL) of sperm was selected for conducting Experiment 3 for practical reasons. Results are provided in Table 2.

3.3. Experiment 3: comparison of different sperm volumes and packaging methods (0.25 mL or 0.5 mL straws) for donkey sperm vitrification

Depicts results after warming of samples vitrified in different volumes and with different packaging methods. There were no differences between volumes (100 or 160 μL) when storage was in 0.25 mL plastic straws ($P > 0.05$). Conversely, values for sperm variables were markedly decreased when 200 μL of sperm were directly loaded in 0.5 mL straws ($P < 0.001$); except for DFI, for which there were no differences between volumes or packaging methods ($P > 0.05$).

4. Discussion

In the present study, sperm concentration, volume and packing method were important considerations regarding success of donkey sperm vitrification, as well as the media composition and sucrose concentration, as has been widely reported for other species (Consuegra et al., 2018a; Diaz-Jimenez et al., 2018a; Hidalgo et al., 2018). The effect of these factors were evaluated in the present study and results were used to optimize aseptic vitrification approaches in straws for donkey sperm. Furthermore, sperm quality after vitrification in the present study was similar to that previously reported using conventional slow freezing of donkey sperm (Ortiz et al., 2015).

Experiment 1 of the present study was performed to ascertain the most suitable combination of non-permeable cryoprotectants for donkey sperm vitrification. Two different extenders conventionally used for stallion sperm were assessed in combination with two different concentrations of sucrose by taking into consideration previous studies of sperm vitrification in mammals (Sánchez et al., 2011; Pradiee et al., 2015; Diaz-Jimenez et al., 2017; Swanson et al., 2017; Caturla-Sánchez et al., 2018). From one perspective, a combination of 0.25 M sucrose prepared in distilled water plus 1% BSA has been used to protect sperm from cryodamage during vitrification (Isachenko et al., 2008), and has been successfully used in human (Isachenko et al., 2008; Sanchez et al., 2009, 2012a; Sanchez et al., 2012b), dog (Sánchez et al., 2011) and donkey (Diaz-Jimenez et al., 2017) sperm vitrification. A concentration of 0.1 M sucrose without inclusion of BSA has been used to protect wild goat (Pradiee et al., 2015), wild sheep (Pradiee et al., 2016) and stallion (Hidalgo et al., 2018) sperm after vitrification. Interestingly, there were no differences in vitrification outcomes when there were different sucrose concentrations used in combination with or without BSA in the present study. In this regard, the positive effect of this sugar might depend not only on its final concentration in the extender, but also on the addition procedures to the extender. This would explain the different sperm quality in the present study using water-diluted sucrose in comparison to results from a previous report with stallion sperm vitrification where the same concentration of sucrose (0.25 M) was used but added directly to the extender as powder (Pérez-Marín et al., 2018). There should be consideration that vitrification was performed in spheres in the stallion study; however, when compared with the same vitrification procedure, values of stallion sperm quality variables were greater after reducing the powdered sugar concentration (0.02 M) (Hidalgo et al., 2018). The relatively higher osmolality of the extender after sugar addition could be balanced by a previous dilution in water, as there were similar osmolality values when 0.25 M water diluted sucrose or 0.1 M powdered sucrose were added to the extender. Conversely, different results were obtained when the two different extenders were compared in the present study: use of the skimmed milk-egg-yolk based extender resulted in improvements

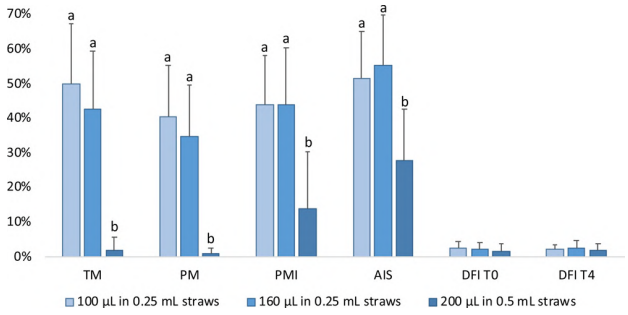

Fig. 1. Sperm variables from vitrified-warmed samples (n = 15) using different sperm volumes and packaging vessels; Samples were diluted in the extender containing egg − yolk and 0.1 M sucrose at 300 million sperm/mL; Different superscripts (a, b) indicate differences ($P < 0.05$); TM = total motility; PM = progressive motility; PMI = plasma membrane integrity; AIS = acrosome-intact sperm; DFI = DNA fragmentation index.

in values for sperm motility and velocity variables in comparison to use of the extender without egg-yolk. Vitrification of human sperm have been conventionally achieved using human tubal fluid enriched with sucrose and human serum albumin (Sanchez et al., 2012a) but in the absence of egg-yolk due to bio-security reasons. More recently, the addition of lipoproteins (from seminal plasma) was found to improve plasma and mitochondrial membrane integrity after human sperm vitrification (Isachenko et al., 2017a). Similarly, in other mammalian species, different cryopreservation extenders containing egg-yolk and skim milk as sources of lipoprotein has been used, which interact with the cell membrane during the freezing-thawing process in a species-specific way, protecting sperm cells from cold shock (Dong and VandeVoort, 2009; Ustuner et al., 2016; Bateman and Swanson, 2017). In donkeys, egg-yolk seems to protect sperm against cold shock during cryopreservation, as described by Zhang et al. (2018). In the present study, the use of the extender containing egg-yolk resulted in greater sperm motility after vitrification but there were no differences for PMI, AIS or DFI percentages. Similar results were previously reported by Dorado et al. (2014) when these extenders were compared for donkey sperm cooling up to 72 h. It appears sperm motility is favourably affected by the use of egg-yolk extenders after cold storage or cryopreservation (Parks and Graham, 1992; White, 1993; Cottorello et al., 2002); nevertheless, acrosome reaction, plasma membrane or DNA integrity were unaffected by the sucrose concentration or medium composition, which is consistent with findings in previous studies (Esteves et al., 1998; Isachenko et al., 2011a) (Fig. 1).

Sperm vitrification in straws has been performed in a wide range of sperm concentrations: from 1 million sperm/mL in humans (Sanchez et al., 2012a), to 1148 million sperm/mL in goats (Daramola et al., 2016), obtaining good sperm quality after warming in both studies. Nevertheless, in the study of Jiménez-Rabadán et al. (2015) there was poor sperm quality after ram sperm vitrification at concentrations of 20 and 50 million sperm/mL. Appropriate sperm concentrations for vitrification in straws is apparently different for animal species, which could be related to the sperm cell characteristics of each species, as previously described with use of slow freezing procedures (Alvarez et al., 2012). Human sperm heads are smaller in size in comparison with other mammalian species, therefore, human sperm have maximal cryostability (Gao et al., 1997). In addition, assisted reproductive techniques such as IVF or ICSI allow human sperm vitrification in low concentration ranges: from 1 (Sanchez et al., 2012a) to 15 million sperm/mL (Sanchez et al., 2012b); however, vitrification of donkey sperm requires greater concentrations for its use in artificial insemination of jennies (Acha et al., 2015; Oliveira et al., 2016; De Oliveira et al., 2017). In addition, it has been hypothesized that increasing the number of sperm per dose, combined with the use of the deep-horn insemination technique, could improve pregnancy rates in jennies (Miró and Papas, 2018). In the present study, donkey sperm could be vitrified with desirable outcomes at a very high concentration of 300 million sperm/mL. Similarly, in other species such as fish, dogs and sheep, there was a desirable sperm quality after cryopreservation at high sperm concentrations (Alvarez et al., 2012). Although there were no differences in the sperm quality after warming of sperm vitrified at 200 or 300 million sperm/mL in the present study, the greatest sperm concentration was selected to conduct Experiment 3 because of practical considerations and general convenience.

Experiment 3 was also conducted to assess the use of a similar insemination dose that is conventionally used in donkeys, through increasing the vitrified sperm volume. Two storage approaches and different sperm volumes were evaluated: with 0.25 into 0.5 mL straws being filled with A) 100 µL of semen, as previously described in humans (Sanchez et al., 2012a, b; Sanchez et al., 2013; Merino et al., 2015; Mansilla et al., 2016; Schulz et al., 2017), rams (Jiménez-Rabadán et al., 2015), stallions (Consuegra et al., 2018b) and donkeys (Diaz-Jimenez et al., 2017) sperm; and B) 160 µL of semen, as the maximum volume that could be placed in the inner straw. With the second approach, 200 µL of sperm were directly loaded in 0.5 mL French straws (Isachenko et al., 2011b; Slabbert et al., 2015). Unexpectedly, use of the 0.5 mL straw method resulted in almost no motile sperm after warming, in contrast to successful large volume vitrification of human sperm using this procedure (Isachenko et al., 2011b; Slabbert et al., 2015). Inconsistent with this finding, the use of the two-straw packaging method resulted in the greatest values for all the sperm variables assessed, as previously described for humans (Sanchez et al., 2012a, 2013; Merino et al., 2015; Mansilla et al., 2016; Schulz et al., 2017). These inconsistent results between species using the 0.5 mL straw technique might be a consequence of the greater cryostability of human sperm (Gao et al., 1997), and poor repeatability of the technique, as described by Katkov et al. (2012). The greater distribution of the sperm sample along the thinner straw, allow for cooling by the liquid nitrogen at more rapid rates using the two-straw method, resulting in the most suitable technique for donkey sperm vitrification. In this direction, sperm vitrification success is probably not completely related to the volume of semen stored because there were no differences with the use of 100 and 160 µL, but rather with the

distribution of the liquid nitrogen on the straw surface. Similar results were reported by Diaz-Jimenez et al. (2018b) where there were greater values for the sperm quality variables with use of the two-straw method when compared with the use of spheres for donkey sperm vitrification. Furthermore, the main inconvenience with use of the sphere method is the risk of cross-contamination during cryopreservation in liquid nitrogen tanks (bacteria transfer from infected semen pellets to sterile pellets), as was previously described by Isachenko et al. (2017a). The two-straw method of sperm vitrification not only allows for rapid cooling and for greater vitrification volumes, but also isolation of the sperm from liquid nitrogen.

In conclusion, the process of donkey sperm vitrification is affected by the factors analyzed in the present study: extender, sperm concentration, volume and storage vessel. Donkey sperm could be vitrified using an extender containing egg-yolk and 0.1 M sucrose, at 300 million sperm/mL in 0.25 mL straws with outer covers.

Author declaration

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Conflicts of interest

The authors declare no conflicts of interest.

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SHORT COMMUNICATION

WILEY Reproduction in Domestic Animals

Vitrification in straws conserves motility features better than spheres in donkey sperm

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Contents

Sperm vitrification as alternative to conventional freezing is increasing in popularity in many species. It has been achieved by direct exposure of diluted semen to liquid nitrogen in spheres or straws. Both techniques have been successfully developed, but they had not been compared yet in donkeys. The aim of this study was to compare these two methods of vitrification for donkey semen. Ejaculates from six Andalusian donkeys were collected and extended in Gent without glycerol supplemented with sucrose 0.1 M (Molar). Samples were slowly cooled at 5°C. For vitrification, 30 μl suspensions (spheres) were dropped directly into liquid nitrogen (LN_2) or filled in covered 0.25 ml straws and then plunged into the LN_2 (straws). For warming, straws and spheres were directly immersed in 3 ml of INRA-96 at 43°C. Total (TM, %) and progressive motility (PM, %) were objectively evaluated by computer-assisted sperm analysis and plasma membrane integrity (PMI, %) by epifluorescence microscopy. Results showed the straw method resulted in significantly higher values than spheres for: TM (54.7% ± 10.1 vs. 28.6% ± 6.5) and PM (44.2% ± 9.4 vs. 17.7% ± 6.4), but no significant differences were found between straws or spheres for PMI (31.5 ± 10.7 vs. 41.6 ± 14.3) respectively. In conclusion, donkey sperm could be vitrified in straws obtaining better sperm motility parameters after warming in comparison to the sphere method.

1 | INTRODUCTION

Sperm vitrification as alternative to conventional freezing is increasing in popularity in many species. This is a simple, quick, cost-efficient technique consisting of ultra-rapid freezing of the sperm suspension by direct immersion in liquid nitrogen. It has showed a strong cryoprotective effect and has been achieved in two different ways: dropping sperm directly in LN_2, obtaining spheres; or in filled straws and then immersed in LN_2. The sphere method has shown to protect sperm parameters after vitrification and warming in human (Isachenko et al., 2011), dog (Sánchez et al., 2011), fish (Merino et al., 2011), wild goat (Pradiee et al., 2015) and cat (Swanson et al., 2017). Likewise, vitrification in straws has obtained good results in human (Sanchez et al., 2012), domestic goat (Daramola et al., 2016) and donkeys (Diaz-Jimenez et al., 2017). Even though both techniques have the same principle: an ultra-rapid cooling rate of small volume samples (30–100 μl); results may be altered depending on the device used, as was previously showed for fish vitrification (Zilli et al., 2018). Therefore, the aim of this study was to compare these two methods of vitrification for donkey semen regarding sperm motility and plasma membrane integrity after warming.

2 | MATERIALS AND METHODS

All the experiments were performed in accordance with the Ethical Committee for Animal Experimentation of the University of Cordoba (project no. 31/08/2017/105) and the Spanish law for animal welfare

and experimentation (RD 53/2013). Two ejaculates per animal (n = 12) were collected from six healthy, mature Andalusian donkeys using a Missouri-model artificial vagina. Gel-free semen samples were assessed for plasma membrane integrity and motility in order to determine the seminal quality before cryopreservation, as previously described (Ortiz et al., 2014), and were randomly assigned to each vitrification method. Vitrification solution was prepared for both techniques using a skimmed milk-egg yolk based extender without glycerol, Gent (G, Minitübe, Germany) supplemented with sucrose 0.1 M. Semen samples were centrifuged and resuspended with the vitrification media until a final concentration of 200 × 10^6 spermatozoa/ml was reached. Thereafter, extended samples were slowly cooled for 1 hr at 5°C and cryopreserved as follows: for spheres vitrification, 30 µl aliquots of spermatozoa suspension were dropped directly into LN_2 and stored until analysis (Isachenko et al., 2011; Pradiee et al., 2015). For straws procedure, 100 µl of the sperm solution was packed in 0.25 ml French plastic straws, which were horizontally inserted into 0.5 ml CBS^{TM} straws (Cryo Bio System, France). Thereafter, the covering straw was sealed, plunged directly into LN_2 and stored until post-warming analysis (Diaz-Jimenez et al., 2017).

For the warming procedure, a total of three spheres were directly submerged one by one in 3 ml of INRA-96 (I, IMV, L'Aigle, France) at 43°C; and 0.5 ml straws were opened and each 0.25 straw with the vitrified sperm was directly immersed in the same warming media until melting. Thawed samples were then centrifuged and re-extended with INRA-96 after supernatant removal, to reach final concentration of 25 × 10^6 spermatozoa/ml for sperm evaluation.

