UNIVERSIDAD D CÓRDOBA

TESIS DOCTORAL

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OPTIMIZACIÓN DE LAS TÉCNICAS DE EVALUACIÓN Y VITRIFICACIÓN DE OVOCITOS DE ÉQUIDOS

"Optimization of equine oocyte evaluation and vitrification techniques"

Blasa del Carmen Pereira Aguilar

2021

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TITULO: *Optimization of equine oocyte evaluation and vitrification techniques*

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UNIVERSIDAD DE CÓRDOBA

FACULTAD DE VETERINARIA

Tesis Doctoral con Mención Internacional

Programa de Doctorado

Biociencias y Ciencias Agroalimentarias

OPTIMIZACIÓN DE LAS TÉCNICAS DE EVALUACIÓN Y VITRIFICACIÓN DE OVOCITOS DE ÉQUIDOS

"Optimization of equine oocyte evaluation and vitrification techniques"

Doctoranda:

Blasa del Carmen Pereira Aguilar

Directores:

Manuel Hidalgo Prieto

Jesús M. Dorado Martín

Córdoba, noviembre de 2021

FINANCIACIÓN

- La Doctoranda, durante el período de realización de su Tesis Doctoral, ha sido contratada temporal como Personal Joven Investigador con Titulación Superior Universitaria, a través del Sistema Nacional de Garantía Juvenil (Programa Operativo de Empleo Juvenil 2014-2020) y de la 2.º Convocatoria de Contratos de Investigación con cargo a Proyectos, Grupos, Contratos y Convenios de Investigación (2019) de la Universidad de Córdoba.

- La estancia realizada, para la obtención de la Mención Internacional de la Tesis Doctoral, durante tres meses en el Department of Equine Sciences of the Faculty of Veterinary Medicine of the University of Utrecht (Netherlands), fue parcialmente financiada por el Instituto de Estudios de Postgrado de la Universidad de Córdoba.

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

TÍTULO DE LA TESIS:

Optimización de las técnicas de evaluación y vitrificación de ovocitos de équidos "Optimization of equine oocyte evaluation and vitrification techniques"

DOCTORANDA: Blasa Carmen Pereira Aguilar

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

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

INFORMAN:

Que el trabajo de tesis presentado por D.ª Blasa Carmen Pereira Aguilar, titulado "Optimización de las técnicas de evaluación y vitrificación de ovocitos de équidos" ha sido realizado por la doctoranda bajo nuestra dirección y cumple con la normativa reguladora de los Estudios de doctorado de la Universidad de Córdoba para su presentación como compendio de publicaciones, así como para obtener la mención internacional.

El objetivo principal de la misma consistió en optimizar la técnica actualmente empleada para la evaluación del estado de maduración nuclear del ovocito de équido, desarrollar y optimizar una técnica de vitrificación de ovocitos de équidos, así como optimizar y desarrollar técnicas de evaluación de la viabilidad de los ovocitos. Parte de los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral han resultado en cuatro publicaciones científicas, siendo ésta elaborada por compendio de publicaciones.

Tesis por compendio de publicaciones:

1. **Blasa Pereira**, Isabel Ortiz, Jesus Dorado, Maria Diaz-Jimenez, Cesar Consuegra, Sebastian Demyda-Peyras, Manuel Hidalgo. *The Effect of Different Vitrification and Staining Protocols on the Visibility of the Nuclear Maturation Stage of Equine Oocytes*. Journal of Equine Veterinary Science (2020). 90: 103021.

2. **Blasa C. Pereira**, Isabel Ortiz, Jesus Dorado, Cesar Consuegra, Maria Diaz-Jimenez, Sebastian Demyda-Peyras, Jaime Gosalvez, Manuel Hidalgo. *Evaluation of DNA Damage of mare Granulosa Cells Before and After Cryopreservation Using Chromatin Dispersion* Test. Journal of Equine Veterinary Science (2019). 72: 28-30.

3. **Blasa Pereira**, Jesus Dorado, Maria Diaz-Jimenez, Cesar Consuegra, Isabel Ortiz, Jaime Gosalvez, Manuel Hidalgo. *Relationship between DNA fragmentation of equine granulosa cells and oocyte meiotic competence after in vitro maturation*. Reproduction in Domestic Animals (2019). 54 Suppl 4: 78-81.

4. **Blasa Carmen Pereira**, Isabel Ortiz, Jesús Manuel Dorado, Maria Ángeles Diaz-Jimenez, Cesar Consuegra, Jaime Gosalvez, Manuel Hidalgo. *Effect of permeable cryoprotectant-free vitrification on DNA fragmentation of equine oocyte-cumulus cells*. Reproduction in Domestic Animals (2019). 54 Suppl 3: 53-56.

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 Ciencia Equina (Reproducción) de la Universidad de Utrecht (Holanda).

La presente Tesis Doctoral, por compendio de publicaciones, ha sido revisada, reuniendo a nuestro juicio todos los requisitos necesarios para su lectura y defensa, 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, 10 de noviembre de 2021

Firma de los directores

Fdo.: D. Manuel Hidalgo Prieto

Fdo.: D. Jesús M. Dorado Martín

INTERNATIONAL DOCTORS REPORT
DOCTORAL THESIS

REFEREE REPORT ON THE PhD TESIS PRESENTED IN THE UNIVERSITY OF CÓRDOBA (SPAIN) BY **BLASA CARMEN PEREIRA AGUILAR**.

TITLE OF THE THESIS:	Optimization of equine oocyte evaluation and vitrification techniques

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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):

I am writing to provide my evaluation of Ms. Blasa Carmen Pereira Aguilar's doctoral thesis entitled "Optimization of equine oocyte evaluation and vitrification techniques" Efficient and robust equine reproductive biotechnologies such as in vitro oocyte maturation, cryopreservation or vitrification are greatly needed for the equine industry. The studies presented in this thesis have carefully examined the effects of different cryopreservation and vitrification protocols on equine oocyte nuclear maturation, DNA damage and fragmentation of granulosa or cumulus cells. The design of the different experiments is clear and allows a coherent response to the hypothesis of departure in each case. The thesis covers a wide range of techniques including cryopreservation, vitrification, staining, oocyte in vitro maturation, DNA analyses, among others. The data

have been analyzed with correct statistical procedures and are presented clearly. The results are brilliantly discussed and justify the conclusions of the thesis. The experiments are very well connected to each other and, all together, make it coherent and cohesive very complete and interesting. All this shows that the candidate has been able to master different techniques to develop the different investigations, which has clarified some interesting biological problems. The value of the results achieved in this doctoral thesis is evidenced by its publication in prestigious international journals of the JCR. The thesis is made up of a total of four scientific articles, published in the journals: Journal of Equine Veterinary Science (2), and Reproduction in Domestic Animals (2). The candidate is listed as first author in all articles.

In summary, this thesis contains valuable information on the improvement of critical equine reproductive biotechnologies, and therefore I strongly recommend a doctoral degree being awarded to Ms. Blasa Carmen Pereira Aguilar.

DATE:16/11/2021

SIGNATURE:

INTERNATIONAL DOCTORS REPORT
DOCTORAL THESIS

REFEREE REPORT ON THE PhD TESIS PRESENTED IN THE UNIVERSITY OF CÓRDOBA (SPAIN) BY **BLASA CARMEN PEREIRA AGUILAR**.

TITLE OF THE THESIS:	Optimization of equine oocyte evaluation and vitrification techniques

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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):

A wide variety of assisted reproductive techniques (ARTs) are available now to aid in managing aspects of equine reproduction. Oocytes can be collected from maturing or immature follicles from live mares or harvested ovaries. These oocytes can be shipped under defined conditions to a central facility for ICSI. To obtain success with these procedures, the clinician needs to be knowledgeable in the proper methods to collect, identify, and handle oocytes. Therefore, the improvement of techniques to identify developmental competence of oocytes seems to be of the greatest importance for subsequent production of embryos.

Additionally, efficient methods for vitrification of equine oocytes are also important for conservation and shipment of genetic material at any convenient time. Vitrification as a cryopreservation method bases on ultra-rapid freezing rates using high concentrations of cryoprotectant agents which prevent the formation of intracellular ice crystals. This technique is widely used on human oocytes with obtaining live offspring, however in horses producing extremely low blastocyst rates after ICSI. Therefore, establishing methods for equine oocyte

vitrification would be a great benefit to the equine industry, for both clinical and research purposes.

In horses, the high amount of lipid compounds in oocytes makes the assessment of their nuclear maturation stage difficult. To overcome this obstacle, DNA-specific fluorescent labels are used, making the nuclear chromatin visible without the removal of the cytoplasmic lipids.

The present work discusses these mentioned most current aspects related to ART in horses. This thesis proposes a new approach of oocyte staining protocol combining low Hoechst 33342 concentration with propidium iodide to optimize the oocyte nuclear chromatin visualization maintaining suitable nuclear chromatin fluorescence intensity as well as reducing autofluorescence background. Moreover, it was found that DNA fragmentation analysis of equine granulosa cells can be a valuable test to identify equine oocytes showing the best meiotic competence after in vitro maturation; and vitrification only with sucrose cannot be considered as alternative to conventional vitrification solution for equine oocytes.

1. Format

The thesis is clear and well organized. The title and abstract reflect the content and the introduction clearly states the problem being investigated and provides an adequate background. The present thesis consists: summary, introduction, objectives, three chapters with published papers (one with two publications), conclusions, references, quality indexes and other scientific contributions derived directly from the Doctoral Thesis.

Despite the lack of a general discussion, each paper contains discussion section that strongly support the results collected from the different experiments carried out. It would be very difficult to construct one compact discussion for each experiment performed in these four papers.

2. Methodology

The materials & methods in each paper are described in detail allowing repeat such experiments. Methods used in this thesis includes: staining with Hoechst 33342 and propidium iodide to evaluate oocyte nuclear chromatin and their maturation stage; and chromatin dispersion assay with using prototype D3-MAX or Ovoselect kit for DNA damage of granulosa cell.