Total (TM, %) and progressive (PM, %) sperm motility were objectively evaluated after vitrification by the Sperm Class Analyzer (SCA v.5.4, Microptic S.L., Spain). Plasma membrane integrity (PMI, %) was assessed using the Vital-Test® commercial kit (Halotech DNA S.L., Spain) for sperm staining according to the manufacturer′ instructions. All data were analysed using SPSS Statistics 22.0 (SPSS Institute Inc. Headquarters, USA); ejaculates and results were compared between techniques using the Student′s t test for independent samples and expressed as mean ± *SEM*. Significant differences were considered when $p < 0.001$.

3 | RESULTS

Ejaculates used for both methods (straws and spheres) showed no significant differences ($p > 0.05$) before vitrification for TM (89.11% vs 86.23%), PM (58.28% vs 73.36%) and PMI (65.37% vs 61.39%), respectively. Sperm parameters assessed after vitrification and warming are represented in Figure 1.

3.1 | Motility results

The straw method resulted in significantly higher values than spheres for: TM (54.7% ± 10.1 vs. 28.6% ± 6.5) and PM (44.2% ± 9.4 vs. 17.7% ± 6.4).

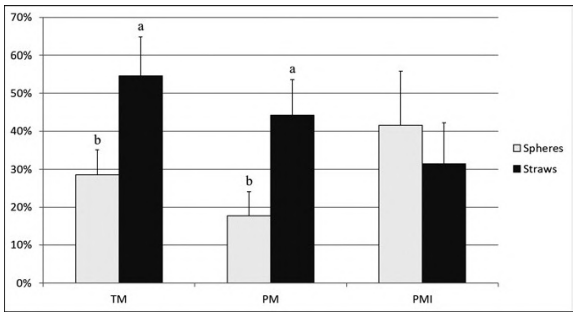

FIGURE 1 Percentage of total (TM) and progressive (PM) motility and plasma membrane integrity (PMI) in donkey spermatozoa after vitrification in spheres or straws methods. Data is represented as mean ± SEM (n = 12). Different letters indicate significant differences between techniques evaluated ($p < 0.001$).

3.2 | Plasma membrane integrity results

No significant differences were found between straws or spheres for PMI (31.5 ± 10.7 vs. 41.6 ± 14.3) respectively.

4 | DISCUSSION

According to the results obtained in this study, donkey sperm vitrification using 0.25 ml straws significantly improves motility results after warming than spheres method. Nevertheless, both techniques were able to protect sperm cell membrane from cryodamage. To the best of our knowledge, there are no previous reports comparing sperm vitrification techniques in donkeys but only two reports assessing parameters after warming between droplets and cut straws vitrification in fish (Zilli et al., 2018) and four different devices in human (Isachenko et al., 2005). However, their results were in disagreement with those presented in this study: higher motility and membrane integrity results were obtained using dropping method by Zilli et al. (2018), while no differences between methods employed where found by Isachenko et al. (2005). Results presented in this study might be explained because of different LN_2 distribution between techniques: although less sperm volume was used in spheres method (30 µl) than in straws (100 µl), smaller straw diameter leads to a higher suspension surface exposition, thus achieving higher cooling rates. Motility sperm parameters after vitrification using the straw method were higher than those previously reported for donkey sperm using slow freezing without permeable cryoprotectant (Diaz-Jimenez et al., 2018). In addition, straws method is an aseptic technique which avoids direct contact with NL_2, minimizing therefore the potential microbial contamination (Isachenko et al., 2005). Since no differences were found between techniques in PMI, vitrification itself may be able to protect sperm membrane, as previously described (Sánchez et al., 2011).

In this paper, a fixed sucrose concentration has been employed, but it is known carbohydrate requirements seems to be different regarding animal species (Caturla-Sánchez et al., 2018; Merino et al., 2012; Pradiee et al., 2015; Swanson et al., 2017). In this sense, it

would be necessary to carry on further studies including different sucrose concentrations in the extenders as well as different volume and concentration of the sperm suspension required for each vitrification technique.

In conclusion, the present study has demonstrated donkey sperm vitrification in straws preserves total and progressive motility better than the spheres method in absence of permeable cryoprotectants.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

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CHAPTER 4

Effect of warming temperatures on donkey sperm vitrification in 0.5mL straws in comparison to conventional freezing

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RESEARCH ARTICLE

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Effect of warming temperatures on donkey sperm vitrification in 0.5 mL straws in comparison to conventional freezing

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Abstract

Aim of study: There is little information about vitrification of sperm in large volumes (up to 0.5 mL). This study aimed to develop the vitrification technique in 0.5 mL straws in donkey sperm, evaluating the effect of three warming temperatures.

Area of study: Cordoba, Spain.

Material and methods: Ejaculates from five donkeys were divided in four groups: one control subjected to conventional slow freezing (C) and three vitrified in 0.5 mL straws and warmed using different protocols (W1: 37ºC/30s, W2: 43ºC/20s and W3: 70ºC/8s + 37ºC/52s). Sperm motility, kinematic parameters, plasma membrane and acrosome integrity were evaluated. Conventional freezing resulted in significantly higher values for total (42.7 ± 19.6%), and progressive motility (30.3 ± 16.7%), plasma membrane (49.1 ± 10.4%) and acrosome integrity (39.6 ± 14.5%) respect to vitrification method.

Main results: Values after warming ranged between 0.2-2.8% for total motility; 0.2-2.1% for progressive motility; 5.5-20.0% for plasma membrane integrity and 14.5-29.8% for acrosome integrity in all warming protocols after sperm vitrification. However, no differences were found between W3 and C for kinematic parameters; and W3 resulted in significantly higher values for membrane integrity (20.0 ± 11.0%) in comparison to W1 (5.5 ± 3.6%) and W2 (9.3 ± 8.4%).

Research highlights: High warming rates seem to be better for donkey sperm vitrification in large volumes; but this methodology is still not an alternative to conventional sperm freezing.

Additional keywords: equine; cryopreservation; large-volume vitrification; aseptic; thawing.

Abbreviations used: ALH (amplitude of lateral head displacement); AO (acridine orange); BCF (beat cross frequency); CASA (computer-assisted sperm motility analysis); FITC (fluorescein isothiocyanate); LIN (linear coefficient); PI (propidium iodide); PM (progressive motility); PMI (plasma membrane integrity); PNA (peanut agglutinin); STR (straightness coefficient); TM (total motility); VAP (average path velocitiy); VCL (curvilinear velocity); VSL (straight line velocity); WOB (wobble coefficient).

Authors' contributions: MDJ and MH contributed to all sections. JD, IO, EI and VI contributed to the study design, data analysis and interpretation. CC, BP and CV performed the experiment. All authors read and approved the final version of the manuscript.

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Introduction

Since sperm vitrification in straws was first described in human by Isachenko *et al.* (2005), it has been investigated in different animals including ram (Jiménez-Rabadán *et al.*, 2015), goat (Daramola *et al.*, 2016), fish (Zilli *et al.*, 2018) and horse (Consuegra *et al.*, 2018; Restrepo *et al.*, 2019). This technique has also been developed in donkey sperm in absence of permeable cryoprotectants, showing promising

results regarding *in vitro* parameters (Diaz-Jimenez *et al.*, 2017, 2018b). In this sense, vitrification in straws would be an interesting alternative to conventional slow freezing in this species, whose results after artificial insemination in jennies using frozen sperm are very poor, with pregnancy rates ranging from 0 to 36% (Rota *et al.*, 2012; Oliveira *et al.*, 2016). However, the relatively small volume employed (100 µL) seems to be a major disadvantage in comparison to conventional freezing (Slabbert *et al.*, 2015). To solve this limitation, vitrification of large volumes of sperm (up to 0.5 mL) has been developed in human beings, showing similar (Slabbert *et al.*, 2015) and higher (Isachenko *et al.*, 2011) sperm motility parameters after warming when compared to conventional freezing. Nevertheless, to our knowledge, there is little information about its use in animals, and if so, no motile sperm was recovered after warming (Restrepo *et al.*, 2019).

Temperature and time during thawing have shown to affect sperm motility, plasma membrane and acrosome integrity after conventional freezing (Athurupana *et al.*, 2015); and they seem to be also key factors for sperm vitrification success (Mansilla *et al.*, 2016). A few studies comparing warming temperatures after vitrification could be found in the literature: Mansilla *et al.* (2016) reported optimum results in vitrification of human sperm in 0.25 mL straws after warming at 42ºC in comparison to lower temperatures; however, heterogeneous results were obtained after sperm vitrification following the spheres method. In this sense, high warming temperatures (up to 60ºC) seemed to be more adequate for mouflon (Pradiee *et al.*, 2016) and ibex (Pradiee *et al.*, 2015) than for dog (Caturla-Sánchez *et al.*, 2018) sperm, in which slower warming rates (37ºC) showed better sperm quality after vitrification. To the best of our knowledge, there are no previous studies comparing different temperatures and times for warming after vitrification in donkey sperm. Therefore, the aim of this study was to vitrify donkey sperm in 0.5 mL straws using different temperatures and rates for warming in comparison to conventional freezing regarding sperm motility parameters, plasma membrane and acrosome integrity.

Material and methods

Semen collection and processing

Samples were obtained from five healthy, fertile, Andalusian donkeys from 3 to 8 years of age. All the experiments were performed in accordance with the Ethical Committee for Animal Experimentation of the University of Cordoba (project no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (BOE, 2013). Ejaculates (three per donkey, n=15) were collected using a Missouri artificial vagina and assessed before freezing or vitrification for sperm volume, concentration, motility and plasma membrane integrity as previously described (Diaz-Jimenez *et al.*, 2018a). After that, gel-free semen samples were diluted 1:1 (v/v) with a commercial milk fraction-based extender (INRA-96, IMV Technol., L'Aigle, France), centrifuged at 400 × g for 7 min at 22°C in a corning-adapted centrifuge (Eppendorf, model 5702 RH, Eppendorf AG, Hamburg, Germany) and resuspended to achieve a final concentration of 200 × 10^6 total sperm/mL. A skimmed milk-egg-yolk based extender for equine sperm was used (Gent, Minitüb, Tiefenbach, Germany) (i) containing glycerol (De Oliveira *et al.*, 2017) for conventional slow freezing (control, C); or (ii) without glycerol, but adding instead sucrose (S) to reach a final concentration of 0.1 M (437 mOsm/kg) for sperm vitrification (Diaz-Jimenez *et al.*, 2018b).

Conventional freezing and vitrification procedures

Conventional freezing and thawing were performed following a previous protocol for donkey sperm cryopreservation (Diaz-Jimenez *et al.*, 2018a). Briefly, extended semen was slowly cooled to 5ºC for 2 hours into an Equitainer (Hamilton Research, Inc. Ipswich, MA, USA), and loaded in 0.5 mL plastic French straws. Thereafter, straws were placed in racks 2.5 cm above the surface of liquid nitrogen (LN_2) for 5 min and then stored in tanks. Thawing was performed by immersion in a 37ºC water bath for 30s. For the vitrification technique, sperm extended in S was cooled at 5ºC during 1 hour (Diaz-Jimenez *et al.*, 2017) and loaded into 0.5 mL straws (CBS™, Cryo Bio System, Paris, France). Straws were hermetically sealed at both sides and directly plunged into LN_2 for vitrification (Isachenko *et al.*, 2011; Slabbert *et al.*, 2015). Vitrified samples were stored in LN_2 tank until use. For warming, straws were immersed in a water bath at different temperatures and times according to the following protocols: (W1) 37ºC for 30s, (W2) 43ºC for 20s and (W3) 70ºC for 8s and immediately maintained at 37ºC for 52s. After thawing or warming, all samples were extended with INRA-96 to a final concentration of 25 × 10^6 sperm/mL for sperm evaluation.

Computer-assisted sperm motility analysis (CASA)

Sperm motility was objectively evaluated by the Sperm Class Analyzer (SCA v5.4, Microptic S.L.,

Barcelona, Spain). For each evaluation, two 5 µL drops and three random microscopic fields per drop were evaluated. A minimum of 200 spermatozoa were analysed. The trajectory of each spermatozoa was determined by the software and the following parameters of sperm motion were measured: total (TM, percentage of spermatozoa with a mean average path velocity >10 µm/s) and progressive motility (PM, percentage of motile spermatozoa with >75% of the straightness coefficient); curvilinear (VCL, µm/s), straight line (VSL, µm/s) and average path velocities (VAP, µm/s); linear coefficient (LIN, VSL/VCL×100); straightness coefficient (STR, VSL/VAP×100); wobble coefficient (WOB, VAP/VCL×100); amplitude of lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz).

Assessment of sperm plasma membrane integrity

Plasma membrane integrity (PMI, %) was assessed using the double stain propidium iodide (PI) with acridine orange (AO) from the VitalTest stain (Halotech DNA SL, Madrid, Spain) as previously described (Diaz-Jimenez *et al.*, 2018a). Briefly, a 10 µL aliquot of diluted semen was mixed with 1 µL PI and 1 µL AO and evaluated under epifluorescence microscopy (Olympus BX40, Tokyo, Japan) using a U-ND25-2 filter (a 460–490 nm excitation filter). At least 200 sperm were counted and those with green emission (viable spermatozoa) were recorded.

Assessment of acrosome integrity

The sperm acrosomes were evaluated using the peanut agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC) and PI staining, as described by Dorado *et al.* (2014). In brief, 10 µL of diluted sample (25 × 10^6 sperm/mL) was spread on a slide and permeabilized with 70% ethanol for 30 s. A mixture of 10 µL PI and 20 µL FITC-PNA was spread over each smear after 5 min incubation at 38ºC, and the slides were incubated in a dark, moist chamber for 30 min at 4ºC. Slides were then evaluated under epifluorescence microscopy (Olympus BX40, Tokyo, Japan) using the 100x oil immersion objective and the U-ND25-2 filter (a 460–490 nm excitation filter). Sperm acrosomes were classified as intact (spermatozoa displaying intensively bright green fluorescence of the acrosomal cap) and damaged (spermatozoa displaying disrupted fluorescence, fluorescent band at the equatorial segment or no fluorescence, indicating damages to the outer acrosomal membrane). A total of 200 spermatozoa were evaluated, and the percentage of acrosome-intact sperm was recorded (AIS, %).

Statistical analysis

Statistical analysis was performed using the Statistical Analysis Software (SAS, v.9.0, SAS Institute Inc., Cary, NC, USA) and data was presented as mean ± standard deviation (SD). Results were compared between treatments using a general lineal model procedure (PROC GLM) followed by Duncan multiple comparison test as *post-hoc* test. Animals and ejaculates were considered as random factors. Significant differences were considered when $p<0.05$.