Statistical procedures were adequate for data analysis from each experiment. There were used chi-squared analysis for comparison of visibility grades and nuclear maturation stages among staining protocols and vitrification systems; analysis of variance for comparison of fragmentation DNA in granulosa cells before and after cryopreservation; ANOVA and Duncan test for evaluation of differences in DNA fragmentation of granulosa cells between levels of oocyte maturation; and Student's test for comparison of differences in DNA fragmentation rates before and after oocyte vitrification in the absence of permeable cryoprotectant agents.

3. Publications

This thesis includes three thematic chapters, which each consists of valuable results.

Chapter 1st and 3rd contains one publication and chapter 2nd is divided into 2 subsections with 1 publication each. The division is very logical and corresponds to three presented objectives.

In the first paper authors recommend a staining protocol with a low concentration of Hoechst 33342 to evaluate the nuclear chromatin stage of equine oocytes after in vitro maturation. Moreover, for the first time in horse oocytes was assessed the efficiency of permeable cryoprotectant-free vitrification protocol, indicating that nonpermeable cryoprotectants are not suitable for oocyte vitrification in mares.

In the second article the evaluation of the four cryopreservation protocols combining different storage temperatures or cryoprotectants on granulosa cells DNA damage was performed showing that in any of these protocols DNA is adequately preserved for further analysis.

The third publication presents very important results confirming that DNA status of granulosa cells represents a valuable indicator of oocyte competence thereby reflecting oocyte quality.

The last publication concerns the subject of the effect of absence of permeable cryoprotectant vitrification on equine cumulus cells and shows that vitrification with non-permeable agents was able to protect the cumulus cells from the chromatin dispersion pattern of high fragmented DNA, but not from low DNA fragmentation.

4. Scientific value

The journals where the manuscripts have been published are recognized as having high scientific reputation. Reproduction in Domestic Animals is the official organ of the European Society for Domestic Animal Reproduction (ESDAR), the European Veterinary Society of Small Animal Reproduction (EVSSAR) and the Spanish Society of Animal Reproduction. This journal belongs to leaders journals on the field of animal reproduction with high impact and citation indices. Journal of Equine Veterinary Science is an international journal designed for the practicing equine veterinarians, equine researchers and other equine health care specialists with high range. Therefore, all journals confirm a high scientific relevance of the presented thesis. Additionally, many posters and oral presentations shown during national and international conferences also confirm the significant scientific value of presented work.

For all the evidence provided, I can strongly recommend the PhD candidate, Blasa Carmen Pereira Aguilar, for the PhD degree.

DATE: 18.11.2021 **SIGNATURE:** Agnieszka Partyka

European/International Mention in the Doctorate Degree

Certificate of stay

I hereby confirm that Mrs Blasa Carmen Pereira Aguilar has steadily stayed at the Department of Equine Sciences of the Faculty of Veterinary Medicine of the Utrecht University, Netherlands from 01/05/2019 to 31/07/2019 and has successfully developed research activities in equine embryo evaluation and clinical cases, that is directly related with her PhD thesis in evaluation and vitrification of equine oocytes.

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Signed in Utrecht, 31 of July, 2019

AGRADECIMIENTOS

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El desarrollo de esta Tesis Doctoral no podría catalogarla como algo sencillo, lo que sí puedo es afirmar que durante estos cuatro años he podido disfrutar de la mejor compañía posible, de rodearme de gente maravillosa, que siempre me ha apoyado (sobre todo en los momentos más difíciles) y ha confiado en mí desde el primer momento. Por todo ello, me gustaría mi más sincero agradecimiento a:

- A mi director Manuel Hidalgo, por haber sido un pilar fundamental durante todos estos años. Gracias por haber sido no solo guía en este arduo camino, si no también por ser familia y preocuparte por mí en todo momento. Gracias tener siempre propuestas, planes, proyectos…en definitiva, solución para todo. Jamás podré agradecer todo el esfuerzo e infinita paciencia que siempre has mostrado para que aprendiera a redactar, las eternas e interminables correcciones que siempre acababan en textos borrados… hasta que por fin leí la frase que más ilusión me ha podido hacer en todos estos años: "me gusta cómo has orientado la introducción". Gracias por tener siempre palabras de apoyo, confiar en todos nosotros y contar con nosotros para absolutamente todo y mandarnos mil oportunidades para continuar formándonos. Gracias por haberme convertido en quien hoy soy.
- A mi director Jesús Dorado, por mostrarme un corazón enorme. Gracias por haber contado conmigo para cada proyecto y haberme hecho sentir un componente más del grupo desde el primer momento. Por la confianza que has depositado en mí durante estos años (sobre todo con los embriones). Gracias por estar siempre disponible tras la puerta de tu despacho y tras la pantalla de tu ordenador, respondiendo inmediatamente a todas y cada una de las dudas que te he preguntado, prestando tu ayuda y guiándome a lo largo de los años. Gracias también por tu esfuerzo en enseñarme a redactar (he sido bastante dura en captar la idea). Gracias por inculcarnos orden, limpieza y el ser meticulosos tanto en el laboratorio como en la vida. En definitiva, gracias por todas as enseñanzas y apoyo que me has brindado durante estos años.

Me alegro de haberos tenido a ambos como directores y como familia.

- A toda mi familia, a mis tíos y primos por todo su apoyo. Gracias en especial a mi madre y a mi padre. Por haberme apoyado incondicionalmente y sin dudarlo en todas y cada una de mis decisiones desde que empecé a tomarlas. Gracias por haberme inculcado la importancia de la lucha por conseguir mis objetivos, por enseñarme que todo esfuerzo tiene su recompensa y por estar siempre a mi lado. A mi madre por haberme escuchado ("y soportado") con infinita paciencia durante estos años de tesis, y a mi padre, que desde esa estrella mágica, he sentido que me has guiado, apoyado y alentado de una forma única y especial, sintiendo y disfrutando cada palabra como si me la dijeras apretándome fuerte la mano. A mi chon, cómplice de travesuras y enemigo de batallas durante toda la infancia. Gracias por estar siempre sin pedirlo y por mantenerte tan cerca aún en la distancia, mi teniente.

- A mis compañeros de vida durante estos años,

  A Isa, por haberme dirigido y guiado en el maravilloso y laborioso mundo "ovocitario", por haberme acompañado en cada viaje a por material poniendo las calles y por enseñarme el delicado manejo que un ovocito necesita (incluso en fin de semana). Gracias de corazón por por tener mejores respuesta que "google", por convertirte en una amiga y fiel contrincante de padel.

  A mi pollo (mari). Por comenzar teniendo siempre buena predisposición para ayudarme en cada momento, y por acabar teniendo parte de mi corazón. No puedo estar más agradecida de que el doctorado me pusiera en tu camino. Gracias por cada punto de vista que me has dado de la vida y por haber sido (y seguir siendo) una hermana para mí.

  A César, compañero desde los 18 años. Haber coincidido en el doctorado me ha permitido conocer la gran persona currante que eres. Gracias por tu infinita disponibilidad para todo tipo de ayuda que haya necesitado, por mostrar siempre entusiasmo y aptitud positiva tras la gran cantidad de horas que hemos compartido.

- A Fran, por haberme apoyado desde el primer momento. Gracias por saber manejar a la perfección mi estrés, por hacerme más llevadero los momentos más difíciles, consiguiendo hacerme desconectar de la tesis y de las entregas.
- A mis amigos, en especial a Isa Lalay, David Jimena, Álvaro, Cristina, Carmen, María, Lorena, Isa, por sacar lo mejor de mí y dar los mejores consejos de ánimo y apoyo, aún sin conocer mucho de eso de la "vitrificación". A Eli, mi arquitecta y diseñadora gráfica y de portada y de todo lo que te haya podido pedir, siempre dispuesta a tenderme tu mano independientemente del momento y de la hora, por ser tan parecidas de carácter y por apoyarnos tanto en la ambición que siempre ponemos en nuestros objetivos de vida. Muchas gracias a todos.
- A mi nuevo equipo de trabajo, por animarme y apoyarme tanto en estos últimos meses de tesis combinado con trabajo. En especial a Irene, mi flaca "larguirucha". Gracias por estar a mi lado tan bien como lo haces, por escucharme y animarme en todo, incluso en nuestros "fin de semana de la muerte" (y por supuesto por la ayuda como estilista para la defensa de la tesis). Gracias por aparecer en mi vida.
- Al Dr. Sebastián Demyda-Peyrás, por haberme ayudado siempre que lo he necesitado, desde las revisiones para la primera publicación hasta análisis de ADN de hermafroditas. Gracias por ser una gran persona y tratarme siempre con cercanía y respeto.
- A Fani, nuestra infalible técnico de laboratorio. Gracias por la ayuda, por tu permanente buen estado de ánimo, siempre con una sonrisa en la cara, y por tus ganas de trabajar y entusiasmo, ha hecho que el tiempo que estuviste con nosotros fuera mucho más ameno.
- Thanks to Dr. Juan Cuervo-Arango, Dr. Tom Stout, Dr. Anthony Claes, Dr. Marta de Ruijter-Villani and all the team of the research and clinical group of equine reproduction of Service Equine Sciences. Thanks for letting me be a part of your team and for all you have taugh me during my stay in Urtecht. It has been an honor for me to work with you all.
- Thanks to Dr. Agniezska Partyka, for all her valuable help with my english during her stay in Cordoba. You were one of us.
- Thank Ms Thuel for the beautiful photo that illustrates the cover.

- A HALOTECH DNA, en particular a Jaime Gosálvez, por haberme facilitado los medios y la inestimable ayuda de Paqui para poner a punto la técnica de la fragmentación del ADN de células de esta tesis doctoral.
- A la empresa de Miguel, por su colaboración al cedernos el material para llevar a cabo los experimentos de esta tesis. Sin su ayuda esta tesis no hubiera sido posible.
- Gracias a la Universidad de Córdoba por la "Ayuda de Movilidad Internacional para la obtención de la Tesis con Mención Internacional.
- A todas esas personas que han formado parte de mi vida durante estos años de tesis doctoral.

ÍNDICE

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SUMMARY

SUMMARY

Equine oocyte cryopreservation is an assisted reproductive technology that allows to preserve female genetic material from valuable mares. The main objective of this thesis was to optimize the evaluation and vitrification of equine oocyte. In chapter 1, two concentrations of the fluorochrome Hoechst 33342 (HO) were compared to assess the nuclear chromatin stage of the oocyte after vitrification using nonpermeable and permeable cryoprotectant agents (CPAs). A higher percentage of oocytes showing upper visibility grade was found when using a lower concentration of fluorescent labels. The assessment of a vitrification solution free of permeable CPAs was performed for the first time in equine oocytes. Unfortunately, no cryoprotectant-free-vitrified oocytes reached metaphase II after warming, therefore cryoprotectant-free vitrification cannot be considered an alternative to conventional equine oocyte vitrification. Chapter 2 was designed to evaluate the DNA fragmentation of equine granulosa cells (GCs) as a biomarker to evaluate the developmental competence of the equine oocyte after cryopreservation. The use of cryopreservation protocols combining different storage temperatures (-80ºC/-196ºC) and CPAs (ethylene glycol or dimethyl sulfoxide), adequately preserved the DNA of equine GCs. The chromatin dispersion test was found as a reliable method to assess DNA fragmentation in equine GCs. The percentage of GCs-DNA fragmentation was higher in GCs from oocytes able to mature. In chapter 3, the effect of permeable cryoprotectant-free vitrification was evaluated on DNA fragmentation of equine cumulus cells. Low and total fragmentation rates of vitrified equine cumulus cells were higher when compared to control group. The use of sucrose as CPA increase the total DNA fragmentation rates of equine cumulus cells but protected them against high DNA fragmentation rates during equine oocyte vitrification. In conclusion, according to the results obtained in this Doctoral Thesis; a low concentration of Hoechst 33342 is recommended to assess the nuclear chromatin stage of equine oocytes; DNA fragmentation analysis of equine granulosa cells can be a valuable test to identify equine oocytes showing the best meiotic competence after in vitro maturation; and cryoprotectant-free vitrification cannot be considered an alternative to conventional vitrification solution for equine oocytes.

RESUMEN

La criopreservación de ovocitos equinos es una técnica de Reproducción Asistida que permite preservar el material genérico de yeguas valiosas. El objetivo de la presente tesis fue optimizar las técnicas de evaluación y vitrificación de ovocitos equinos. En el capítulo 1, se compararon dos concentraciones del fluorocromo Hoechst 33342 (HO) para evaluar el estado de la cromatina nuclear del ovocito tras la vitrificación usando agentes crioprotectores (CPAs) permeables y no permeables. Empleando menores concentraciones de sustancias fluorescentes, se obtuvo un mayor porcentaje de ovocitos mostrando mayor grado de visibilidad. Medios de vitrificación sin CPAs permeables se han empleado por primera vez en ovocitos equinos. Desafortunadamente, ningún ovocito consiguió alcanzar el estado de metafase II tras el calentamiento en ausencia de crioprotectores permeables, por lo que la vitrificación sin CPAs permeables no puede ser considerada una alternativa a la vitrificación convencional de ovocitos. El capítulo 2 fue diseñado para evaluar la fragmentación del ADN de las células de la granulosa (GCs) como biomarcador para el desarrollo de competencia de los ovocitos de équidos tras la crioconservación. El uso de cualquier protocolo de crioconservación combinando diferentes temperaturas (-80ºC/-196ºC) y CPAs (etilenglicol y dimetilsulfóxido) preservó adecuadamente el ADN de las GCs de équidos. El test de la dispersión de la cromatina resultó en una técnica válida para evaluar el ADN de las GCs de ovocitos equinos. Los ovocitos que consiguieron madurar mostraron mayor fragmentación del ADN de las GCs. En el capítulo 3, se evaluó el efecto de la vitrificación en ausencia de CPAs permeables sobre la fragmentación del ADN de las células del cúmulo de ovocitos equinos. La tasa de fragmentación baja y total de las células del cúmulo del ovocito equino fue mayor cuando se comparó con el grupo control. El uso de medios de vitrificación compuestos por sacarosa como único CPA, aumenta la tasa de fragmentación total del ADN de las células del cúmulo, pero las protege frente a la alta tasa de fragmentación del ADN durante la vitrificación de ovocitos equinos. En conclusión, de acuerdo a los resultados obtenidos en esta Tesis Doctoral; una concentración baja de fluorocromo Hoechst 33342 es recomendada para evaluar el estado nuclear de la cromatina de ovocitos equinos; el análisis de la fragmentación del ADN de las células de la granulosa de équidos puede ser

un método valioso para identificar ovocitos equinos mostrando la mejor competencia meiótica tras ser madurados *in vitro*; la vitrificación sin agentes crioprotectores permeables no puede ser considerada una alternativa a la vitrificación convencional.

INTRODUCTION

INTRODUCTION

Nowadays, there is an extensive interest in assisted reproductive techniques (ARTs) in equine species. A wide variety of ARTs are available to aid in managing different aspects of equine reproduction. For example, oocyte recovery and transfer to the oviduct of inseminated mares, or intracytoplasmatic sperm injection (ICSI), embryo culture and subsequent transfer or even nuclear transfer (cloning), are well-established equine ARTs (Hinrichs 2018). Moreover, the creation of oocyte banks to indefinitely preserve valuable genetics of mares is advisable.

In this sense, establishing methods for equine oocyte vitrification would be a great benefit to the equine industry, for both clinical and research purposes (Canesin *et al.* 2017). The cryopreservation of oocytes from mares would allow conservation and shipment of the genetics in competition mares, younger mares prior to age-associated changes in oocyte viability (Carnevale & Ginther 1995), in valuable mares after unexpected death, delaying stallion selection at any convenient date, or in endangered equids (Smits *et al.* 2012). However, vitrification of unfertilized equine oocytes still has low success (Hinrichs 2018).

Vitrification is a cryopreservation method based on ultra-rapid freezing rates using high concentrations of cryoprotectant agents (CPAs) which prevent the formation of intracellular ice crystals (Fuku *et al.* 1995; Vajta 2000). This technique has been widely performed on human oocytes, in which live offspring have been obtained from cryopreserved oocytes more than 15 years ago (Yoon *et al.* 2003; Kuwayama *et al.* 2005; Kuwayama 2007). In horses, oocyte vitrification has been successfully used, but producing extremely low blastocyst rates after ICSI (Canesin *et al.* 2017; Canesin *et al.* 2018). Moreover, the first live foaling using this technology in horses was reported only a few years ago (Ortiz-Escribano *et al.* 2018).

It is well known that the high concentrations of permeable CPAs required for vitrification are toxic for oocytes (Yamada *et al.* 2007; Szurek & Eroglu 2011). In horses, several studies have been performed combining permeable and nonpermeable CPAs to develop an optimal equilibrium between the protective effect and the chemical toxicity level (Rosati *et al.* 2006). In this sense, it has been shown that a reduction in the exposure time of equine oocytes to high

concentrations of permeable CPAs before vitrification (<2 minutes) yielded improve maturation and blastocyst rates after ICSI (Tharasanit *et al.* 2006). However, the overall success of equine oocyte vitrification, combining permeable and non-permeable CPAs, resulted in low embryo production rates (Canesin *et al.* 2018; Ortiz-Escribano *et al.* 2018).

A relatively new vitrification technique, avoiding permeable CPAs, has been recently developed during the last years in sperm from different species, including horses or donkeys (Diaz-Jimenez *et al.* 2018; Consuegra *et al.* 2019), and also in mouse oocytes and embryos, obtaining satisfactory percentages of morphologically normal oocytes developing to 2-cell embryos after *in vitro* fertilization (Jin & Mazur 2015). However, this permeable cryoprotectant-free vitrification has not yet been explored in mares, so the first step towards developing a vitrification protocol for equine oocyte would be the comparison between permeable and non-permeable CPAs for oocyte vitrification.

The identification of oocyte nuclear chromatin stage, using staining techniques is a common method to assess the oocyte maturation stage in mares. This procedure is used following different ARTs, such as oocyte vitrification. Unfortunately, the high amount of lipid compounds in the equine oocyte makes the assessment of their nuclear maturation stage difficult (Ambruosi *et al.* 2009). To overcome this hindrance, DNA-specific fluorescent labels are used, making the nuclear chromatin visible without the removal of the cytoplasmic lipid (Hinrichs *et al.* 1993). In horses, permeable, and impermeable fluorescent probes, such as such as Hoechst 33342 (HO) and propidium iodide (PI) respectively, have been used to assess the nuclear maturation status of oocytes with acceptable results (Hinrichs *et al.* 2005; Vernunft *et al.* 2013). However, the differences in their absorption rates can modify the intensity of the fluorescence emitted (Darzynkiewicz 2011). Consequently, a fine optimization of fluorescent probe concentrations and combinations, as well as the temperature and incubation time, is important to assess properly the maturation stage of stained equine oocytes.

In this sense, we proposed a new approach of oocyte staining protocol combining low HO concentration with PI to optimize the oocyte nuclear chromatin visualization. We hypothesized that this HO-PI combination would

reach a suitable nuclear chromatin fluorescence intensity as well as a reduced HO-autofluorescence background.

The development of techniques to identify meiotic and developmental potential of immature oocytes is of the utmost importance for subsequent production of embryos after *in vitro* fertilization (Ruvolo *et al.* 2013). Several studies have been focused on identifying equine oocyte characteristics associated with meiotic competence and cytoplasmic maturation (Del Campo *et al.* 1995); however, somatic cells within the follicular environment (granulosa and cumulus cells) play also an important role in the development of oocytes (Del Collado *et al.* 2018). In this sense, these cells are commonly stored to perform the DNA analysis in a later time when instantaneous evaluation is unfeasible. The effect of cryopreservation on DNA stability of reproductive tissue have been reported not only in ovarian tissue (Maffei *et al.* 2013; Fabbri *et al.* 2016), but also in somatic cells of human beings and animal species (Lindley *et al.* 2001; Dell'Aquila *et al.* 2003; Jiang *et al.* 2010), including the use of CPAs and different temperatures of storage (-80ºC/-196ºC).