Results and discussion

Sperm parameters before cryopreservation had the following average values: gel-free volume = 61.1 ± 25.3 mL, sperm concentration = (354.5 ± 101.2) × 10^6 sperm/mL, TM = 89.5 ± 6.4%, PM = 61.0 ± 15.7% and PMI = 61.8 ± 12.7%. Conventional freezing showed significantly ($p<0.001$) higher results than vitrification for TM = 42.7 ± 19.6%, PM = 30.3 ± 16.7%, PMI = 49.1 ± 10.4% and AIS = 39.6 ± 14.5% despite the warming procedure employed (Fig. 1). No differences ($p>0.05$) were observed among warming temperatures after vitrification for TM (W1: 0.2 ± 0.4%, W2: 2.8 ± 4.3% and W3: 2.4 ± 2.1%) and PM (W1: 0.2 ± 0.4%, W2: 1.2 ± 1.8% and W3: 2.1 ± 1.9%). However, PMI was significantly higher in W3 (20.0 ± 11.0%) than W1 (5.5 ± 3.6%) and W2 (9.3 ± 8.4%) ($p<0.05$). The percentage of AIS resulted also in higher values in W3 (29.8 ± 4.5) than W1 (14.5 ± 3.7) ($p<0.05$).

Parameters regarding velocity features showed no differences ($p>0.05$) between W3 and C for VCL VSL and VAP. Protocol W1 resulted in the lowest values for LIN, STR, WOB, ALH and BCF. Mean values of kinematic sperm parameters assessed are represented in Table 1.

Vitrification of sperm in large volumes has been mostly researched in human showing similar (Slabbert *et al.*, 2015) or higher (Isachenko *et al.*, 2011) sperm quality after warming when compared to conventional freezing. However, in the present study conventional freezing conserved motility, plasma membrane and acrosome integrity better than vitrification in 0.5 mL straws in donkey sperm. A possible explanation for this difference is the higher cryostability of the human sperm due to its structural composition (Isachenko *et al.*, 2003). Cryostability of sperm cells has been negatively correlated with the size, probably due to higher water content and lower internal compaction of the bigger ones (Katkov *et al.*, 2012). Human sperm head size is smaller in comparison to other mammalian species, including equine (Garner, 2006;

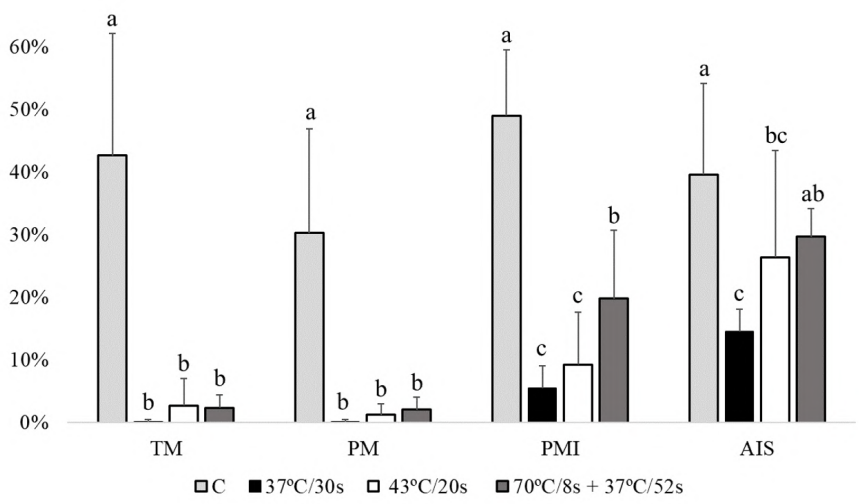

Figure 1. Percentages of total (TM) and progressive (PM) motility, plasma membrane integrity (PMI) and acrosome integrity (AIS) from donkey sperm after conventional slow freezing (C) compared to vitrification in 0.5 mL straws warmed following different protocols. Different superscripts (a, b, c) indicate significant differences between treatments (p<0.05). Values are expressed as mean ± SD.

Jiménez-Rabadán *et al.*, 2015; Arraztoa *et al.*, 2017). This characteristic probably makes human sperm less vulnerable to cryopreservation damage (Arraztoa *et al.*, 2017), which allows successful vitrification in higher volumes. Additionally, differences between these species exist also in plasma membrane composition of the sperm (cholesterol and phospholipid types) and they are also determinants of sperm cryostability. Besides, sucrose concentration employed in the present study was selected from preliminary tests in donkey sperm vitrification in spheres (30 µL), in which 0.1 M sucrose conserved sperm parameters better than the extender commonly used for human sperm vitrification: 0.25 M sucrose prepared in distilled water plus serum albumin (Isachenko *et al.*, 2008; Sanchez *et al.*, 2012 a,b). In this sense, the extender requirements for guaranteeing vitrification of donkey sperm packed in 0.5 mL straws may be different in comparison to spheres method. Therefore, further studies testing higher non-permeable cryoprotectant concentration; other sugars, such as trehalose (El-Badry *et al.*, 2017; Schulz *et al.*, 2017); as well as other substances supplementation, such as

Table 1. Mean values of kinematic parameters from samples subjected to conventional slow freezing (C) and vitrification in 0.5 mL straws warmed using different protocols.

Kinematic parameters	C	Warming procedures after vitrification		
		37ºC/30s	43ºC/20s	70ºC/8s+37ºC/52s
VCL, µm/s	75.3±11.8^{a}	13.6±30.4^{b}	29.5±38.5^{b}	79.3±56.6^{a}
VSL, µm/s	64.0±10.9^{a}	13.1±29.4^{b}	25.2±32.9^{b}	70.2±49.2^{a}
VAP, µm/s	69.4±11.8^{a}	13.3±29.7^{b}	23.4±34.5^{b}	72.6±51.0^{a}
LIN, %	84.9±2.8^{a}	19.3±43.1^{c}	34.1±44.0^{bc}	62.5±43.5^{ab}
STR, %	92.3±1.4^{a}	19.8±44.2^{c}	38.3±49.4^{bc}	67.8±46.8^{ab}
WOB, %	92.0±2.2^{a}	19.5±43.6^{c}	35.6±46.0^{bc}	64.4±44.7^{ab}
ALH, µm	1.8±1.3^{a}	0.2±0.4^{c}	0.9±1.1^{bc}	1.6±1.3^{ab}
BCF, Hz	8.8±0.4^{a}	1.3±2.9^{c}	4.1±5.3^{bc}	7.7±4.7^{ab}

VCL: curvilinear velocity. VSL: straight line velocity. VAP: average path velocity. LIN: linear coefficient. STR: straightness. WOB: wobble. ALH: amplitude of head displacement. BCF: beat cross frequency. Different superscripts $^{(a, b)}$ indicate significant differences between protocols (p<0.05). Values are expressed as mean ± SD.

cyclodextrins (Madison *et al.*, 2013; Partyka *et al.*, 2016; Lone, 2018) would be interesting to improve results after sperm vitrification in 0.5 mL straws in this species.

In addition, human sperm vitrification has been traditionally performed after sperm selection of motile and viable cells; whereas sperm selection techniques prior to freezing in stallions has shown no improvements on sperm motility after thawing (Hidalgo *et al.*, 2017). In this regard, it is also important to point out that vitrification in 0.5 mL straws has obtained despair results regarding species, even among studies in human sperm, as previously described by Katkov *et al.* (2012). Conventional freezing has shown better results than sperm vitrification in other domestic animals as dog (Caturla-Sánchez *et al.*, 2018), and ram (Pradiee *et al.*, 2016); but the spheres method was used in both.

In order to maximize the possibilities of sperm vitrification success in large volumes in donkeys, different warming protocols were compared. Unexpectedly, no differences in motility percentages between warming rates were found; although higher temperatures during shorter periods of time (W3, 70ºC/8s + 37ºC/52s), showed significantly higher membrane integrity and velocity features than lower temperatures during longer periods of warming (W1 and W2). Moreover, W3 warming protocol outcomes similar sperm velocity parameters (VSL, VCL and VAP) after vitrification than those obtained after conventional freezing. In this regard, hyperactivation of sperm cells might be suspected; however, it has been generally defined as a change in the pattern of sperm motility (Hinrichs & Loux, 2012), which has been set in stallion sperm as (i) VCL>180 µm/s and mean ALH>12 µm (Rathi *et al.*, 2001) or (ii) VCL between 225 and 350 µm/s and ALH between 8 and 13 µm (Hinrichs & Loux, 2012). In this sense, none of the kinematic sperm parameters assessed in this study had those characteristics, so sperm hyperactivation after vitrification could be dismissed. On the other hand, sperm velocity parameters ascertained by CASA assessments have been previously correlated with in vivo fertility in mammals (Gomendio & Roldan, 2008). Unfortunately, fertility has not been assessed in this study.

Additionally, the better sperm quality found in the present study using high warming rates (W3), is in agreement to those results previously reported by Pradiee *et al.* (2016), in which rapid warming prevented damage after vitrification in spheres in mouflon sperm (60ºC better than 37ºC). Similarly, Athurupana *et al.* (2015) found higher motility and membrane integrity sperm values using 70ºC/8s + 39ºC/52s in comparison to 39ºC/60s in conventional freezing of boar sperm. On the contrary, a recent work comparing warming procedures after dog sperm vitrification in spheres (Caturla-Sánchez *et al.*, 2018), showed better sperm quality after warming at 37ºC rather than 65ºC. In the present study, almost no motile sperm were recovered using the conventional thawing protocol for 0.5 mL French straws (W1, 37ºC/30s), which may be explained because the warming rate for sperm vitrification in large volumes must be raised in comparison to conventional freezing. In this sense, W2 (43ºC/20s) was expected to outcome good sperm quality after vitrification because similar warming protocols were successfully employed in donkey (Diaz-Jimenez *et al.*, 2017, 2018b) and human (Mansilla *et al.*, 2016) sperm vitrification using 0.25 mL straws. Nevertheless, volume and package were modified in this report in comparison to the previous ones, so the vitrification procedure was completely different. The ratio between volume and surface occupied by the semen samples in 0.5 mL straws was different in comparison to 0.25 mL straws, reducing thereby the cooling rates achieved in this study. According to this, the ultra-rapid cooling rates required for successful 'kinetic vitrification' of equine sperm may not occur (Hidalgo *et al.*, 2018), which could explain the poor sperm quality obtained after warming.

Sperm motility after vitrification yielded substantially lower percentages in comparison to conventional freezing, being therefore deficient for its use in routine artificial insemination practices. However, percentage of sperm viability using the W3 warming protocol was higher than other warming rates tested, reaching values around 20%. In this regard, selection of sperm cells after warming might be performed (Ortiz *et al.*, 2015) and possibly be used for advanced reproduction techniques such as "intracytoplasmic sperm injection" (ICSI) (Gonzalez-Castro & Carnevale, 2019).

In conclusion, high warming rates seem to be better for donkey sperm vitrification in large volumes; but this methodology is still not an alternative to conventional sperm freezing.

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CHAPTER 5

CHAPTER 5.1

Vitrification of donkey sperm using straws as an alternative to conventional slow freezing

Diaz-Jimenez et al., 2020. Reproduction in Domestic Animals

CHAPTER 5.2

First pregnancies in jennies with vitrified donkey semen using a new warming method

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SUPPLEMENT ARTICLE

Reproduction in Domestic Animals WILEY

Vitrification of donkey sperm using straws as an alternative to conventional slow freezing

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Abstract

Cryoprotectant-free vitrification of donkey sperm using 0.25 ml straws has been recently developed, but the obtained results have not been directly compared to conventional slow freezing yet. The aim of this study was to compare sperm quality parameters after cryopreservation using both methods. Semen samples were collected from three Andalusian Donkeys. Semen was centrifuged and pellets resuspended with an extender with glycerol for conventional freezing or the same extender without glycerol, but with sucrose 0.1 mol/L for vitrification. Conventional freezing was performed in nitrogen vapours and thawed in a water bath (30s/37°C). Vitrification was performed in covered 0.25 ml straws plunged directly into liquid nitrogen and warmed in 3 ml of a milk-based extender at 43°C. Total (TM, %) and progressive motility (PM, %) were evaluated by computer-assisted sperm analysis, and plasma membrane (PMI, %) and acrosome (AIS, %) integrities by epifluorescence microscopy. No differences ($p > .05$) were found between slow freezing and vitrification methods for any of the parameters assessed: TM (58.2 ± 16.1% vs. 52.7 ± 15.6%), PM (44.7 ± 18.2% vs. 44.3 ± 15.0%), PMI (55.4 ± 9.0% vs. 49.2 ± 11.2%) and AIS (38.4 ± 19.6% vs. 45.0 ± 11.0%), respectively. In conclusion, donkey sperm vitrification in straws presented similar sperm quality after thawing in comparison to conventional freezing. Therefore, it could be considered as an alternative to slow freezing regarding the sperm parameters assessed.

KEYWORDS
cryopreservation, jack, semen

1 | INTRODUCTION

Long-term storage of germ cells at sub-zero temperatures is considered an important tool in assisted reproductive technologies, which also permits creation of genetic banks of valuable or endangered animal species (Gandini, Pizzi, Stella, & Boettcher, 2007). Research on different sperm cryopreservation procedures aiming to reduce cell damage and costs have increased in the last few years (Hezavehei et al., 2018). Sperm vitrification has been developed as a simpler and cheaper alternative to conventional freezing method, and it has resulted in good sperm quality parameters after warming in several animal species (Sánchez et al., 2011; Swanson, Bateman, & Vansandt, 2017; Zilli et al., 2018). Due to the current situation of endangerment of donkey breeds in Europe (Camillo et al., 2018), research in reproductive technologies in this species has increased in recent years. As such, freezing cryoprotectants (Acha et al., 2015), post-thaw sperm quality (Ortiz et al., 2015) and fertility (Rota, Panzani, Sabatini, & Camillo, 2012) have been studied. Donkey sperm vitrification has been also developed, with sperm

quality percentages similar to those obtained with slow freezing (Diaz-Jimenez, Dorado, Consuegra, Ortiz, et al., 2019). However, a direct comparison between this cryoprotectant-free method of vitrification in 0.25 ml straws and conventional slow freezing with glycerol has not been performed yet. In order to fully understand if sperm vitrification using straws could be an alternative to slow freezing, this study aimed to compare sperm quality parameters after cryopreservation using both methods.

2 | MATERIALS AND METHODS

All the experiments were performed in accordance with the Ethical Committee for Animal Experimentation of the University of Cordoba (project no. 31/08/2017/105) and the Spanish law for animal welfare and experimentation (RD 53/2013). Three healthy Andalusian Donkeys, aged from 4 to 7 years, were used as semen donors. Four ejaculates per animal ($N = 12$) were collected using an artificial vagina. Gel-free semen samples were first diluted 1:1 with INRA-96 (IMV, L'Aigle, France) for centrifugation (400 ***g***/7 min). Sperm pellets were then resuspended with the freezing extender: a skimmed milk–egg yolk-based extender containing glycerol (Minitübe, Germany), for conventional freezing (CF) or the same extender without glycerol, but containing sucrose 0.1 M for vitrification (V). Conventional freezing and thawing were performed following a previously described protocol for donkey sperm cryopreservation (Diaz-Jimenez et al., 2018). In brief, samples were slowly cooled for 2 hr at 5°C, filled in 0.5 ml straws, frozen in nitrogen vapours and thawed in a water bath (37°/30s). For sperm vitrification, samples were cooled (1 hr at 5°C) and 160 µl of the sperm solution was packed in 0.25 ml French plastic straws, which were horizontally inserted into 0.5 ml straws (CBS, Cryo Bio System). Thereafter, the covering straw was sealed and plunged directly into liquid nitrogen (LN_2) (Diaz-Jimenez, Dorado, Consuegra, Ortiz, et al., 2019). For warming, each 0.25 ml straw with the vitrified sperm was directly immersed in a tube containing 3 ml of the milk-based extender at 43°C.