On the other hand, a modified chromatin dispersion test (prototype D3-MAX; Ovoselect, Halotech DNA SL, Madrid, Spain) has been shown as a reliable, fast and reproducible method to assess DNA damage in human cumulus cells (Barcena *et al.* 2015). However, this chromatin dispersion test has not been tested in equine GCs.

A critical step in oocyte vitrification is the assessment of oocyte competence to determine the viability for subsequent embryo production. In this sense, the quality of the oocyte is largely dependent on its follicular environment (Hunter *et al.* 2005; Thomas & Vanderhyden 2006). It is well known that folliculogenesis requires a carefully orchestrated cross talk between the oocyte and the surrounding somatic cells through gap junctions (Uyar *et al.* 2013). During this process, granulosa cells (GCs) surrounding the oocyte proliferate and form multiple layers of somatic cells that surround the oocyte. Under influence of follicle stimulating hormone (FSH), which stimulates follicular growth (Fritz & Speroff 2011), preovulatory (also called antral or late antral) follicle is formed. Inside this follicle, the oocyte is surrounded by a specialized type of GCs, called cumulus cell, distinct from the mural GCs that line the antrum (Diaz *et al.* 2007). When preovulatory follicle is activated, cumulus cells undergo cumulus

expansion, which is the final stage of follicular development (Uyar *et al.* 2013). In this sense, the morphology of cumulus cells, which are surrounding immature oocytes, is related to equine follicle viability; equine oocytes originating from viable follicles are surrounded by compact cumuli while those oocytes obtained from follicles with different stages of atresia are surrounded by expanded cumuli (Hinrichs & Williams 1997). However, this morphology criteria of cumulus cells are not completely reliable considering that oocytes from early atresia follicles may be surrounded by compact or expanded cumuli. Furthermore, one of the features of follicular atresia is the fragmentation of GCs DNA into oligonucleosomal DNA fragments. Thus, the DNA status of somatic cells represents a potentially valuable indicator of oocyte competence which has been proposed as a useful non-invasive biomarker for oocyte quality evaluation in different ARTs in animals and human beings (Dell'Aquila *et al.* 2003; Uyar *et al.* 2013). Hence, the evaluation of the relationship between DNA fragmentation of GCs, using the chromatin dispersion test, and equine oocyte meiotic development after *in vitro* maturation, would be a challenging area in the field of equine oocyte cryopreservation in order to find the most appropriate non-invasive biomarker for oocyte quality for this species.

Last but not least, the limited information about the effect of permeable cryoprotectant-free vitrification cumulus cells as well as the vital role of cumulus cells for immature oocyte and the effect of equine oocyte vitrification using non-permeable CPAs must be determined in equine species.

OBJECTIVES

OBJECTIVES

The main objective of this Doctoral Thesis was to optimize the evaluation and vitrification techniques for equine oocyte. To achieve this goal, the following specific objectives were addressed:

Objective 1. To compare two staining protocols to assess the nuclear chromatin stage of equine oocytes after vitrification using permeable and nonpermeable cryoprotectants.

This objective has been addressed in **chapter 1**, which has been focused on the examination of different concentrations of fluorescent labels to assess the nuclear chromatin stage of equine oocyte after *in vitro* maturation; and to evaluate the effect of vitrification using permeable and nonpermeable cryoprotectants on the nuclear maturation stage of equine oocytes.

Blasa Pereira, Isabel Ortiz, Jesus Dorado, Maria Diaz-Jimenez, Cesar Consuegra, Sebastian Demyda-Peyras, Manuel Hidalgo. The Effect of Different Vitrification and Staining Protocols on the Visibility of the Nuclear Maturation Stage of Equine Oocytes. Journal of Equine Veterinary Science (2020). 90: 103021.

Objective 2. To evaluate the DNA fragmentation of equine granulosa cells as a biomarker for developmental competence of the equine oocyte.

This objective has been addressed in chapter 2, which includes:

Chapter 2.1. To compare the effect of four cryopreservation protocols combining different storage temperatures (-80ºC/-196ºC) or cryoprotectants (ethylene glycol and dimethyl sulfoxide) on the DNA damage of granulosa cells using the chromatin dispersion test.

Blasa C. Pereira, Isabel Ortiz, Jesus Dorado, Cesar Consuegra, Maria Diaz-Jimenez, Sebastian Demyda-Peyras, Jaime Gosalvez, Manuel Hidalgo. Evaluation of DNA Damage of mare Granulosa Cells Before and After Cryopreservation Using Chromatin Dispersion Test. Journal of Equine Veterinary Science (2019). 72: 28-30.

Chapter 2.2. To assess the relationship between the DNA fragmentation of granulosa cells, using the chromatin dispersion test, and the meiotic development of equine oocytes after *in vitro* maturation.

Blasa Pereira, Jesus Dorado, Maria Diaz-Jimenez, Cesar Consuegra, Isabel Ortiz, Jaime Gosalvez, Manuel Hidalgo. Relationship between DNA fragmentation of equine granulosa cells and oocyte meiotic competence after in vitro maturation. Reproduction in Domestic Animals (2019). 54 Suppl 4: 78-81.

Objective 3. To evaluate the effect of permeable cryoprotectant free-vitrification on DNA fragmentation of equine cumulus cells.

This objective has been addressed in **chapter 3**, which was designed to compare DNA fragmentation of equine cumulus cells before or after oocyte vitrification, using sucrose as cryoprotectant agent.

Blasa Carmen Pereira, Isabel Ortiz, Jesús Manuel Dorado, Maria Ángeles Diaz-Jimenez, Cesar Consuegra, Jaime Gosalvez, Manuel Hidalgo. Effect of permeable cryoprotectant-free vitrification on DNA fragmentation of equine oocyte-cumulus cells. Reproduction in Domestic Animals (2019). 54 Suppl 3: 53-56.

OBJETIVOS

El objetivo general de la presente Tesis Doctoral fue optimizar las técnicas evaluación y vitrificación de ovocitos de équidos,. Para la consecución de este objetivo general, se plantearon los siguientes objetivos específicos:

Objetivo 1. Comparar dos protocolos de tinción para evaluar el estado de dispersión de la cromatina nuclear de ovocitos de équidos tras ser vitrificados usando crioprotectores permeables y no permeables.

Este objetivo ha sido abordado en el **capítulo 1**, que pretendió examinar el efecto de diferentes concentraciones de sondas fluorescentes para evaluar el estado de la cromatina nuclear de los ovocitos de équidos tras ser sometidos a maduración *in vitro*; y evaluar el efecto de la vitrificación en el estado de maduración nuclear de ovocitos de équidos, empleando crioprotectores permeables y no permeables.

Blasa Pereira, Isabel Ortiz, Jesus Dorado, Maria Diaz-Jimenez, Cesar Consuegra, Sebastian Demyda-Peyras, Manuel Hidalgo. The Effect of Different Vitrification and Staining Protocols on the Visibility of the Nuclear Maturation Stage of Equine Oocytes. Journal of Equine Veterinary Science (2020). 90: 103021.

Objetivo 2. Evaluar la fragmentación del ADN de células de la granulosa como biomarcador de la competencia para el desarrollo de ovocitos de équidos.

Este objetivo ha sido abordado en el **capítulo 2**, que incluye:

Capítulo 2.1. Comparar el efecto de cuatro protocolos de crioconservación, mediante la combinación de diferentes temperaturas de almacenamiento (-80ºC/-196ºC) o crioprotectores (etilenglicol o dimetilsulfóxido) en el daño del ADN de las células de la granulosa usando el test de dispersión de la cromatina.

Blasa C. Pereira, Isabel Ortiz, Jesus Dorado, Cesar Consuegra, Maria Diaz-Jimenez, Sebastian Demyda-Peyras, Jaime Gosalvez, Manuel Hidalgo. Evaluation of DNA Damage of mare Granulosa Cells Before and After Cryopreservation Using Chromatin Dispersion Test. Journal of Equine Veterinary Science (2019). 72: 28-30.

Capítulo 2.2. Evaluar la relación entre la fragmentación del ADN de las células de la granulosa, usando el test de dispersión de la cromatina, y el desarrollo de

competencia meiótica de ovocitos de équidos tras ser sometidos a maduración *in vitro*.

Blasa Pereira, Jesus Dorado, Maria Diaz-Jimenez, Cesar Consuegra, Isabel Ortiz, Jaime Gosalvez, Manuel Hidalgo. Relationship between DNA fragmentation of equine granulosa cells and oocyte meiotic competence after in vitro maturation. Reproduction in Domestic Animals (2019). 54 Suppl 4: 78-81.

Objetivo 3. Evaluar el efecto de la vitrificación sin crioprotectores permeables en la fragmentación del ADN de las células del cúmulo de ovocitos de équidos.

Este objetivo ha sido abordado en el **capítulo 3**, que fue diseñado para comparar la fragmentación del ADN de células del cúmulo de ovocitos de équidos antes y después de la vitrificación, usando sacarosa como agente crioprotector.

Blasa Carmen Pereira, Isabel Ortiz, Jesús Manuel Dorado, Maria Ángeles Diaz-Jimenez, Cesar Consuegra, Jaime Gosalvez, Manuel Hidalgo. Effect of permeable cryoprotectant-free vitrification on DNA fragmentation of equine oocyte-cumulus cells. Reproduction in Domestic Animals (2019). 54 Suppl 3: 53-56.