Sperm parameters were evaluated after thawing/warming, as previously described (Diaz-Jimenez, Dorado, Consuegra, Pereira, et al., 2019). Briefly, total (TM, %) and progressive (PM, %) sperm motility were objectively evaluated by the Sperm Class Analyzer (SCA v.5.4, Microptic S.L., Spain); while plasma membrane integrity (PMI, %) was assessed using the Vital-Test (Halotech DNA S.L., Spain) double stain commercial kit, according to the manufacturer´ instructions. Sperm acrosome integrity (AIS, %) was evaluated using peanut agglutinin–fluorescein isothiocyanate/propidium iodide staining with an epifluorescence microscope. All data were analysed using SPSS Statistics 22.0 (SPSS Institute Inc. Headquarters, USA). Normality of the data distribution and homogeneity of variances was assessed using the Kolmogorov–Smirnov and Levene test, respectively. Sperm parameters after vitrification or conventional freezing were compared using the Student´s *t* test for independent samples and expressed as mean ± standard deviation (*SD*). Significant differences were considered when $p < .05$.

3 | RESULTS

Sperm parameters assessed after cryopreservation are represented in Figure 1. No differences ($p > .05$) were found between vitrification and conventional freezing methods for any of the parameters assessed: TM (52.7% ± 15.6 vs. 58.2% ± 16.1), PM (44.3% ± 15.0 vs. 44.7% ± 18.2), PMI (49.2%±11.2 vs. 55.4%±9.0) and AIS (45.0%±11.0 vs. 38.4%±19.6), respectively.

4 | DISCUSSION

Vitrification of donkey sperm in 0.25 ml straws has been studied regarding vitrification extenders, and sperm volume and concentration (Diaz-Jimenez, Dorado, Consuegra, Ortiz, et al., 2019; Diaz-Jimenez et al., 2017). Due to the good sperm quality after warming, this technique is regarded as one of the most promising alternative for donkey sperm cryopreservation. However, to the best of the authors knowledge, this is the first study in which donkey sperm vitrification in 0.25 ml straws has been directly compared with conventional slow freezing with glycerol. According to our results, no differences regarding sperm quality after thawing was found between methods.

To date, there is considerable lack of agreement among studies performed in other species when vitrification in straws is compared to conventional freezing. Similar results between methods regarding sperm parameters after warming/thawing were found in human using 0.25 ml (Mohamed, 2015) or 0.5 ml straws (Slabbert, du Plessis, & Huyser, 2015), but significantly higher sperm quality after vitrification in 0.5 ml straws was found in other study by Isachenko et al. (2011). In stallion, vitrification in 0.25 ml straws resulted in higher sperm quality parameters when compared to slow freezing (Consuegra et al., 2019b), but almost no motile sperm was recovered after vitrification in 0.5 ml straws (Consuegra et al., 2019a; Restrepo, Varela, Duque, Gómez, & Rojas, 2019). Similar results were found in donkeys using 0.5 ml straws (Diaz-Jimenez, Dorado, Consuegra, Pereira, et al., 2019), and in ram with 0.25 ml straws (Jiménez-Rabadán et al., 2015), in which conventional freezing resulted in significant higher sperm parameters after thawing compared to vitrification.

It seems that there are differences between methods regarding species, although if sperm vitrification is performed in large volumes (from 300 to 500 µl), low sperm quality parameters are generally found (Katkov et al., 2012). Those differences when comparing techniques may be caused by different cryoprotectant requirements regarding cryostability of sperm cells among animal species, as previously hypothesized (Diaz-Jimenez, Dorado, Consuegra, Pereira, et al., 2019; Hidalgo et al., 2018).

The vitrification technique has some advantages in comparison to conventional freezing, such as avoiding toxicity of permeable agents, simplicity, cost-effectiveness and being less time consuming.

In conclusion, donkey sperm vitrification in straws resulted in similar sperm quality after thawing in comparison to conventional freezing. This is an interesting cryopreservation method to be

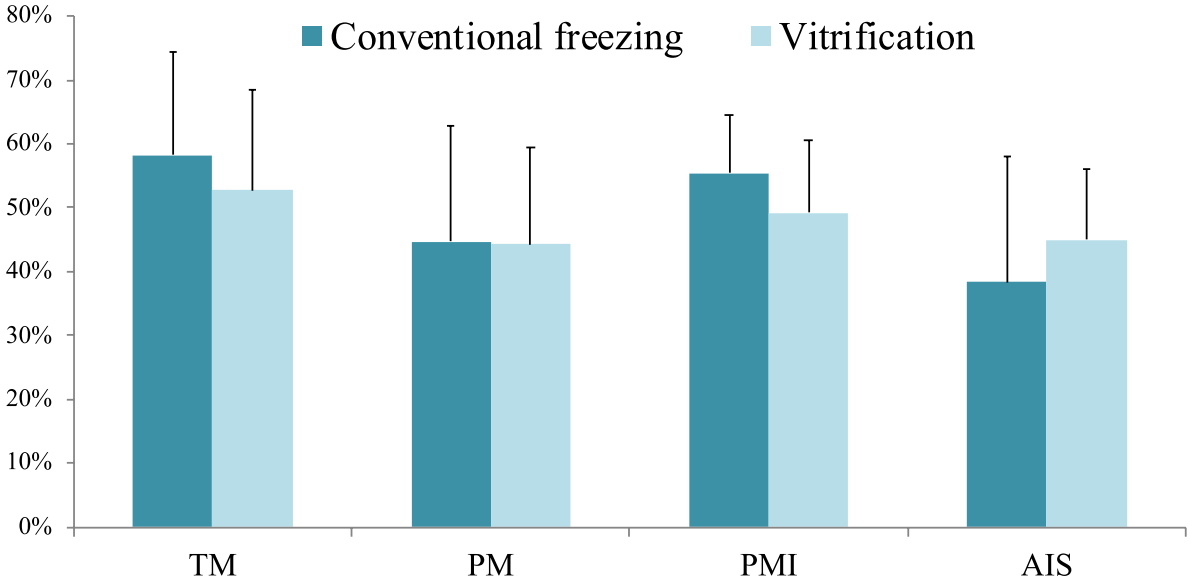

FIGURE 1 Percentage of total (TM) and progressive (PM) motility; plasma membrane (PMI) and acrosome integrities (AIS) in donkey sperm after conventional freezing or vitrification in 0.25 ml straws. Data are represented as mean ± *SD*. No significant differences between techniques were found ($p > .05$)

included into the assisted reproductive technologies for donkey species, and it could be considered as an alternative to slow freezing regarding the sperm parameters assessed.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHORS CONTRIBUTION

M Hidalgo and M Diaz-Jimenez contributed to all sections. J Dorado contributed to the study design, preparation and final approval of the manuscript. C Consuegra and B Pereira performed the experiments. Head of the Project: M Hidalgo and J Dorado.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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First pregnancies in jennies with vitrified donkey semen using a new warming method

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ABSTRACT

Sperm vitrification has been recently developed, but fertility trials have not been performed yet in equine species. In this study, a new warming technique for vitrified donkey semen was developed and the uterine inflammatory response and fertility were compared to conventional freezing. In Experiment 1, sperm was vitrified in straws and warmed in 3 ml of extender or in a water bath at: 37 °C/30 s; 43 °C/10 s; and 60 °C/5 s. Sperm motility, plasma and acrosome membranes and DNA integrity were compared between treatments. In Experiment 2, jennies were inseminated twice (500×10^6 sperm) in the uterine body either with vitrified or frozen semen (2 cycles/jenny). Pregnancy rates and the uterine inflammatory response (polymorphonuclear neutrophil concentration; **PMN**) were evaluated after artificial insemination (**AI**). No differences between warming in extender/water bath were found and 43 °C/10 s was better than lower temperatures in terms of total (53.8 ± 13.2%) and progressive sperm motility (41.4 ± 11.4%). No differences in PMN concentration ($\times 10^3$ PMN/ml) were found between vitrified (276.8 ± 171.6) or frozen (309.7 ± 250.7) semen after AI. However, PMN decreased faster ($P < 0.05$) using vitrified semen. Pregnancy rates were greater for vitrified (22%) than frozen semen (10%) but not statistically different. In conclusion, donkey sperm vitrified in straws could be directly warmed in a water bath at 43 °C/10 s, reducing the uterine inflammatory response obtained after AI and promoting positive pregnancy outcomes. These findings confirm the possibility to use vitrified semen as an alternative for AI in jennies.

Implications

Pregnancy rates in jennies after artificial insemination with frozen–thawed donkey semen are poor. The permeable agent presence was suggested to provoke high post breeding endometritis affecting fertility. However, no studies have been performed using vitrified semen without permeable agents for artificial insemination in equines. Double pregnancy rates in jennies inseminated with vitrified donkey semen in comparison to frozen–thawed semen were obtained. Vitrified semen also reduced the post breeding uterine inflammatory response. A new methodology for warming vitrified straws was also developed, making intrauterine artificial insemination in field conditions possible, avoiding post-warming dilution and centrifugation, and improving the methodology in terms of cost effectiveness and time to process.

Introduction

During the last century, the donkey population has considerably diminished due to the industrial revolution and the mechanization of agriculture (Kugler et al., 2008; Camillo et al., 2018). Actually, although these animals are still employed in some regions (mostly in less industrialized countries), the majority of the European donkey breeds are threatened by extinction; as stated by the Food and Agriculture Organization (FAO), 2018. Given the importance of biodiversity and domestic animal resource preservation, research should be focused on reproductive procedures aiming to preserve genetically valuable donkey breeds. The use of frozen semen in artificial insemination (**AI**) of domestic species has numerous advantages in comparison to fresh and chilled semen (Loomis and Graham, 2008; Oliveira et al., 2016). Among them, the availability of semen at any time, semen shipping worldwide (which also increases genetic variability) and the long-term preservation of genetic material (through the creation of genetic banks) of valuable males or endangered breeds (Sanchez et al., 2009).

Cryopreservation of donkey semen has been traditionally performed using slow freezing techniques and glycerol is largely applied as a

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permeable cryoprotectant agent (**CPA**). Recent studies analyzed the effect of: other permeable agents and combinations (Oliveira et al., 2006; Acha et al., 2015), addition of different nonpermeable substances (Oliveira et al., 2014; Álvarez et al., 2019), and even cryopreservation with the sole use of nonpermeable agents (Diaz-Jimenez et al., 2018a). Although the excellent quality of donkey sperm is usually obtained after thawing, with sperm motility percentages up to 65% (Ortiz et al., 2015a; Oliveira et al., 2016) and pregnancy rates in mares of 53% (Canisso et al., 2011); pregnancy rates in jennies after AI with frozen–thawed donkey semen are still very disappointing (Vidament et al., 2009; Serres et al., 2014). The presence of glycerol in the insemination dose has been hypothesized to be the cause of the poor results obtained with frozen semen in jennies (Trimeche et al., 1998). Vitrification is a cryopreservation method recently developed as an alternative to conventional sperm freezing. The kind of vitrification technique employed for sperm is different than that used for embryos or oocytes, where high concentrations of permeable cryoprotectants are needed and both the intracellular milieu and the extracellular environment must become vitrified (Katkov et al., 2006). This technique has been named "kinetic vitrification" as it requires high cooling and warming rates but avoiding the use of permeable CPAs. Moreover, it does not imply that the extracellular medium is vitrified (Shaw and Jones, 2003). However, there is still some controversy surrounding the use of this terminology when compared to the conventional term for vitrification associated with oocytes and embryos (Pradiee et al., 2015; Hidalgo et al., 2018). Sperm vitrification has been employed by several authors in different species in the last few years (Merino et al., 2011; Sánchez et al., 2011; Rosato and Iaffaldano, 2013; Jiménez-Rabadán et al., 2015; Pradiee et al., 2016; Isachenko et al., 2017; Swanson et al., 2017; Arraztoa et al. 2017b and 2017c; Caturla-Sánchez et al., 2018; Pradieé et al., 2018; Consuegra et al., 2018b; Diaz-Jimenez et al., 2018b). Different procedures have been developed (cryoloops, spheres, straws), and among them, vitrification using 0.25 into 0.5 ml straws is an aseptic technique that has showed satisfactory post-warming sperm parameters in humans (Sanchez et al., 2012; Mansilla et al., 2016; Schulz et al., 2017; Uribe et al., 2017), stallions (Consuegra et al., 2018a), and donkeys (Diaz-Jimenez et al. 2017 and 2018b). Despite this growing interest, there are only a few studies in which AI has been performed with vitrified semen with no permeable CPAs added: rabbits (Rosato and Iaffaldano, 2013), humans (Sanchez et al., 2012), and mouflons (Pradiee et al., 2016), but it has not been performed in any equine species yet.

The warming protocol commonly employed after sperm vitrification in the two-straws method consisted on the immersion of the inner straw in 2–5 ml of pre-warmed semen extender (37–43 °C), and then centrifuged. This procedure was first described by Sanchez et al. (2012), and quite good sperm quality after vitrification has been obtained. Even so, the warming procedure is one of the major drawbacks of this method of vitrification, as it could be expensive and time consuming. For one single insemination in equines, a high quantity of warming extender and a great number of thawed straws would be required, which must be centrifuged separately after warming. Despite its unfeasibility, no one to the best of our knowledge has researched alternative warming procedures.

During the last year, the sperm vitrification process has been optimized in donkeys regarding sperm concentration, volume, and package (Diaz-Jimenez et al., 2019a), and different warming rates have been tested for vitrification in large volumes (0.5 ml) in the same species (Diaz-Jimenez et al., 2019b). Nevertheless, warming approach and rates in the two-straws method of vitrification have been poorly studied: there is only one study performed in human sperm (Mansilla et al., 2016), but there are no reports in any equine species yet. In this paper, we analyzed a new approach of warming after sperm vitrification in order to simplify the process: direct immersion of the inner straw into a water bath. We hypothesized that this method would maintain the rate of warming and guarantee warmth to be distributed equally in all the straw surface. It is easier, quicker, and cheaper, as the use of extender for warming and following centrifugation is avoided. The dose would be ready to use right after warming. To our knowledge, there are no previous reports testing this technique in sperm vitrification.