CHAPTERS

CHAPTER 1

Effect of permeable cryoprotectant-free vitrification on DNA fragmentation of equine oocyte-cumulus cells

B. Pereira et al., 2019. Reproduction in Domestic Animals

Journal of Equine Veterinary Science 90 (2020) 103021

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Original Research

The Effect of Different Vitrification and Staining Protocols on the Visibility of the Nuclear Maturation Stage of Equine Oocytes

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ARTICLE INFO

Article history:
Received 16 December 2019
Received in revised form
1 April 2020
Accepted 2 April 2020
Available online 10 April 2020

Keywords:
Cryoprotectant
Cryopreservation
Mare
Meiotic competence
Hoechst 33342

ABSTRACT

In this study, we compared two staining protocols assessing the nuclear chromatin stage of equine oocytes after vitrification using permeable and nonpermeable cryoprotectants. Slaughterhouse-derived oocytes (n = 155) were obtained from a total of 32 mares and in vitro matured in M199 medium for 42 hours at 38.5°C in 5% CO_2. In the first experiment, two concentrations of Hoechst 33342 (HO) were tested (10 μg/mL; P1 and 2.5 μg/mL; P2) combined with 50 μg/mL of propidium iodide as staining protocols to evaluate the visibility of matured oocytes (n = 44). In the second experiment, 111 oocytes were evaluated using the staining protocol P2, before (C, control) and after vitrification following a two-step conventional protocol with (15% dimethyl sulfoxide, 15% ethylene glycol, and 0.5 M sucrose; V1) or without (1 M sucrose; V2) using permeable cryoprotectants. Our results showed that P2 provided a higher percentage of oocytes with outstanding visibility of the nuclear chromatin stage (52.17%; $P < .05$) in comparison with P1 (19.04%). In the second experiment, no cryoprotectant-free vitrified oocytes reached the metaphase II maturation stage. This result was significantly lower ($P < .05$) than conventional vitrification (15.38%) and both lower in comparison with the nonvitrified control group (42.11%). In conclusion, permeable cryoprotectant-free vitrification of equine oocytes obtained poor results and therefore cannot be considered an alternative to vitrification using permeable cryoprotectants. In addition, a staining protocol with a low concentration of HO is recommended to evaluate the nuclear chromatin stage of equine oocytes after in vitro maturation.

1. Introduction

Vitrification is a cryopreservation method based on ultra-rapid freezing rates using high concentrations of cryoprotectant agents which prevent the formation of intracellular ice crystals [1,2]. This technique has been widely performed on human oocytes, in which live offspring have been obtained from cryopreserved oocytes more than 15 years ago [3–5]. In horses, oocyte vitrification has been successfully used, but producing extremely low blastocyst rates after intracytoplasmic sperm injection [6,7]. Moreover, the first live foaling using this technology in horses was reported only two years ago [8]. It is well known that the high concentrations of permeable cryoprotectants required for vitrification are toxic for oocytes [9,10]. In horses, several studies have been performed combining permeable and nonpermeable cryoprotectants to develop an optimal equilibrium between the protective effect and the chemical toxicity level [11]. But also, it was shown that a reduction in the exposure time of equine oocytes (<2 minutes) to high concentrations of permeable cryoprotectants before vitrification yielded improved results [7,8,12]. Recently, a new strategy to avoid permeable cryoprotectants has been developed on mice oocytes, using nonpermeable cryoprotectants (such as sucrose and ficoll) as

Animal welfare/ethical statement: This study was 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 and Decision, 2012/707/UE).

Conflict of interest statement: None of the authors have any conflict of interest to declare.

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https://doi.org/10.1016/j.jevs.2020.103021
0737-0806/

sole agents, reporting high survival rates [13]. However, this permeable cryoprotectant-free vitrification has not yet been explored in equines.

The staining of nuclear chromatin is a common method to assess the oocyte maturation stage in mares. This procedure is used following different assisted reproduction techniques, such as oocyte vitrification. Unfortunately, the high amount of lipid compounds in the equine oocyte makes the assessment of their nuclear maturation stage difficult [14]. To overcome this hindrance, DNA-specific fluorescent labels are used, making the nuclear chromatin visible without the removal of the cytoplasmic lipid [15]. In horses, permeable, such as Hoechst 33342 (HO) and impermeable fluorescent probes, such as propidium iodide (PI), have been used to assess the nuclear maturation status with acceptable results [16,17]. However, the differences in their absorption rates can modify the intensity of the fluorescence emitted [18]. Consequently, a fine optimization of fluorescent probe concentrations and combinations, as well as the temperature and incubation time, is important to assess properly the maturation stage of stained equine oocytes.

In this study, we proposed a new approach of oocyte staining protocol combining low HO concentration with PI to optimize the oocyte nuclear chromatin visualization. We hypothesized that this HO-PI combination would reach a suitable nuclear chromatin fluorescence intensity as well as a reduced HO-autofluorescence background. To our knowledge, even though different staining protocols containing HO and PI were published, a comparative study between a proposed and tested staining protocol to identify the optimum one to assess the equine oocyte nuclear chromatin maturation stage remains unexplored.

Therefore, the aims of this study were to 1) compare two staining protocols to assess the nuclear chromatin stage of equine oocytes after in vitro maturation (IVM); 2) evaluate the effect of vitrification using permeable and nonpermeable cryoprotectants on the nuclear maturation stage of equine oocytes.

2. Materials and Methods

All procedures were approved by the Ethical Animal Experimentation Committee of the University of Cordoba and by the Regional Government of Andalusia (project no. 31/08/2017-105), according to the Spanish law for animal welfare and experimentation (RD 53/2013 and Decision, 2012/707/UE).

2.1. Reagents

All reagents were purchased from Sigma-Aldrich (San Luis, MO) and Gibco (Life Technologies, Inc, Grand Island, NY) unless otherwise was stated.

2.2. Collection of Ovaries and Cumulus-Oocyte Complexes

In the present study, ovaries from 32 mares were recovered at the local slaughterhouse. After collection, ovaries were stored at 30°C and transported to the laboratory within 2–3 h, where they were trimmed with a scalpel blade and washed in freshly saline solution at room temperature (≈23°C) using sterile procedures. Thereafter, a total number of 155 cumulus-oocyte complexes (COCs) were obtained using the scraping method [19]. Before IVM, all the COCs showing signs of deformed cytoplasm, zona pellucida disruption, or lack of a complete cumulus were discarded. Finally, the remaining COCs were maintained in holding medium (HEPES-buffered M199 with Hank's salts supplemented with 25 μg/mL gentamicin and 10% fetal bovine serum) until maturation.

2.3. Oocyte In Vitro Maturation

COCs were transferred to a maturation medium (M199 with Earle's salts supplemented with 25 μg/mL gentamicin, 5 mU/mL FSH and 10% FBS) and cultured under mineral oil for 42 hours at 38.5°C in a humidified atmosphere of 5% CO_2 in air according to Canesin et al. [7].

2.4. Nuclear Maturation Assessment

In vitro matured COCs were denuded by aspiration using modified glass pipettes in denudation solution (holding medium supplemented with 80 IU hyaluronidase/mL). Then, oocytes were fixed in pH = 7 buffered solution containing 4% formaldehyde (Panreac, Barcelona, Spain) and stained according to experimental design. Finally, nuclear chromatin stage was evaluated using an epifluorescence microscope (360–370 nm exciter filter; Olympus Corporation, Tokyo, Japan) and classified as a germinal vesicle (GV), metaphase I (MI), metaphase II (MII) or degenerated, according to Hinrichs et al. [20].

2.5. Experimental Design

2.5.1. Experiment 1: Comparison of Two Staining Protocols to Evaluate Oocyte Nuclear Chromatin After In Vitro Maturation

In this experiment, 44 oocytes from 16 ovaries were matured, fixed, and randomly processed according to two different staining protocols: Protocol 1 included a mixture of 10 μg/mL HO + 50 μg/mL propidium iodide (PI) in PBS for 10 minutes at 38.5°C (P1; n = 21). Protocol 2 was similar but with a lower concentration of HO (2.5 μg/mL + 50 μg/mL PI in PBS) and incubation time (5 minutes at 38.5°C; P2; n = 23). Stained oocytes were classified into four visibility grades (G) according to the ability to identify the nuclear chromatin and the maturation stage (Table 1).

2.5.2. Experiment 2: Effect of Vitrification Using Permeable and Nonpermeable Cryoprotectants on the Nuclear Maturation Stage of Equine Oocytes

In this experiment, a total of 111 oocytes were obtained from 39 ovaries. First, 54 immature COCs were held base solution (containing 40% M199 with Earle's, 40% HEPES-buffered M199 with Hank's salts, 20% FBS and supplemented with 0.3 mM sodium pyruvate at room temperature (20°C–24°C) during 17–20 hs [6]. Thereafter, COCs were divided into two groups and randomly submitted to two vitrification protocols: V1 (n = 26), containing permeable and nonpermeable cryoprotectants; and V2 (n = 28), containing only nonpermeable cryoprotectants, as described further. In addition, 57 oocytes were in vitro matured after

Table 1
Quality score visualization grade of nuclear chromatin and maturation stage on stained mare oocytes.

Grade of visibility	Description	Nuclear chromatin identification	Maturation stage assessment
GI	Outstanding	Excellent	Precise
GII	Good	Satisfactory	Suitable
GIII	Moderate	Weak	Difficult
GIV	Poor	No identification	Invaluable

Table 2
Grade of visibility of the nuclear chromatin of the stained oocytes allocated to each of the four staining protocols.

Staining protocols	No. oocytes	Grades of visibility			
		Outstanding	Good	Moderate	Poor
P1	21	4 (19.04%)[b]	9 (42.86%)	5 (23.81%)	3 (14.29%)
P2	23	12 (52.17%)[a]	5 (21.74%)	4 (17.39%)	2 (8.70%)
P-value		<.05	>.05	>.05	>.05

Staining protocol 1 (10 μg/mL HO + 50 μg/mL propidium iodide: PI); staining protocol 2 (2,5 μg/mL HO + 50 μg/mL PI).
Different superscripts (a-b) indicate significant differences between staining protocols.

collection as nonvitrified control. After the treatment, maturation rates were determined and compared among groups using the staining protocol 2 (2.5 μg/mL HO for 5minutes + 50 μg/mL PI).