As previously stated, the presence of glycerol in the AI dose seems to be toxic to the sperm cell (Watson, 2000), but also a high post breeding endometritis is provoked in jennies, having a detrimental effect on fertility (Vidament et al., 2009). In this regard, different strategies have been developed in order to improve pregnancy rates when AI with jack frozen semen is performed in jennies: glycerol removal before insemination (Trimeche et al., 1998); glycerol susbstitution with dimethylformamide (Vidament et al., 2009); or its reduction by combination with other permeable CPAs: dimethylsulfoxide, dimethylformamide, and dimethylacetamide for sperm freezing (Oliveira et al., 2006); and performing deep horn AI or increasing the number of inseminations (Oliveira et al., 2016). However, to the best of our knowledge, there are no previous studies of AI with cryopreserved jack semen in the absence of permeable agents. To date, the highest overall jennies pregnancy rate described in the literature using frozen–thawed jack semen is still 36%, reported by Rota et al. (2012). They reported a tendency to obtain higher pregnancy results when glycerol was used as permeable agent and post-thaw extended with seminal plasma (**SP**). In this sense, the role of glycerol in the low success of AI with frozen–thawed sperm in jennies is not so clear, as was previously suggested by Vidament et al. (2009). Consequently, it is advisable to further research in jennies AI using jack semen cryopreserved with the sole use of nonpermeable agents. This would contribute to increase the knowledge about those poor pregnancy results in jennies and if the cause of it is related to the freezing extender composition.

Therefore, the aims of this study were to: 1) evaluate the effect of warming technique and temperature after donkey sperm vitrification in straws and 2) compare the post breeding uterine inflammatory response and fertility of jennies after AI with vitrified or frozen jack semen.

Material and methods

Animals

Four Andalusian and two Amiata donkeys (5 to 9 years old) were used as semen donors. Andalusian donkeys were individually housed in stalls in the Equine Sports Medicine Centre (**CEMEDE**) of the University of Cordoba (Spain) and Amiata donkeys were housed in the same conditions at the Department of Veterinary Sciences of the University of Pisa (Italy). A total of thirteen cyclic Amiata jennies, aged from 7 to 13 years and in good body condition, were used for the AI protocol. Males and females used in the study were known to be fertile. Animals were fed with meadow hay and a commercial feed for horses and water was freely available.

Semen collection and processing

In order to deplete the extragonadal sperm reserves, an initial semen collection was performed in sexually rested donkeys (daily collection for five days). Thereafter, a total of 32 ejaculates were used in this study: six ejaculates from each jack in first experiment ($n = 24$) and four ejaculates per jack in the second one ($n = 8$). Semen collection was performed on a regular basis (twice a week) using an artificial vagina in the presence of a jenny in estrus. Gel-free semen volumes were measured in the graduated collector, sperm concentration was determined using a spectrophotometer, and morphology and acrosome integrity were assessed on stained smears as previously described (Hidalgo et al., 2017; Diaz-Jimenez et al., 2019b). Thereafter, semen was extended (INRA-96, IMV Technologies, L'Aigle, France) and processed as described in each experiment.

Sperm vitrification

Semen samples were vitrified following an optimized protocol described for donkeys (Diaz-Jimenez et al., 2019a). Briefly, after collection and preliminary evaluation, semen was extended 1:1 (v:v) with INRA-96 and centrifuged (7 min/400 ×*g*/22 °C) in a corning-adapted centrifuge (Eppendorf, model 5702 RH, Eppendorf AG, Hamburg, Germany). The supernatant was removed and the sperm pellet resuspended in a skimmed milk-egg-yolk base extender without glycerol (Gent, Minitüb GmbH, Tiefenbach, Germany) supplemented with sucrose 0.1 M until a concentration of 300×10^6 sperm/ml. Thereafter, samples were equilibrated 10 min at room temperature (22 °C) and slowly cooled at 5 °C for 1 h into an Equitainer (Hamilton Research, Inc. Ipswich, Massachusetts, USA). Vitrification was performed applying the two-straw technique: a 160 μl aliquot of the cooled extended semen was packed in 0.25 ml French plastic straws; these were horizontally placed in 0.5 ml straws and hermetically sealed for both ends. Filled straws were then vitrified in a horizontal position by direct plunging into liquid nitrogen. After at least 24 h of storage, straws were warmed as subsequently described in the experimental design.

Post-warming sperm evaluation

Sperm motility

Sperm motility was objectively evaluated using the Sperm Class Analyser system (SCA v5.4, Microptic S.L., Barcelona, Spain). The system consists of an optical phase-contrast microscope (Eclipse 50i; Nikon, Tokyo, Japan), a warm plate at 37 °C (OK 51–512, Osaka, Digifred SL, Barcelona, Spain), and acquisition was performed using high-speed digital camera (A312fc, BaslerTM AG, Ahrensburg, Germany). The settings parameters of the software analysis were as follows: frame rate of 25 frames/s and 25 images captured, cell size from 15 to 75 μm^2, connectivity 12, spermatozoa were considered motile with a mean average path velocity (VAP) > 10 μm/s, and progressive with a straightness coefficient (VSL/VAP × 100) greater than 75%. For each evaluation, two 5 μl drops and three random microscopic fields per drop including a minimum of 200 spermatozoa were evaluated. The trajectory of each spermatozoa was determined by the software and the following parameters of sperm motion were measured: total motility (TM; %); progressive motility (PM, %); curvilinear velocity, or total distance traveled by the spermhead per unit time (VCL; μm/s); straight line velocity, or net distance gain of the sperm head per unit time (VSL; μm/s); average path velocity, or length of a derived "average" pathof sperm head movement per unit time (VAP; μm/s); wobble (WOB, as VAP/VCL × 100), beat cross frequency, number of times the curvilinear path crosses the average path per unit time (BCF; Hz), and amplitude of lateral head displacement, or width of the head movement envelope (ALH; μm).

Plasma membrane integrity

Sperm plasma membrane integrity was examined with fluorescence microscopy using the VitalTest stain (Halotech DNA S.L.), as previously described (Dorado et al., 2014). Briefly, an aliquot of 10 μl of diluted semen was mixed with 1 μl propidium iodide stock solution and 1 μl of acridine orange stock solution and evaluated under epifluorescence microscopy (Olympus BX40, Tokyo, Japan) using a U-ND25-2 filter (a 460–490 nm excitation filter). At least 200 sperms were counted and those with green emission (spermatozoa with an intact plasma membrane) were recorded. The results are expressed as plasma membrane integrity percentage (PMI; %).

Acrosome membrane integrity

For sperm acrosome evaluation, the double stain propidium iodide (PI)/peanutagglutinin–fluorescein isothiocyanate (FITC-PNA) (Sigma-Aldrich, Sant Louis, USA) was used as described by Diaz-Jimenez et al. (2019b). In short, a mixture of 10 μl PI and 20 μl FITC-PNA was spread over pre-permeabilized smears of the samples and slides were incubated in a dark, moist chamber at 4 °C for 30 min. Thereafter, a minimum of 200 spermatozoa were evaluated under epifluorescence microscopy, and sperm acrosomes were classified as follows: intact (spermatozoa displaying intensively bright green fluorescence of the acrosomal cap) and damaged (spermatozoa displaying disrupted fluorescence, fluorescent band at the equatorial segment or no fluorescence, indicating damages to the outer acrosomal membrane). The percentage of acrosome-intact sperm was recorded (AIS, %).

Sperm DNA analysis

Sperm DNA integrity was assessed with the sperm chromatin structure assay (**SCSA**). Data were acquired and analyzed using a CytoFLEX S high-resolution flow cytometer (Beckman Coulter, Life Sciences Division Headquarters, Indianapolis, USA) with subsequent data analysis using CytExpert software (Beckman Coulter Life Sciences, Indianapolis, IN, USA). Sperm samples were managed before analysis as described by Diaz-Jimenez et al. (2018b): an aliquot from each treatment was stored at −80 °C (Jackson et al., 2010). The individual semen samples were thawed in a water bath at 37 °C prior to DNA assessment. A 5 μl aliquot of semen was diluted in 195 μl of a buffered solution and then mixed with 400 μl of a low pH solution for 30 s. Then, 1.2 ml of a stock solution (4.0 g/ml) of acridine orange was added to the sample and immediately processed using flow cytometer (Salazar et al., 2011). The analysis was restricted to the sperm cells based on size and granularity using forward (**FSC**) and side scatter (**SSC**), respectively, and uniformity of the cell suspension. Each sample was allowed to pass through the flow cytometer system in a flow rate of 10 μl/min, and at least 10000 cells/sample were studied, after exclusion of the non-sperm cells. The percentage of sperm cells with fragmented DNA was recorded as DNA fragmentation Index (**DFI**, %).

Experimental design

Experiment 1. Effect of warming technique and temperature after donkey sperm vitrification in straws

This experiment was performed in order to improve and simplify warming protocol after vitrification in straws by testing different variables: warming procedure and warming rate.

Comparison of different methods of warming and centrifugation effect after sperm vitrification

A comparison between a previously described warming protocol (Diaz-Jimenez et al., 2017; Diaz-Jimenez et al. 2018b and 2019a) and a new direct method of warming was performed. First, the covering straw was opened with forceps and each 0.25 straw with the vitrified sperm was pulled out. The inner straw was either (i) immersed into a tube containing 3 ml of extender (INRA-96) at 43 °C and then centrifuged (Control; as described by Diaz-Jimenez et al. (2019a)) or directly warmed in a water bath at 43 °C during 10 s. Since the inner straw was not sealed, immersion was done in an upside-down way in order to avoid contact with water (Fig. 1). After warming in the water bath samples were (ii) directly diluted for evaluation (WB) or (iii) subjected to centrifugation as performed in the control (WB + centrifugation). Finally, sperm parameters were evaluated as described above and compared between protocols.

Effect of different warming rates after donkey sperm vitrification

The effect of different temperatures and warming rates on donkey sperm quality after vitrification in the two-straws method was evaluated. The warming approach with the most desirable results obtained was employed. For that purpose, the covering straw was opened with forceps and each 0.25 ml straw with the vitrified sperm was pulled out and directly immersed in a water bath at (i) 37 °C during 30 s; (ii) 43 °C during 10 s; and (iii) 60 °C during 5 s. Warmed samples were not centrifuged and directly re-extended with INRA-96 for sperm evaluation as described in the followings sections.

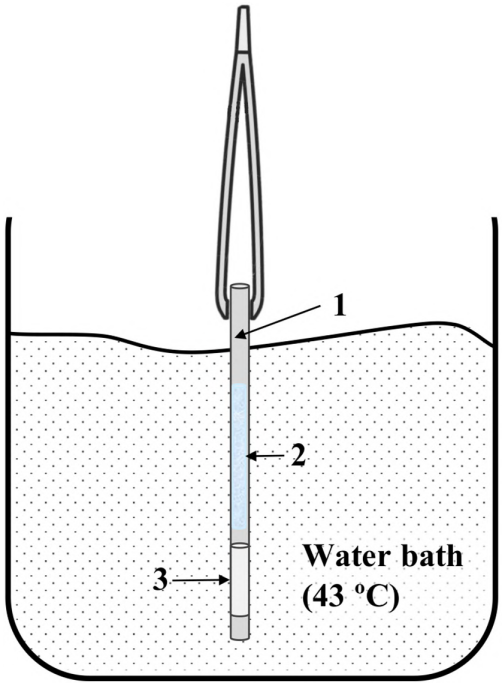

Fig. 1. Scheme of the mode of warming in an upside-down way: (1) opened 0.25 ml straw, (2) vitrified donkey sperm suspension, (3) cotton plug.

Experiment 2. Comparison between the post breeding uterine inflammatory response and fertility of jennies after artificial insemination with vitrified and frozen jack semen

The second experiment consisted of the comparison between pregnancy rates and post breeding uterine inflammatory response of jennies inseminated with vitrified sperm in comparison to a control using conventionally frozen sperm.

Sperm processing

After semen collection and evaluation, each ejaculate was divided into two aliquots and subjected to vitrification and conventional slow freezing.

Vitrification was performed using the two-straws method (as described before) followed by the optimized warming protocol from Experiment 1: in a water bath at 43 °C/10 s with no centrifugation (WB). The slow freezing and thawing procedures were performed exactly as described by Rota et al. (2012), without modifications. In brief, extended semen was centrifuged (10 min/600 $\times g$/22 °C) and pellet was resuspended in two steps until a final concentration of 168 $\times$ 10^6 sperm/ml. The freezing extender was previously prepared using INRA-96 containing 2% egg yolk and 2.2% of glycerol. Sperm suspension was maintained at 4 °C during 1 h and loaded in 0.5 ml straws. Freezing was performed in a programmable freezer (Mini-Digitcool, IMV Technologies) at 60 °C/min from 4 to 140 °C and then straws were plunged into liquid nitrogen. Straws were thawed in a water bath at 37 °C for 30 s.

Artificial insemination and breeding management

A total of 20 estrus cycles from 10 jennies were used for inseminations. Two estrus cycles per animal were randomly submitted to AI: one cycle with the vitrified and the other with the frozen semen, from the same ejaculate.

Ovarian activity was monitored weekly by transrectal palpation and ultrasonography using a linear transducer (Mindray DP30, Shenzhen, China) until a growing follicle bigger than 28 mm was detected. Then, ultrasound evaluations were performed daily: when the dominant follicle reached 35 mm, in the presence of uterine edema and estrous behaviour, ovulation was induced with 0.4 ml sc of GnRH agonist (buserelin acetate, Suprefact, Sanofi Spa, Milano, Italy).

wA fixed-time insemination protocol previously described by Rota et al. (2012) was followed. Jennies were inseminated twice, 18 and 38 h post-induction. Each breeding dose consisted of approximately 500 $\times$ 10^6 sperm of pooled sperm from both jacks, in order to avoid male effect. Therefore, ten 0.25 ml straws from vitrified (five straws from each jack) or six 0.5 ml straws from frozen semen (three straws from each jack) were used in each AI. Post-thaw re-extension was performed, for frozen or vitrified semen, with respectively either 2 or 3 ml of INRA-96, to obtain a final insemination volume of 5 ml in both treatments. All inseminations were performed in the uterine body using an equine insemination pipette (IMV Technologies) after subjective motility evaluation of thawed doses under a phase contrast microscope. The uterus was flushed after each AI for cytological examination, as described in section "*Uterine inflammatory response evaluation*".

The schedule regarding breeding management was previously described (Rota et al., 2012) and it is showed in Table 1. Cycles in which jennies ovulated before 14 h or later than 62 h post-induction were not included in the study. If a jenny had ovulated by 24 h post-induction of ovulation, she was not reinseminated at 38 h.

Uterine inflammatory response evaluation

Six hours after the last AI, jennies were examined ultrasonographycally for evaluation of the uterine edema, intrauterine fluid accumulation and follicle diameter or ovulation. Uterine lavages for endometrial cytology were performed 6 and 10 h after the first and second AI, respectively (Table 1). The jenny's uterus was infused with 1 l of Ringer Lactate (Galenica Senese, Siena, Italy) using a sterile one-way 30 cm French equine embryo flushing catheter (Bivona, Kruuse, Denmark). Fluid was recovered back to the bag and concentration and proportion of polymorphonuclear cells (**PMN**) was recorded.