2.5.2.1. Conventional Vitrification Protocol (V1). This procedure was performed using a combination permeable (dimethyl sulfoxide (DMSO) and ethylene glycol (EG)) and nonpermeable (sucrose) cryoprotectants, according to the methodology described by Canesin et al. [6]. First, COCs were washed thrice in a 250 μL droplet of standard solution (SS; HEPES-buffered M199 with Hank's salts supplemented with 25 μg/mL gentamicin and 20% FBS). Thereafter, COCs were equilibrated in three 250 μL droplets of SS with an increased concentration of DMSO and EG (3.25%, 4.67%, and 7.5%) for 2, 2, and 10 minutes, respectively. Finally, the COCs were transferred to 250 μL droplet of vitrification solution (VS: SS supplemented with 15% DMSO, 15% EG and 0.5 M sucrose) for 1 minute, loaded in a 1 μL droplet on a calibrated sterile inoculation loop and immediately submerged into liquid nitrogen (LN_2) using a sterile open pulled straw cap. Oocytes were kept in cryo-tanks until further processing.

2.5.2.2. Permeable Cryoprotectant-free Vitrification Protocol (V2). This procedure was performed using sucrose as sole cryoprotectant according to the methodology described by Jin and Mazur [13] in mice. Briefly, COCs were washed thrice in a 250 μL droplet of SS and subsequently equilibrated in a 250 μL droplet of SS supplemented with 1M sucrose for 2 minutes. Thereafter, COCs were placed individually on a sterile inoculating loop and stored in cryo-tanks as previously described for V1.

2.5.2.3. Oocyte Warming. Warming was performed on a heated surface of a thermal plate at 38.5°C. Inoculating loops containing COCs were uncapped under LN_2 and subsequently equilibrated in three Petri dishes containing 3 mL of SS supplemented with descendant concentrations of sucrose: 1.25 M, 0.62 M and 0.31 M for 1, 5, and 5 minutes for V1 or 0.5 M and 0.25 M for 2 and 5 minutes for V2, respectively. Afterward, the COCs were transferred to SS and in vitro matured following the protocol described previously. Finally, oocytes were stained with the protocol selected in experiment 1 Section 3.1.

2.6. Statistical Analysis

Statistical analysis was performed using the Statistical Analysis System (SAS 9.0 version; SAS Institute Inc, Cary, NC). Visibility grades and nuclear maturation rated were compared among staining protocols (P1 and P2) and vitrification systems (V1, V2, and control group), respectively, using chi-squared analysis. Difference was considered statistically significant at $P < .05$.

3. Results

3.1. Experiment 1: Comparison of Two Staining Protocols to Evaluate Oocyte Nuclear Chromatin After In Vitro Maturation

No differences were found between staining protocols showing good, moderate, and poor visualization quality ($P > .05$; Table 2). However, a higher percentage of oocytes showing an outstanding visibility grade was found after using P2 ($P < .05$).

3.2. Experiment 2: Effect of Permeable and Nonpermeable Cryoprotectants in Equine Oocyte Vitrification

The percentage of oocytes reaching the MI and MII stage was higher in the control group (15.79% and 42.11%, respectively) in comparison with vitrification procedures ($P < .05$). However, oocytes cryopreserved using V1 showed increased MII rates (15.38%) in comparison with those vitrified using nonpermeable cryoprotectants (V2, 0%), whereas no differences were found between both vitrification procedures in MI maturation stage ($P > .05$). The rate of degenerated oocytes was similar between treatments (V1 = 57.69% vs. V2 = 60.71%; $P > .05$); however, both vitrification procedures showed a higher proportion of degenerated oocytes in comparison with the control group (17.54%). Similar results were observed in GV stage among treatments ($P > .05$) (Table 3).

4. Discussion

In this study, the efficiency of a permeable cryoprotectant-free vitrification protocol (V2) was assessed in comparison with a conventional protocol (V1) containing both permeable and nonpermeable cryoprotectants for the first time in horse oocytes.

Table 3
Maturation status in control and vitrified immature equine oocytes using the staining protocol 2 (2.5 μg/mL of Hoechst + 50 μg/mL of propidium iodide).

Vitrification protocol	No. oocytes	Maturation stage			
		VG	MI	MII	DEG
Control	57	14 (24.56%)	9 (15.79%)[a]	24 (42.11%)[a]	10 (17.54%)
V1	26	7 (26.92%)	0 (0%)[b]	4 (15.38%)[b]	15 (57.69%)
V2	28	10 (35.71%)	1 (3.57%)[b]	0 (0)[c]	17 (60.71%)
P-value		>.05	<.05	<.05	>.05

Abbreviations: VG, germinal vesicle; MI, metaphase I; MII, metaphase II; DEG, degenerate; Control, no vitrification; V1, vitrification protocol using permeable and nonpermeable cryoprotectants; V2, vitrification protocol using nonpermeable cryoprotectants.
Different superscripts (a-c) indicate significant differences between protocols.

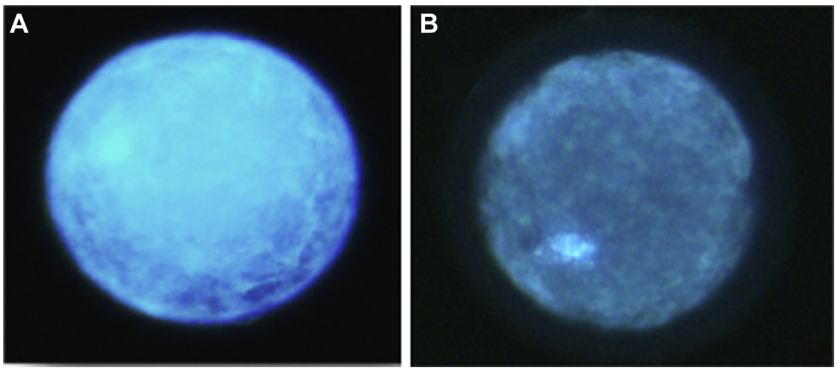

Fig. 1. Photomicrographs of nuclear chromatin configuration from mare oocytes labeled with four staining protocols using fluorescence microscopy. (A) Protocol 1 (10 μg/mL HO + 50 μg/mL propidium iodide: PI); (B) Protocol 2 (2,5 μg/mL HO + 50 μg/mL PI). Abbreviations: HO, Hoechst 33342.

Despite the reduced number of oocytes analyzed, our results have shown that in the present conditions, nonpermeable cryoprotectants are not suitable for oocyte vitrification in mares. It is known sucrose has been conventionally added to vitrification solutions because of its effect in increasing osmotic force and cell dehydration, one of the main critical factors affecting oocyte survival after vitrification techniques [21–23]. However, the sole use of sucrose on mouse oocytes, in which oocyte vitrification is a common technique, was also reported as unsuccessful [24], in agreement with the lack of results obtained in our V2 treatment. This lack of success, according to the authors, could be more explained by using slow warming rates than by the absence of permeable cryoprotectants by increasing crystallization of intracellular glassy water during warming but also by producing the recrystallization of small intracellular ice crystals that have been formed during cooling [25]. This hypothesis was also supported by the fact that a recently developed technique, which allows achieving ultra-high rates of temperature increase during the warming of oocytes based on high-intensity very-short pulses of a laser beam, was reported as able to reduce those problems [13]. However, it was not yet tested in horse oocytes, which are well known for its low suitability in assisted reproductive techniques in comparison with other domestic and laboratory animals [26]. Considering that the warming process has been suggested as the most important factor correlated with the successful vitrification using nonpermeable cryoprotectants [25,27], further studies combining permeable cryoprotectant-free vitrification with this key step of ultra-high warming rates are needed.

It was noteworthy that oocyte maturation rates obtained in V1 were similar to a previous study in which a similar combination of permeable (DMSO and EG) and nonpermeable cryoprotectants (sucrose) was used [6], as well as the maturation rates observed in the nonvitrified oocytes (42.11%), which can also be considered as normal from slaughterhouse-derived oocytes. The oocytes obtained from slaughterhouse include young and old mares, as well as mares that have experienced poor care and stress before slaughter, which may reflect differences in oocyte quality. Therefore, the overall results obtained in this experiment support the idea that the toxicity observed in horse oocytes using the V2 vitrification protocol was not related to nonexperimental factors.

In mares, different conditions for immature oocytes vitrification have been tested, including not only permeable and nonpermeable cryoprotectant combinations but also exposure times to vitrification and warming solutions [6]. Permeable cryoprotectants used in this paper (DMSO and EG) have reported in previous studies a minimal toxicity effect on maturation rates when equine oocytes were expose to cryoprotectants without vitrification/warming process [12], but drastically reduced blastocyst development [6]. In addition, when immature oocytes were exposed to these cryoprotectants and subsequent vitrification, the maturation rates from the present study only reached 15.38% in comparison with nonvitrified oocytes group (42.11%; neither vitrification nor cryoprotectant exposure). These results are similar to previous study where the maturation rate from vitrified group (13.3%) was lower than those from nonvitrified group (48.87%; control) [6]. As equine oocyte cryopreservation by either method continues to be not efficient enough to recommend for clinical use, we hypothesized that successful vitrification procedures may be based on the dominance of warming rate over cooling rate and the use of different cryoprotectant/exposure times [27].

In the previous experiment, a new staining protocol for equine oocytes was proposed (P2), showing an increase in visibility of the nuclear chromatin after IVM. Despite that both staining protocols were able to identify the nuclear chromatin and recognize the maturation status of most of the oocytes evaluated (GI + GII: P1 = 61.9%; P2 = 73.91%), this new proposed methodology shortened the incubation time on HO by half (5 min/38.5°C). Similar conditions of time and temperature incubation but different concentrations of HO and PI were proposed for ram spermatozoa staining (37°C/6 minutes) to obtain an optimum sperm chromatin fluorescence intensity and a reduced autofluorescence background [28]. Even though the highest concentrations of HO used in P1 also produced great chromatin-fluorescent intensity, the contrast between the nuclear DNA and cytoplasm was reduced, impairing the visibility of the nuclear chromatin stage (Fig. 1). Therefore, our experiment suggests that the evaluation of IVM in horse oocytes could be improved by combining low concentrations of HO and low incubation time.