For that purpose, the bag was first mixed to homogenise cell suspension and obtain a representative sample of cells. A 10 ml sample of the fluid was drawn with a syringe and cell concentration was measured using a Thoma counting chamber and number of cells per ml was recorded. For a proportion of PMN evaluation, the bag was suspended for 30 mins to help cell precipitation. Thereafter, 20 ml were collected from the bottom of the bag and centrifuged (10 min/600 $\times g$). The supernatant was discarded except for a small quantity in which the pellet was re-suspended. Smears were prepared onto glass microscope slides, air-dried and stained with Diff-Quick (Dade Berhing SPA, Milano, Italy). At least 200 cells were counted and the proportion of PMN was calculated (PMN, %). The PMN concentration ($\times 10^3$ cells/ml, [PMN]) was calculated as described by Rota et al. (2012), as follows:

Table 1
Breeding management schedule of jennies inseminated with cryopreserved donkey semen.

Time (h)	Procedure
0	Induction of ovulation (buserelin)
14	Ultrasound
18	Ultrasound and artificial insemination
24	Ultrasound and uterine flushing
38	Ultrasound and artificial insemination
42	Ultrasound
48	Ultrasound and uterine flushing
62	Ultrasound

$$[\mathrm{PMN}] = cellconcentration \times \frac{\mathrm{PMN}\%}{100}$$

Pregnancy diagnoses

Pregnancy diagnosis was made by ultrasonography 14 days after ovulation using a linear transducer (Mindray DP30, Shenzhen, China). After being rechecked at 16 days to confirm the pregnancy, luteolysis was induced with 3 mg (intramuscular) of alfaprostol (Gabbrostim, CEVA, VETEM, Milan, Italy) to bring all jennies back into estrus. Pregnancy rates (%) were calculated as the total number of pregnancies divided by the total number of oestrus cycles bred (Vidament et al., 2009).

Statistical analyses

Analyses were performed using IBM SPSS Statistics 20 (IBM Corp., Armonk, NY, USA). All data was first tested for normality of the data distribution and homogeneity of variances using the Kolmogorov–Smirnov and Levene test, respectively. When necessary, data were transformed using logarithmic scale before analysis.

The effect of warming technique and temperature after vitrification on sperm quality parameters was analysed by a general linear model (GLM) followed by the Duncan and Tukey test for *post hoc* analysis. Animals and ejaculates were considered as random factors. The percentage of pregnancy rates between vitrified and frozen semen and the relationship between uterine fluid accumulation and cryopreservation method, pregnancy diagnosis and uterine inflammatory response was analysed by Chi-square and Fisher's exact test. Differences in number of PMN between the first and the second flushing were evaluated using the paired two samples *t*-test. Differences in number of PMN between cryopreservation methods were evaluated by two-sample *t*-test. Data are shown as mean ± standard deviation (SD). In all the statistical analyses, the level of significance was set at $P < 0.05$.

Results

Average sperm parameters obtained after fresh sperm evaluation from ejaculates used in both experiments were: gel-free volume 49.7 ± 17.4 ml (range: 20–85 ml), sperm concentration 345.7 ± 216.3 × 10^6 sperm/ml (range: 153.0 – 1273.0 × 10^6 sperm/ml), total motility 88.1 ± 8.8% (range 70.0–98.1%), progressive motility 80.4 ± 9.1% (range 59.5–93.1%), normal forms 87.5 ± 11.7% (range 58.0–98.1%) and acrosome-intact sperm 70.2 ± 18.2% (range 35.7–97.0%). All males were known to be fertile and all ejaculates were within the values considered as physiologic when evaluating donkey sperm (Miró et al., 2005; Ortiz et al., 2014).

Experiment 1. Effect of warming technique and temperature after donkey sperm vitrification in straws

Comparison of different methods of warming and centrifugation effect after sperm vitrification

No significant ($P > 0.05$) differences between methods of warming nor centrifugation process were found for TM, PMI, AIS and other sperm kinematic parameters (ALH, WOB and BCF). However, warming in the extender (control) and direct warming in a water bath without centrifugation (WB) showed statistically ($P < 0.05$) higher percentages for PM and numerically greater ($P > 0.05$) sperm velocities (VSL, VCL and VAP) than direct warming and centrifugation. Direct warming in a water bath, with or without centrifugation also resulted in significantly ($P < 0.05$) lower DNA fragmentation percentage.

The direct warming protocol in a water bath without centrifugation (WB) was selected for the subsequent experiments instead of the control because the DNA results and simplicity of the technique. Results are provided in Table 2.

Effect of different warming rates after donkey sperm vitrification

Semen quality data from comparison between warming rates after sperm vitrification is provided in Fig. 2. Warming at 43 °C/10 s showed significantly higher ($P < 0.05$) results for TM (53.8 ± 13.2%) and PM (41.4 ± 11.4%) in comparison to 37 °C/30 s (TM: 40.8 ± 11.4% and PM: 30.2 ± 11.9%); but no significant differences were found when compared to 60 °C/5 s (TM: 47.3 ± 15.4% and PM: 37.5 ± 11.7%; $P > 0.05$). Parameters regarding membrane integrity (PMI and AIS) and DNA integrity showed no differences between warming rates ($P > 0.05$). Variables regarding sperm motility features are summarized in Table 3. No differences ($P > 0.05$) between 43 °C/10 s and 60 °C/5 s warming rates were found for sperm velocities (VSL, VCL and VAP), but 37 °C/30 s showed a tendency to obtain the lowest percentages. No differences ($P > 0.05$) were found for the remaining parameters assessed.

The warming protocol 43 °C/10 s was selected for the following experiment instead of 60 °C/5 s for the tendency to obtain higher total and progressive motility and practical reasons.

Experiment 2. Comparison between the post breeding uterine inflammatory response and fertility of jennies after artificial insemination with vitrified and frozen jack semen

A total of 19 cycles were considered in this study, as one cycle from one jenny was not included because failure to ovulate within 72 h after induction.

Only those straws showing at least 30% of total sperm motility after thawing were used for artificial insemination in this experiment. After

Table 2
Sperm parameters from vitrified-warmed samples ($N = 12$) using different methods of warming with and without centrifugation in donkey.

Sperm parameters	Warming procedure (43 °C)			*P*-values
	Extender + centrifugation (control)	Water Bath	Water Bath + centrifugation	
TM (%)	51.1 ± 15.8	50.7 ± 13.0	42.1 ± 15.5	>0.05
PM (%)	41.8 ± 14.8^{a}	39.5 ± 10.5^{a}	31.51 ± 12.3^{b}	<0.05
PMI (%)	49.1 ± 8.9	48.0 ± 11.1	46.1 ± 9.9	>0.05
AIS (%)	44.0 ± 12.2	43.8 ± 11.1	49.7 ± 10.6	>0.05
VCL (μm/s)	90.9 ± 13.1^{ab}	95.7 ± 19.4^{a}	84.2 ± 14.1^{ab}	<0.05
VSL (μm/s)	82.0 ± 11.4^{a}	83.9 ± 17.8^{a}	73.4 ± 12.8^{b}	<0.05
VAP (μm/s)	85.0 ± 11.6^{ab}	89.2 ± 18.7^{a}	78.0 ± 13.4^{b}	<0.05
ALH (μm)	1.9 ± 0.3	1.9 ± 0.3	1.8 ± 0.3	>0.05
WOB (%)	93.5 ± 2.1	93.1 ± 1.8	92.6 ± 1.5	>0.05
BCF (Hz)	9.0 ± 0.7	9.0 ± 0.6	8.8 ± 5.4	>0.05
DFI (%)	21.0 ± 14.6^{b}	12.7 ± 4.3^{a}	11.3 ± 4.7^{a}	<0.05

Extender (control): vitrified sperm warmed in 3 ml of extender (INRA) at 43 °C and then centrifuged; Water Bath: vitrified sperm directly warmed in a water bath at 43 °C; Water Bath + centrifugation: vitrified sperm directly warmed in a water bath at 43 °C and then centrifuged. TM = total motility; PM = progressive motility; PMI = plasma membrane integrity; AIS = acrosome-intact sperm; VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; ALH = amplitude of lateral head displacement; WOB = wobble; BCF = beat cross frequency; DFI = DNA fragmentation index. Values are expressed as mean ± SD. ^{a,b}Different superscripts in the same row indicate significant differences between warming methods.

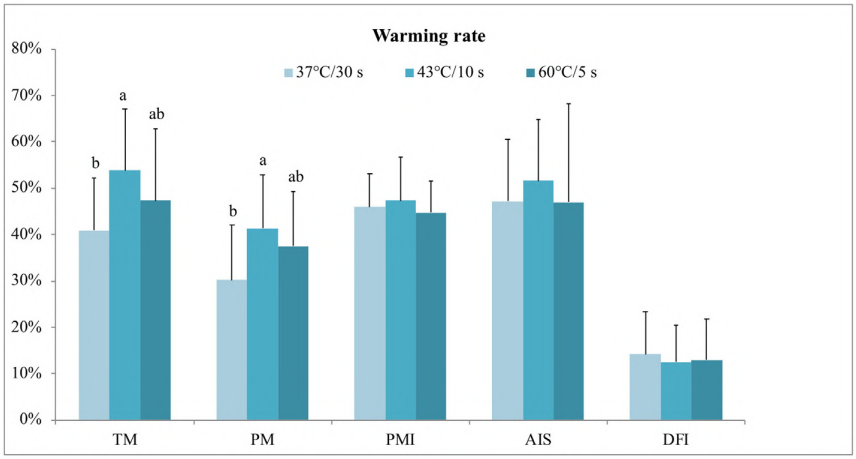

Fig. 2. Percentages of total (TM) and progressive (PM) motility, plasma membrane integrity (PMI), acrosome intact sperm (AIS) and DNA fragmentation index (DFI) from donkey sperm after sperm vitrification and warming at different rates. Different superscripts (a, b) indicate significant differences between warming rates ($P < 0.05$). Values are expressed as mean ± SD.

AI, intrauterine fluid (always <2 cm) was observed in 12/37 (32.4%) ultrasound examinations at flushing times post-AI and 6/10 (60%) jennies. In one of the positive pregnancy diagnosis uterine fluid was detected. No relation was found between uterine fluid accumulation and cryopreservation method, pregnancy diagnosis nor uterine inflammatory response ($P > 0.05$).

Pregnancy rates diagnosed at day 16 post ovulation are summarized in Table 4. AI using vitrified sperm obtained double values in positive pregnancy diagnosis percentages than frozen semen, but there were no statistical differences ($P > 0.05$).

The mean concentration of PMN is shown in Table 5. AI using vitrified semen showed a significant ($P < 0.05$) reduction in the concentration of PMN at the second flushing in comparison to the first one. No significant differences ($P > 0.05$) were found between first and second flushing using frozen semen. The comparison of PMN concentration after AI between treatments (in first and second flushing) showed no significant ($P > 0.05$) differences.

Discussion

In the present study, donkey sperm vitrification in straws has been optimized regarding the method of warming, and it has been employed for the first time in AI of jennies. To the best of the authors knowledge, there are no previous reports of AI in equine species using vitrified semen.

Sperm vitrification using the straw method has showed some advantages in comparison to other vitrification procedures. It is an aseptic technique which avoids direct contact with the liquid nitrogen and therefore preventing the risk of cross-contamination (Isachenko et al., 2017), as might occur using open carriers (open-pulled straws, cryoloops or spheres). Moreover, it allows cryopreservation of greater sperm volumes than all mentioned devices. In order to maximize the vitrified semen sample volume, vitrification in straws in larger volumes (up to 0.5 ml) has been successfully developed in humans (Isachenko et al., 2011; Slabbert et al., 2015), obtaining high sperm quality after warming. However, this technique has been recently tested in stallion (Consuegra et al., 2019; Restrepo et al., 2019) and donkey (Diaz-Jimenez et al. 2019a and 2019b) semen cryopreservation, and results were very disappointing: total motility ranged from 0 to 19%. In this sense, sperm from equine species seemed to have higher outcomes of quality when the straw system is employed. Although excellent sperm quality after vitrification in the straw method in the absence of permeable agents is obtained, the warming procedure is not quite practical; particularly when applied to an AI program: each

Table 3
Mean values of sperm motility features from vitrified samples ($n = 12$) warmed at different temperatures and times (warming rate) in donkeys.

Sperm parameters	Warming rate			*P*-values
	37 °C/30 s	43 °C/10 s	60 °C/5 s	
VCL (μm/s)	90.1 ± 15.4[b]	93.2 ± 17.2[ab]	103.2 ± 12.9[a]	<0.05
VSL (μm/s)	77.1 ± 15.1[b]	81.0 ± 14.6[ab]	90.2 ± 11.1[a]	<0.05
VAP (μm/s)	82.7 ± 15.5[b]	86.2 ± 15.2[ab]	96.5 ± 12.5[a]	<0.05
ALH (μm)	2.0 ± 0.4	1.9 ± 0.4	2.0 ± 0.3	>0.05
WOB (%)	91.6 ± 3.0	92.6 ± 3.0	93.5 ± 1.7	>0.05
BCF (Hz)	9.2 ± 0.3	9.4 ± 1.9	9.4 ± 0.6	>0.05

VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; ALH = amplitude of lateral head displacement; WOB = wobble; BCF = beat cross frequency. Values are expressed as mean ± SD. [a,b]Different superscripts in the same row indicate significant differences between warming rates.

Table 4
Pregnancy rates in jennies ($n = 10$) inseminated with vitrified or conventionally frozen donkey semen.

	Cryopreservation method		Total
	Vitrification	Conventional freezing	
Pregnancy rate	2/9 (22.2%)	1/10 (10%)	3/19 (15.8%)

Differences were not statistically significant $P > 0.05$.

Table 5
Number (showed as $\times 10^3$ cells/ml) of polymorphonuclear neutrophils on recovered post breeding fluid ($n = 32$) after artificial insemination of jennies.

Flushing	Polymorphonuclear neutrophils concentration		*P*-values
	Vitrified	Slow frozen	
1st Flushing	459.1 ± 382.2[a]	347.6 ± 389.5	0.77
2nd Flushing	94.5 ± 76.9[b]	298.2 ± 329.5	0.08
Both flushings	276.8 ± 171.6	309.7 ± 250.7	0.74
P-values	0.03	0.79	

[a,b]Different superscripts in the same column indicate significant differences between flushings (paired *t*-test). Different superscripts within a row indicate significant differences between cryopreservation methods (two-samples *t*-test). Data are presented as mean ± SD.

inner straw must be singly warmed in a tube with semen extender, and subsequently centrifuged in order to remove vitrification medium and concentrate the sample (Sanchez et al., 2012; Uribe et al., 2017). Different extender volumes per straw have been employed in warming: from 2 ml (Sanchez et al., 2012; Schulz et al., 2017; Uribe et al., 2017) to 5 ml (Mohamed, 2015) in human; and 3 ml in stallion (Consuegra et al., 2018a) and donkey sperm (Diaz-Jimenez et al. 2017 and 2018b). This warming protocol is suitable in human reproduction, in which 3 straws with a total of 1.3×10^6 sperm are employed for intrauterine insemination (Sanchez et al., 2012). However, in equine species, approximately 10 straws would be necessary per an AI dose (500×10^6 sperm), which would imply the use of at least 30 ml of equine extender for each insemination. In the present study, an optimized and more practical warming method for sperm vitrification in the two-straws has been developed: the inner straw is directly immersed in a water bath in an upside-down way, and no semen extender is needed for warming (WB). According to our results, no differences were found between the conventionally warming protocol in 3 ml extender and the new WB method without centrifugation in terms of sperm motility or membrane integrities, but significant ($P < 0.05$) less DNA fragmentation was found after warming using WB warming method. This direct warming in a water bath had been previously tested after sperm vitrification in large volumes in sealed 0.5 ml straws, but as mentioned before, poor sperm motility results were obtained when it has been employed in equines (Consuegra et al., 2019; Restrepo et al., 2019; Diaz-Jimenez et al. 2019a and 2019b).

Interestingly, we found that the post-warming sperm centrifugation could be also avoided: progressive motility and sperm velocities (VSL and VAP) were significantly higher in uncentrifugued samples (WB) in comparison to WB + centrifugation protocol. The effect of centrifugation in comparison to direct re-extension in frozen–thawed jack semen was previously evaluated by Ortiz et al. (2015b). They reported a higher sensitivity of the sperm cell and its membranes to centrifugation after freezing, which resulted in higher sperm viability percentages in uncentrifugued samples. In our results, no differences between plasma membrane integrity were found between centrifuged or uncentrifugued samples. This could be due to the different cryopreservation method used in the present study, which has demonstrated to conserve plasma membrane integrity better than conventional slow freezing (Hidalgo et al., 2018). This warming protocol represents a clear improvement on current warming methods in the straws vitrification method in terms of cost effecteness and time-saving.

Despite the fact that the thawing process has demonstrated to influence post-thaw sperm function (Sanchez et al., 2013), the effect of warming rate after vitrification using the two-straws method has been poorly researched. In the study performed by Mansilla et al. (2016) in human sperm after vitrification in the two-straws method, authors showed that plasma membrane integrity percentage was higher at a warming rate of 42 °C/5 s, in comparison to lower temperatures (38 and 40 °C). In our experiment, no differences were found regarding plasma or acrosome membrane between warming rates employed. The importance of DNA integrity and its relation to fertility has been widely described in different animal species including equids (Evenson, 2016). In fact, it has shown to be an independent predictor of fertility (Bungum et al., 2006; Oleszczuk et al., 2013), in spite of apparently normal values of sperm motility, morphology or plasma membrane integrity. In this study, no differences between DNA fragmentation index were found among warming treatments, but in terms of sperm motility, higher temperatures (43–60 °C) were found to obtain significantly greater sperm motility and velocity percentages in comparison to the lower one (37 °C). This is consistent with motility results reported by Mansilla et al. (2016), in which percentages were improved as temperature was increased (42 °C better than 40 and 38 °C). Likewise, it supports previous findings after sperm vitrification in spheres in mouflon (Pradiee et al., 2016) and in large volumes in donkey (Diaz-Jimenez et al., 2019b) and stallion (Consuegra et al., 2019). All mentioned studies reported better sperm quality results after vitrification using high warming rates during short periods of time. This phenomenon was hypothesized by Seki and Mazur (2009) and recently reviewed by Mazur and Paredes (2016): it seems to exist an opposite relation between warming rate and necessary permeable CPA concentration, so in the absence of any permeable agent the warming rate should be high. They also showed cell survival to be highly dependent on the warming rate: a cell that has survived cooling to low sub-zero temperatures is still challenged during warming and thawing, which can exert effects on survival comparable with those of cooling (Mazur, 1984; Gao and Critser, 2000; Johnson et al., 2000). Besides, it has been reported that if a very rapid curve of temperature descent is used, the thawing should be equally fast (Cochran et al., 1984; Mazur, 1984; Gao and Critser, 2000; Johnson et al., 2000).

In the second experiment, a comparison between pregnancy rates obtained after AI of jennies in the uterine body using vitrified or conventionally frozen sperm was performed. In previous studies in other species, vitrified sperm (with and without permeable agents) has been employed for *in vitro* fertilization (**IVF**) in cat (Swanson et al., 2017), mouse (Horta et al., 2017), fish (Zilli et al., 2018) and wild goat sperm (Pradieé et al., 2018) and intracytoplasmic sperm injection in humans (Isachenko et al., 2012) and porcines (Arraztoa et al., 2017a). However, a thorough search of the literature yielded few studies in which intrauterine AI with vitrified sperm has been carried out: in human (Sanchez et al., 2012), rabbit (Rosato and Iaffaldano, 2013), wild goat (Pradiee et al., 2015) and mouflon sperm (Pradiee et al., 2016). Sanchez et al. (2012) reported the first case of children born after intrauterine insemination with vitrified semen, and Pradiee et al. (2015) obtained similar fertility outcomes using vitrified sperm as those obtained with conventionally frozen semen in earlier studies performed in wild goat (Santiago-Moreno et al., 2006). In contrast, Rosato and Iaffaldano (2013) and Pradiee et al. (2016) in their studies performed in rabbit and mouflon, respectively; reported considerably lower pregnancy rates using vitrified in comparison with fresh and/or conventionally frozen semen, but the quality of sperm after vitrification in both studies was very disappointing. Our work represents the first report in which a direct comparison using both techniques for intrauterine insemination has been performed in equine species. Results showed a trend to obtain higher pregnancy percentages using vitrified (22.2%), *versus* frozen semen (10%); but differences were not statistically different. This result is consistent with embryo production and development rates between methods after IVF in mentioned species (Horta et al., 2017; Swanson et al., 2017; Pradieé et al., 2018). In this sense, vitrified semen seems to be, at least, equally able to result in pregnancy as frozen semen is. Larger number of cycles might provide more accurate results, and possibly evince a statistically significant higher pregnancy rates using vitrified semen.

For many years, limiting factors affecting the low fertility results in jennies when cryopreserved jack semen is employed were explored. First investigations on this regard considered glycerol to impair the fertilizing ability of jack sperm, as pregnancy rates were higher after its removal (Trimeche et al., 1998). Later, Vidament et al. (2009) also reported lower pregnancy rates after insemination when glycerol was present in the AI dose, even with cooled semen, which were improved when it was substituted with other molecules. Surprisingly though, when a combination of those permeable CPAs was used (with and without glycerol presence) by Oliveira et al. (2006), no pregnancies were obtained. This was recently reviewed by Rota et al. (2012), who reported no differences between pregnancy rates using ethylene glycol and glycerol, but a tendency to obtain better results when glycerol was resuspended in SP. In that study, pregnancy rates obtained when glycerol was not resuspended with SP (23%) were similar to those obtained in this study. This lends support to the fact that the lack of SP addition in the present study may be the cause of the lower average mean pregnancy rates obtained (15.8%), in comparison to those previously reported by Rota et al. (2012) using treatments adding SP (36%). Authors decided to avoid post-thaw addition of SP before AI because

the main purpose of this study was to evaluate the influence of permeable agent presence in the insemination dose. Therefore, and according to our results, the role of glycerol in the low fertility rates obtained in jennies could not be confirmed yet.

More recent investigations focused research on semen dose and deposition site effect, in order to improve fertility outcomes in jennies. Oliveira et al. (2016) obtained highest pregnancy results when a dose of $1\,000 \times 10^6$ sperm (twice the dose employed in the present study) was deposited in the tip of the uterine horn. Still, only slightly higher pregnancy rates (28.3%) were achieved by Oliveira et al. (2016) using frozen semen in comparison to our results using vitrified semen (22.2%), even if female tract was monitored every 8 h until detection of ovulation. Nevertheless, the effect of a higher semen dose and deep horn insemination protocol in relation to fertility using vitrified semen needs to be further investigated.

A higher uterine inflammatory response after insemination with cryopreserved semen has been also considered to negatively affect fertility in jennies (Vidament et al., 2009; Miro et al., 2011). This physiologic response is necessary for uterine clearance, but the maintenance of inflammatory cells in the uterine lumen results in an undesirable environment, reducing fertility (Katila, 2012). In this sense, SP addition prior to AI has been described as a strategy to partially solve this problem by reducing the duration of inflammatory response in equine species, which has been reviewed for horses (Troedsson et al., 2001) and studied in donkeys *in vivo* (Rota et al., 2012). Although no differences in the uterine inflammatory response were found in previous studies after AI with cryopreserved donkey semen with or without SP addition (Rota et al., 2012), pregnancy rates were higher when AI dose was resuspended with SP. Similarly, in this study, we found no differences between uterine inflammatory response after insemination using vitrified (without permeable CPAs) or frozen semen; however, a significant reduction in PMN concentration between first and second insemination and subsequent flushing was found using vitrified sperm. In this sense, the inflammatory response of the uterus after insemination with frozen–thawed jack semen could be independent of the presence of permeable agents, but the inflammatory condition is shorter if there is no glycerol in the AI dose. The present findings might help to solve the difficulty of treating the endometritis in jennies after frozen–thawed jack sperm insemination, as previously described by Viles et al. (2013). Following our results, the uterus of the jenny could be able to clean itself easier after insemination if no permeable agents are present, in particular glycerol. Nevertheless, whether uterine inflammatory response and fertility were related is still not so obvious. This study is the first step toward enhancing our understanding of the relation between uterine inflammatory response in jennies and permeable CPAs presence.

In summary, a new methodology for direct warming of vitrified straws in a water bath (43 °C/10 s) has been devised, avoiding post-warming sperm centrifugation. Artificial insemination with vitrified donkey sperm promoted positive pregnancy outcomes and reduced the post breeding inflammatory response in jennies. These findings represent an excellent initial step toward to use vitrified semen as an effective alternative for artificial insemination with cryopreserved donkey semen.

Ethics approval

All the experiments were performed in accordance with the Ethical Animal Experimentation Committee of Cordoba University (Project No. 31/08/2017/105) according to the Spanish law for animal welfare and experimentation (Decision 2012/707/UE and RD 53/2013) and approved by the Ethical Committee of the University of Pisa (Prot. n. 45/2017).

Data and model availability statement

None of the data were deposited in an official repository.

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Declaration of interest

None.

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CONCLUSIONS

CONCLUSIONS

According to the objectives proposed and the results obtained, the following conclusion have been achieved:

Conclusion 1. The use of an extender containing sucrose 0.25 M combined with bovine serum albumin at 1 %, as non-permeable cryoprotectant agents, may be considered as an alternative to conventional freezing extenders for fertile donkey sperm. Although promising results were obtained regarding plasma membrane and sperm DNA integrity, it is still not an option for sperm cryopreservation of subfertile donkeys.

Conclusion 2. Donkey sperm vitrification in spheres using sucrose 0.1 M and bovine serum albumin at 5 %, enhanced sperm motility and plasma membrane integrity after warming in comparison to the use of glycerol as permeable cryoprotectant agent.

Conclusion 3. Sperm vitrification in straws has been optimized for donkey using an extender containing egg-yolk and 0.1 M sucrose, at 300 million sperm/mL in 0.25 mL straws with outer covers. This methodology preserves total and progressive motility better than the spheres method in absence of permeable cryoprotectants.

Conclusion 4. High warming rates seem to be better for donkey sperm vitrification in large volumes, but donkey sperm vitrification in 0.5 mL straws resulted in lower sperm quality parameters in comparison to conventional sperm freezing.

Conclusion 5. Sperm vitrified in 0.25 mL straws could be directly warmed in a water bath at 43 ºC during 10 s, resulting in similar sperm quality in comparison to conventional freezing. The use of vitrified semen for AI in jennies resulted in shorter uterine inflammatory response after insemination and similar pregnancy outcomes than frozen semen.

CONCLUSIONES

De acuerdo con los objetivos propuestos y los resultados obtenidos, podemos concluir los siguiente:

Conclusión 1. La adición de sacarosa a 0,25 M, en combinación con albúmina sérica bovina al 1 %, al diluyente de congelación como agentes no permeables, puede ser considerada una alternativa a los diluyentes comúnmente empleados en la congelación de esperma de asnos fértiles. Aunque los resultados de integridad de membrana plasmática y ADN fueron prometedores, la congelación en ausencia de crioprotectores permeables no es una opción para la congelación de esperma de asnos subfértiles.

Conclusión 2. La vitrificación del esperma de asno en esferas puede realizarse empleando diluyentes con sacarosa a 0,1 M y albúmina sérica bovina al 5 %, resultando en parámetros espermáticos de movimiento e integridad de la membrana plasmática superiores a los obtenidos empleando glicerol como agente permeable.

Conclusión 3. La vitrificación de esperma de asno en pajuelas ha sido desarrollada y optimizada. Ésta puede realizarse empleando un diluyente que contenga yema de huevo y sacarosa a 0,1 M; a 300 millones de espermatozoides/mL en pajuelas cubiertas de 0,25 mL. La vitrificación de esperma en pajuelas porporciona mejores resultados de movimiento tras la descongelación que la vitrificación en esferas.

Conclusión 4. Altas velocidades de calentamiento parecen ser más adecuadas para la vitrificación de esperma en grandes volúmenes, pero la vitrificación de esperma en pajuelas de 0,5 mL resultaró en parámetros espermáticos más bajos en comparación con la congelación convencional.

Conclusión 5. El esperma vitrificado en pajuelas de 0,25 mL puede ser directamente descongelado en baño de agua a 43º C durante 10 segundos,

obteniéndose una calidad espermática similar a la obtenida tras la congelación convencional. El uso de esperma vitrificado en IA de asnas provoca una endometritis que se resuelve de forma más rápida que empleando esperma congelado de forma convencional, obteniendo tasas de gestación similares.