In conclusion, permeable cryoprotectant-free vitrification of equine oocytes obtained poor results and therefore cannot be considered an alternative to vitrification using permeable cryoprotectants. In addition, a staining protocol with a low concentration of HO is recommended to evaluate the nuclear chromatin stage of equine oocytes after IVM.

Acknowledgments

This study was funded by MINECO ("Ministerio de Economia y Competitividad), Spain (grant AGL2013-42726-R). B. Pereira was supported by an EJI-17-AGR-275 contract with funding provided by Conserjeria de Conocimiento, Investigacion y Universidad (Junta de Andalucia, Spain), and European Social Fund (ES- F_2014–2020). S. Demyda-Peyrás is supported by a 5.2.b grant of the Universidad de Córdoba research program.

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CHAPTER 2

CHAPTER 2.1

Evaluation of DNA damage of mare granulosa cells before and after cryopreservation using a chromatin dispersion test

B. Pereira et al., 2019. Journal of Equine Veterinary Science

CHAPTER 2.2

Relationship between DNA fragmentation of equine granulosa cells and oocyte meiotic competence after in vitro maturation

B. Pereira et al., 2019. Reproduction in Domestic Animals

Journal of Equine Veterinary Science 72 (2019) 28–30

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Short Communication

Evaluation of DNA Damage of Mare Granulosa Cells Before and After Cryopreservation Using a Chromatin Dispersion Test

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ARTICLE INFO

Article history:
Received 4 July 2018
Received in revised form
10 October 2018
Accepted 13 October 2018
Available online 21 October 2018

Keywords:
Granulosa cell
Mare
Cryopreservation
DNA

ABSTRACT

DNA fragmentation of granulosa cells might be related to developmental competence of the equine oocyte. Granulosa cells are commonly stored before DNA fragmentation assessment, but the effect of preservation methods on this parameter remains unexplored. The aim of this study was to evaluate whether or not cryopreservation of granulosa cells affects the DNA damage. Equine oocytes were recovered from *postmortem* ovaries of five mares. Granulosa cells were washed by centrifugation and then analyzed (control) or stored in cryovials following four different protocols: P1 = directly plunged in liquid nitrogen (LN_2) and then stored at −80°C; P2 = LN_2/−80°C adding cryoprotectants (7.5% ethylene glycol + 7.5% dimethyl sulfoxide); P3 = −80°C; P4 = −80°C + cryoprotectants. Granulosa cell samples were processed with the prototype D3-Ovoselect, Halotech DNA, Spain), and DNA was visualized under fluorescence microscopy. High, low, and total DNA fragmentation percentages were compared among treatments by analysis of variance. Results were expressed as mean ± standard error. No significant differences ($P > .05$) were found among treatments and the control group. Therefore, the four conservation protocols could be considered equally efficient for DNA preservation of granulosa cells from mare oocytes. In conclusion, cryopreservation of granulosa cells in any of the four protocols used adequately preserved the DNA for further analysis.

1. Introduction

The meiotic competence of mare oocytes has been shown to be associated with granulosa cells (GCs) apoptosis rate [1]. In this sense, the DNA damage from GCs has been proposed as a useful biomarker for oocyte quality evaluation in different assisted reproductive technologies [2]. These cells are commonly stored to perform the DNA analysis in a later time when instantaneous evaluation is unfeasible. Several strategies of somatic cells cryopreservation, including the use of cryoprotectants and different temperatures of storage (−80°C/−196°C) have been reported in human beings and animal species [1,3–6]. The effect of cryopreservation on DNA stability of GCs from cryopreserved ovarian tissue was previously assessed in several species [7–10]. In addition, a modified chromatin dispersion test (prototype D3-MAX; Ovoselect, Halotech DNA SL, Madrid, Spain) has been shown as a reliable method to assess DNA damage in human cumulus cells [5]. However, the effect of cryopreservation methods on DNA damage of equine GCs remains unexplored. Therefore, the aim of this study was to evaluate the effect of four cryopreservation protocols combining different storage temperatures (−80°C/−196°C) or cryoprotectants (ethylene glycol and dimethyl sulfoxide) on GCs DNA damage using the prototype D3-MAX.

2. Materials and Methods

All procedures have been approved by the Ethical Animal Experimentation Committee of the University of Cordoba and by

Animal welfare/Ethical statement: All procedures have been approved by the Ethical Animal Experimentation Committee of the University of Cordoba and by the Regional Government of Andalusia (grant no. 31/08/2017-105) according to the Spanish law for animal welfare and experimentation (RD 53/2013 and Decision, 2012/707/UE).

Conflict of interest statement: None of the authors have any conflict of interest to declare.

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https://doi.org/10.1016/j.jevs.2018.10.019
0737-0806/

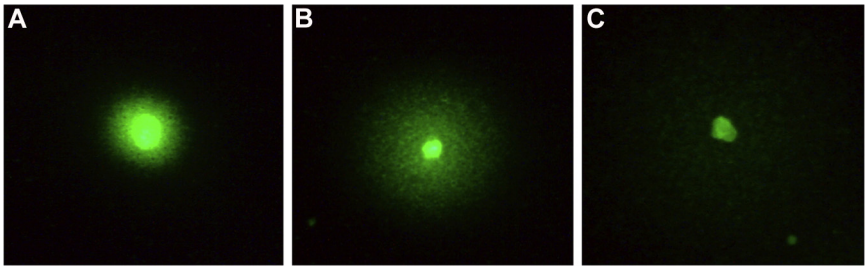

Fig. 1. DNA fragmentation of granulosa cells. Visualization of different DNA damage levels using the prototype D3-MAX under fluorescence microscopy: (A) unfragmented nuclei; (B) low fragmented nuclei; (C) high fragmented nuclei.

the Regional Government of Andalusia (Grant no. 31/08/2017-105) according to the Spanish law for animal welfare and experimentation (RD 53/2013 and Decision, 2012/707/UE). Reactives were purchased from Sigma-Aldrich (San Luis, MO, USA) and Gibco (Life Technologies, Inc, Grand Island, NY, USA). Ovaries from five mares were collected from a local slaughterhouse and transported to the laboratory (30°C; 2–3 hours). The GCs from 57 follicles (8–9 follicles per ovary) were obtained using the scraping method according to Hinrichs et al. [11]. Briefly, follicular contents from each mare were pooled, divided into five groups and cultured individually in 24-well dish plates containing maturation medium (M199 with Earle's salts plus 25 μg/mL gentamycin and 10% fetal bovine serum) for 24hours in a 5% CO_2/38.5°C environment. After incubation, GCs were placed into cryovials of 1.4 mL and centrifuged (800 g per 5 minutes). The supernatant was removed, and the pellets of GCs were submitted to different treatments. One GCs group was immediately assessed before cryopreservation as control, and the other four groups were stored following the four conservation protocols: P1 = GCs were directly plunged in liquid nitrogen (LN_2) for 5 to 10 minutes and then stored at −80°C; P2 = GCs were resuspended in medium 199 with Hank's Balanced Salts adding cryoprotectants (7.5% ethylene glycol + 7.5% dimethyl sulfoxide) and then directly plunged in LN_2 for 5 to 10 minutes and stored at −80°C; P3 = GCs were directly stored at −80°C; and P4 = GCs were resuspended in medium 199 with Hank's Balanced Salts with cryoprotectants (7.5% ethylene glycol + 7.5% dimethyl sulfoxide) and then stored at −80°C.

For DNA analysis, all samples were diluted with PBS to obtain 5 to 7 cells per field under bright microscopy with a 40× objective and then were mounted on pretreated slides according to the methodology described by Barcena et al. [5]. DNA damage of GCs was analyzed using a chromatin dispersion assay (D3-MAX). The pretreated slides, containing GCs, were stained with Fluorgreen (Halotech DNA) and evaluated using an epifluorescence microscope (Olympus Corporation, Japan; 460–490 nm exciter filter). The chromatin dispersion morphology of the fragmented and not fragmented DNA of GCs were classified as previously described by Barcena et al [5] with the following modifications: GCs with unfragmented DNA—nuclei displaying a small regular and compact halo of dispersed chromatin surrounding a regular sized core with intense staining (Fig. 1A); GCs with low fragmented DNA—nuclei displaying halos of variable dimensions of dispersed chromatin surrounding a visible and small size core (Fig. 1B); GCs with high fragmented DNA—nuclei displaying weak staining of the halo surrounding the reduced core (Fig. 1C). A total number of 9,088 GCs were counted (at least 300 cells per slide), and the percentage of GCs containing high, low, and nonfragmented DNA was calculated and compared before (control) and after cryopreservation (P1, P2, P3, and P4) by analysis of variance. Results were expressed as mean ± standard error.

3. Results

The percentage of GCs with fragmented DNA assessed before and after cryopreservation with each protocol is shown in Table 1. There were no significant differences between the control group and the four cryopreservation protocols evaluated ($P > .05$).

4. Discussion

Cryopreservation of GCs enhances tasks organization in the lengthy equine follicular scraping process to obtain oocytes, considering that it is unworkable to perform the DNA analysis of fresh GCs. In addition, the use of frozen samples improves the efficiency of the analysis by allowing the assessment of large number of stored GCs samples at the same time. It is well known that cryopreservation affects the structure of both oocytes and sperm cells [12,13]. Different strategies have been performed to improve cryopreservation success, including the use of different cryoprotectants and cooling rates; however, those cryoprotectants are also responsible for cell toxicity and subsequent cellular membrane damage [14]. A negative effect of cryoprotectant exposure, cooling rate, and the storage temperature is also possible in GCs.

In this study, neither the presence or absence of cryoprotectants (ethylene glycol + dimethyl sulfoxide) nor the temperature of cryopreservation (−80°C/−196°C) affected the DNA fragmentation of GCs from mare follicles before and after cryopreservation. These results are in contrast with Lindley et al. [6], where cryoprotectants increased the DNA integrity of frozen human cumulus cells. A number of studies have been performed using different

Table 1
DNA fragmentation analysis of granulosa cells before (control) and after four cryopreservation protocols.