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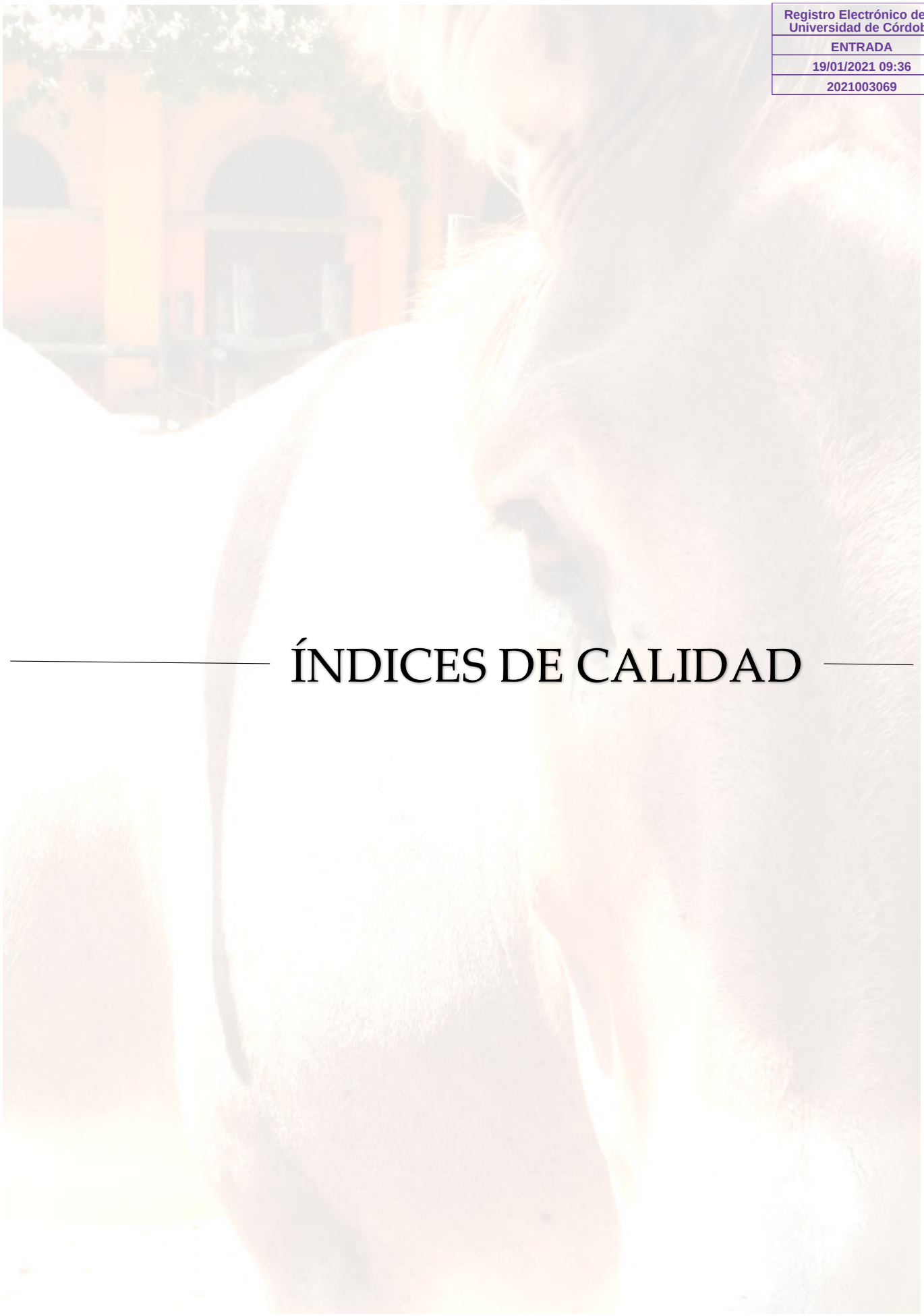

ÍNDICES DE CALIDAD

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Primera publicación

- Título: *Cryopreservation of donkey sperm using non-permeable cryoprotectants*
- Autores (p.o. de firma): *M. Diaz-Jimenez, J. Dorado, I. Ortiz, C. Consuegra, B. Pereira, C.A. Gonzalez-De Cara, R. Aguilera, G. Mari, B. Mislei, C.C. Love, M. Hidalgo*
- Revista (año, vol., pag.): *Animal Reproduction Science (2018), Vol. 189, 103-109*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports, JCR (2018)*
- Área temática en la Base de Datos de referencia: *Agriculture, Dairy and Animal Science*
- Índice de impacto de la revista en el año de publicación del Artículo: 1,817
- Lugar que ocupa/N.º de revistas del Área temática: *12/61 (Q1)*

Segunda publicación

- Título: *Is sperm cryopreservation in absence of permeable cryoprotectants suitable for subfertile donkeys?*
- Autores (p.o. de firma): *M. Diaz-Jimenez, J. Dorado, B. Pereira, C. Consuegra, I. Ortiz, M. Hidalgo*
- Revista (año, vol., pag.): *Reproduction in Domestic Animals (2019), Vol. 54, 102–105*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports, JCR (2019)*
- Área temática en la Base de Datos de referencia: *Agriculture, Dairy and Animal Science*
- Índice de impacto de la revista en el año de publicación del Artículo: 1,641
- Lugar que ocupa/N.º de revistas del Área temática: *23/63 (Q2)*

Tercera publicación

- Título: *Vitrification of donkey sperm: Is it better using permeable cryoprotectants?*
- Autores (p.o. de firma): *M. Hidalgo, M. Diaz-Jimenez, C. Consuegra, B. Pereira, J. Dorado*
- Revista (año, vol., pag.): *Animals (2020), Vol. 10, 1-11*

- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports, JCR (2019)*
- Área temática en la Base de Datos de referencia: *Veterinary Sciences*
- Índice de impacto de la revista en el año de publicación del Artículo: 2,323
- Lugar que ocupa/N.º de revistas del Área temática: *14/142 (Q1, primer decil)*

Cuarta publicación

- Título: *Effect of different extenders for donkey sperm vitrification in straws*
- Autores (p.o. de firma): *M. Diaz-Jimenez, B. Pereira, I. Ortiz, C. Consuegra, A. Partyka, J. Dorado, M. Hidalgo*
- Revista (año, vol., pag.): *Reproduction in Domestic Animals (2017), Vol. 52, 1-3*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports, JCR (2017)*
- Área temática en la Base de Datos de referencia: *Agriculture, Dairy and Animal Science*
- Índice de impacto de la revista en el año de publicación del Artículo: *1,422*
- Lugar que ocupa/N.º de revistas del Área temática: *19/60 (Q2)*

Quinta publicación

- Título: *Optimization of donkey sperm vitrification: effect of sucrose, sperm concentration, volume and package (0.25 and 0.5 mL straws)*
- Autores (p.o. de firma): *M. Diaz-Jimenez, J. Dorado, C. Consuegra, I. Ortiz, B. Pereira, J.J. Carrasco, V. Gomez-Arrones, A. Domingo, M Hidalgo*
- Revista (año, vol., pag.): *Animal Reproduction Science (2019), Vol. 204, 31-38*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports, JCR (2019)*
- Área temática en la Base de Datos de referencia: *Agriculture, Dairy and Animal Science*
- Índice de impacto de la revista en el año de publicación del Artículo: 1,660
- Lugar que ocupa/N.º de revistas del Área temática: *22/63 (Q2)*

Sexta publicación

- Título: *Vitrification in straws conserves motility features better than spheres in donkey sperm*
- Autores (p.o. de firma): *M. Diaz-Jimenez, J. Dorado, B. Pereira, I. Ortiz, C. Consuegra, M. Bottrel, E. Ortiz, M. Hidalgo*
- Revista (año, vol., pag.): *Reproduction in Domestic Animals (2018), Vol. 53, 56-58*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports, JCR (2018)*
- Área temática en la Base de Datos de referencia: *Agriculture, Dairy and Animal Science*
- Índice de impacto de la revista en el año de publicación del Artículo: *1,638*
- Lugar que ocupa/N.º de revistas del Área temática: *17/61 (Q2)*

Séptima publicación

- Título: *Effect of warming temperatures on donkey sperm vitrification in 0.5mL straws in comparison to conventional freezing*
- Autores (p.o. de firma): *M. Diaz-Jimenez, J. Dorado, C. Consuegra, B. Pereira, I. Ortiz, C. Vazquez, E. Isachenko, V. Isachenko, M. Hidalgo*
- Revista (año, vol., pag.): *Spanish Journal of Agricultural Research (2019), Vol. 17, 1-7*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports, JCR (2019)*
- Área temática en la Base de Datos de referencia: *Agriculture, Multidisciplinary*
- Índice de impacto de la revista en el año de publicación del Artículo: *1,037*
- Lugar que ocupa/N.º de revistas del Área temática: *28/58 (Q2)*

Octava publicación

- Título: *Vitrification of donkey sperm using straws as an alternative to conventional slow freezing*
- Autores (p.o. de firma): *M. Diaz-Jimenez, J. Dorado. C. Consuegra, B. Pereira, M. Hidalgo*
- Revista (año, vol., pag.): *Reproduction in Domestic Animals (2020), Vol. 00, 1-4*

- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports, JCR (2019)*
- Área temática en la Base de Datos de referencia: *Agriculture, Dairy and Animal Science*
- Índice de impacto de la revista en el año de publicación del Artículo: 1,641
- Lugar que ocupa/N.º de revistas del Área temática: *23/63 (Q2)*

Novena publicación

- Título: *First pregnancies in jennies with vitrified donkey semen using a new warming method.*
- Autores (p.o. de firma): *M. Diaz-Jimenez, A. Rota, J. Dorado, C. Consuegra, B. Pereira, F. Camillo, D. Panzani, D. Fanelli, M. Tesi, D. Monaco, M. Hidalgo*
- Revista (año, vol., pag.): *Animal Journal. Accepted on October 1st, 2020*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports, JCR (2019)*
- Área temática en la Base de Datos de referencia: *Veterinary Sciences*
- Índice de impacto de la revista en el año de publicación del Artículo*: 2,400*
- Lugar que ocupa/N.º de revistas del Área temática: *11/142 (Q1, Primer decil)*

PRODUCCIÓN CIENTÍFICA

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Otras aportaciones científicas derivadas directamente de la Tesis Doctoral:

Contribuciones a congresos Nacionales

- "Congelación de esperma de asno empleando sucrosa como crioprotector alternativo al glicerol"

M. Diaz-Jimenez, I. Ortiz, C. Gonzalez De Cara, J. Dorado, M. Hidalgo. I Congreso Veterinaria y Ciencia y Tecnología de los Alimentos. Córdoba (España), 12 febrero 2016. Tipo: Comunicación Oral.

- "Vitrificación de esperma de asno en pajuelas empleando crioprotectores no penetrantes"

M. Diaz-Jimenez. VI Congreso Doctorandos en Formación. Creando redes Doctorales Vol. VI. Córdoba (España), 12 febrero 2016. Tipo: Póster

- "Vitrificación de esperma de asno empleando diluyentes con crioprotectores no permeables"

D. Castro, **M. Diaz-Jimenez**, I. Ortiz, B. Pereira, C. Consuegra, J. Dorado, M. Hidalgo-Raya, M. Hidalgo. II Congreso de Veterinaria y Ciencia y Tecnología de los Alimentos. Córdoba (Spain). 9 febrero 2018. Tipo: Comunicación Oral.

Contribuciones a congresos Internacionales con *abstract* publicados en Revistas Indexadas en el JCR:

- "Effect of sucrose as cryoprotectant for donkey sperm cryopreservation"

M. Diaz-Jimenez, I. Ortiz, C. Gonzalez De Cara, R. Aguilera, C. Consuegra, J. Dorado, M. Hidalgo. 20th Annual Conference of the European Society of Domestic Animals Reproduction (ESDAR). Lisboa (Portugal), 27-29 octubre 2016. Tipo: Póster

Revista: Reproduction in Domestic Animals Vol. 51 (s2): 85

- "Donkey sperm vitrification as alternative to conventional freezing: preliminary results"

M. Diaz-Jimenez, B. Pereira, C. Consuegra, I. Ortiz, J. Dorado, M. Hidalgo. 21st Annual Conference of European Society of Domestic Animals Reproduction (ESDAR). Berna (Suiza), 24-26 agosto 2017. Tipo: Póster

Revista: Reproduction in Domestic Animals, Vol. 52 (s3): 80

- "Effect of different extenders for donkey sperm vitrification in straws"

M. Diaz-Jimenez, B. Pereira, I. Ortiz, C. Consuegra, A. Partyka, J. Dorado, M. Hidalgo. 14th International Conference of the Spanish Association for Animal Reproduction (AERA). Barcelona (España), 24-26 agosto 2017. Tipo: Comunicación Oral

Revista: Reproduction in Domestic Animals, Vol. 52 (s4): 1-3

- "Vitrification in straws conserves motility features better than spheres in donkey sperm"

M. Diaz-Jimenez, B. Pereira, C. Consuegra, I. Ortiz, J. Dorado, M. Hidalgo. 22nd Annual Conferenfe of European Society of Domestic Animals Reproduction (ESDAR). Córdoba (España), 27-29 septiembre 2018. Tipo: Comunicación Oral

Revista: Reproduction in Domestic Animals, Vol. 53 (s2): 56-58

- "Comparison between donkey sperm vitrification and conventional freezing using non-permeable cryoprotectants"

M. Diaz-Jimenez, B. Pereira, C. Consuegra, I. Ortiz, J. Dorado, M. Hidalgo. 23rd Annual Conference of European Society of Domestic Animals Reproduction (ESDAR). San Petersburgo (Rusia), 19-22 septiembre 2019. Tipo: Comunicación Oral

Revista: Reproduction in Domestic Animals, Vol. 54 (s3): 77

- "Is sperm cryopreservation in absence of permeable cryoprotectants suitable for subfertile donkeys?"

M. Diaz-Jimenez, B. Pereira, C. Consuegra, I. Ortiz, J. Dorado, M. Hidalgo. 15th International Conference of the Spanish Association for Animal Reproduction (AERA). Toledo (España), 7-9 noviembre 2019. Tipo: Comunicación Oral

Revista: Reproduction in Domestic Animals, Vol. 54 (s4): 102-105

Próximas contribuciones aceptadas en congresos Internacionales

- "Comparison of uterine inflammatory response of jennies after artificial insemination with vitrified or frozen-thawed donkey sperm"

M. Diaz-Jimenez, A. Rota, J. Dorado, D. Panzani, D. Fanelli, M. Tesi, F. Camillo, D. Monaco, B. Pereira, C. Consuegra, M. Hidalgo. 10th International Symposium on Equine Embryo Transfer and Technology. Pisa (Italia), 3-5 julio 2021. Tipo: Comunicación Oral

Revista: Journal of Equine Veterinary Science, Vol. 89.

- "Vitrification of donkey sperm using straws as an alternative to conventional slow freezing"

M. Diaz-Jimenez, J. Dorado, B. Pereira, C. Consuegra, M. Hidalgo. 24th Annual Conference of European Society of Domestic Animals Reproduction (ESDAR). Thessaloniki (Grecia), 12-17 octubre 2021. Tipo: Comunicación Oral

Revista: Reproduction in Domestic Animals.

Publicaciones en revistas de divulgación

- "Inseminación artificial y preparación de dosis seminales". M. Hidalgo, **M. Diaz-Jimenez**, C. Consuegra, J. Dorado. Revista EQUINUS Vol. 57 (2): 23-33.

- "Métodos de recogida de esperma en caballos". J. Dorado, C. Consuegra, **M. Diaz-Jimenez**, M. Hidalgo. Revista EQUINUS Vol. 57 (2): 8-14.

Premios

- Premio a la mejor comunicación oral en el 23rd Annual Conference of European Society of Domestic Animals Reproduction (ESDAR). San Petersburgo (Rusia), 19-22 septiembre 2019.