DNA Fragmentation	Control	Cryopreservation Protocol			
		P1	P2	P3	P4
High	4.0 ± 1.1% (75)	4.8 ± 1.9% (87)	4.9 ± 2.6% (100)	4.6 ± 1.6% (78)	4.8 ± 3.6% (80)
Low	3.1 ± 1.8% (58)	2.0 ± 1.2% (37)	2.7 ± 1.7% (55)	2.5 ± 1.6% (43)	3.2 ± 1.8% (54)
Total fragmented	7.1 ± 1.3% (133)	6.8 ± 2.0% (124)	7.6 ± 2.9% (155)	7.1 ± 2.7% (121)	8.0 ± 4.1% (134)
Total unfragmented	92.9 ± 1.3% (1732)	93.2 ± 2.0% (1688)	92.4 ± 2.9% (1877)	92.9 ± 2.7% (1584)	92.0 ± 4.1% (1540)

Number of cells counted is included in brackets. P1: plunging in liquid nitrogen (LN_2) before storage at −80°C (LN_2/−80°C); P2: LN_2/−80°C adding cryoprotectants; P3: storage at −80°C; P4: storage −80°C adding cryoprotectants. Results are expressed as mean ± standard error. No significant differences were found among protocols ($P > .05$).

cryopreservation protocols for the ovarian tissue, but there is little information using individual GCs. On the other hand, the measurement of sperm DNA fragmentation changed after cryopreservation in several animal species [15–17] but not in human being [18]. All these previous studies have used the comet assay to evaluate different variables of the DNA structure, making comparisons among them difficult to interpret [15]. In this sense, other studies have also shown inconclusive measures of DNA fragmentation on cumulus cells in relation to oocyte fertilization after intracytoplasmic sperm injection using the comet assay [19,20]. Recently, a new reliable, reproducible, and fast DNA test, the prototype D3-MAX has been used to evaluate the ds-DNA breaks of human cumulus cells [5]. Once it has been shown to be an accurate method to detect DNA damage in mare GCs, further studies are needed to evaluate its ability as biomarker for equine oocyte meiotic competence.

In conclusion, cryopreservation of GCs in any of the four protocols used adequately preserved the DNA allowing its evaluation in further analysis.

Acknowledgments

The authors are indebted to Halotech DNA S.L. (Madrid, Spain) for providing the prototype D3-MAX used in the experiments.

Financial disclosure

This study was funded by MINECO ("Ministerio de Economía y Competitividad), Spain (grant AGL2013-42726-R). B. Pereira was supported by a postgraduate fellowships from the ceiA3 (Andalucía, Spain), "II Plan Propio Galileo de Innovación y Transferencia" (University of Cordoba, Spain), and EJI-17-AGR-275 contract with funding provided by Conserjería de Conocimiento, Investigación y Universidad (Junta de Andalucía, Spain), and European Social Fund (ESF_2014–2020).

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Received: 30 April 2019 | Revised: 8 July 2019 | Accepted: 11 July 2019

DOI: 10.1111/rda.13514

SUPPLEMENT ARTICLE

Reproduction in Domestic Animals WILEY

Relationship between DNA fragmentation of equine granulosa cells and oocyte meiotic competence after in vitro maturation

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Funding information
Conserjería de Conocimiento, Investigación y Universidad (Junta de Andalucía, Spain), Grant/Award Number: EJI-17-AGR-275; European Social Fund, Grant/Award Number: ES-F_2014–2020; MINECO, Grant/Award Number: AGL2013-42726-R

Abstract

The acquisition of equine oocyte developmental capacity is ensured by the follicular environment, such as granulosa cells, which could reflect the meiotic development potential of immature oocytes. This study evaluated the relationship between DNA fragmentation of granulosa cells, using the chromatin dispersion test, and equine oocyte meiotic development after in vitro maturation. Granulosa cells and cumulus–oocytes complexes (n = 50) were recovered from slaughterhouse-derived ovaries. Oocytes were in vitro matured, stained and evaluated under fluorescence microscopy. Maturation rates were classified into outstanding, medium and poor levels of maturation using 25th and 75th percentiles as thresholds. For DNA assessment, each sample was processed with the Ovoselect® kit (Halotech DNA). High, low and total DNA fragmentation percentages were compared among levels of maturation rates by ANOVA, followed by Duncan test. Results were expressed as mean ± *SE*. Total and high DNA fragmentation rates of granulosa cells were significantly higher ($p < 0.05$) in follicles whose oocytes had reached outstanding maturation level than those originating from follicles whose oocytes had reached poor maturation level. In conclusion, the DNA fragmentation analysis of equine granulosa cells can be a valuable test to identify equine oocytes showing the best meiotic competence after in vitro maturation.

KEYWORDS
granulosa cells, mare, meiotic competence, oocyte, sperm chromatin dispersion

1 | INTRODUCTION

The development of techniques to identify meiotic and developmental potential of immature oocytes is of the utmost importance for subsequent production of embryos after in vitro fertilization (Ruvolo, Fattouh, Bosco, Brucculeri, & Cittadini, 2013). Several studies have been focused on identifying equine oocyte characteristics associated with meiotic competence and cytoplasmic maturation (Del Campo, Donoso, Parrish, & Ginther, 1995; Pereira et al., 2013); however, somatic cells within the follicular environment (granulosa and cumulus cells) play also an important role in the development of oocytes (Del Collado, Andrade, Meirelles, da Silveira, & Perecin, 2018). In this sense, the morphology of cumulus cells, which are surrounding immature oocytes, is related to equine follicle viability; equine oocytes originating from viable follicles are surrounded by compact cumuli while those oocytes obtained from follicles with different stages of atresia are surrounded by expanded cumuli (Hinrichs & Williams, 1997). However, this morphology criteria of cumulus cells are not completely reliable considering that oocytes from early atresia follicles may be surrounded by compact

TABLE 1 Maturation rates of equine oocytes (n = 50) classified into poor, medium and outstanding levels using 25th and 75th percentiles as thresholds

		Oocyte maturation rate (%)	
Maturation level	Percentile	Range	Mean value
Poor	<25th	0–38	16.67
Medium	25th–75th	38–60	48.27
Outstanding	>75th	61–100	88.89

TABLE 2 Comparison of DNA fragmentation indexes of equine granulosa cells from follicles whose oocytes had reached different maturation levels

	DNA fragmentation of granulosa cells (%)		
Maturation level	High	Low	Total
Outstanding	4.97 ± 2.86[a]	1.81 ± 1.41	6.78 ± 3.14[a]
Medium	3.42 ± 2.95[a,b]	2.11 ± .62	5.53 ± 3.26[a,b]
Poor	1.51 ± 0.55[b]	1.69 ± 1.04	3.20 ± 1.02[b]

Note: Different superscripts (a–b) indicate significant differences (p < 0.05) among maturation levels.

or expanded cumuli. One of the features of follicular atresia is the fragmentation of granulosa cell DNA into oligonucleosomal DNA fragments. Taking into account that DNA status of granulosa cells represents a potentially valuable indicator oocyte competence (Dell'Aquila, Albrizio, Maritato, Minoia, & Hinrichs, 2003), fragmentation of granulosa cells might reflect oocyte quality, providing a non-invasive means to assess oocyte quality. In terms of DNA damage assessment of somatic cells, the chromatin dispersion test provides a reliable, fast and reproducible assay (Barcena et al., 2015). Recently, this test has been successfully performed in mares, allowing the accurate evaluation of DNA fragmentation of granulosa cells (Pereira et al., 2019). Therefore, the aim of this study was to evaluate the relationship between DNA fragmentation of granulosa cells, using the chromatin dispersion test, and equine oocyte meiotic development after in vitro maturation.

2 | MATERIALS AND METHODS

All the procedures 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. A total of 15 ovaries from 10 mares were collected from a local slaughterhouse and transported to the laboratory (30°C; 2–3 hr). Follicular contents from 50 follicles were obtained using the scraping method according to Hinrichs et al. (2005). Cumulus–oocytes complexes from the same ovary were pooled, in vitro matured (42 hr/38.5°C/5% CO_2; Ribeiro, Love, Choi, & Hinrichs, 2008), denuded, fixed in pH = 7 buffered solution containing 4% formaldehyde and then stained (2.5 μg/ml of hoechst 33342 + 50 μg/ml propidium iodide) and classified as: germinal vesicle, metaphase I, metaphase II and degenerating (Hinrichs, Schmidt, & Selgrath, 1993). Maturation rates were classified into outstanding, medium and poor levels of maturation using 25th and 75th percentiles as thresholds.

At the same time, granulosa cells from each collected ovary were pooled and stored according to Pereira et al. (2019) at −80°C until DNA assessment. For DNA analysis, all samples were mounted on pre-treated slides and processed using a chromatin dispersion kit (Ovoselect®; Halotech DNA), according to the methodology described by Barcena et al. (2015), but decreasing to 3 min the time of exposure of granulosa cells to the lysis solution, to obtain optimal protein depletion in equine granulosa cells. The slides, containing granulosa cells, were stained with Fluorgreen (Halotech DNA) and evaluated using an epifluorescence microscope. The chromatin dispersion morphology of DNA of granulosa cells was classified as: unfragmented DNA, low fragmented DNA and high fragmented DNA (Pereira et al., 2019). At least 300 cells were counted per slide and, the percentage of granulosa cells containing high-, low- and total (low + high)-fragmented DNA was recorded.

Normality of the data distribution and homogeneity of variances were assessed using the Kolmogorov–Smirnov and Levene's tests, respectively. Arcsine transformation was applied when values were not normally distributed. Differences in DNA fragmentation between levels of maturation were evaluated by ANOVA followed by Duncan test. Results were expressed as mean ± *SE*.

3 | RESULTS

Mean and range values of maturation rate of equine oocytes classified as poor, medium and outstanding maturation levels are shown in Table 1. Total and high DNA fragmentation rates of granulosa cells were significantly higher (p < 0.05) in follicles whose oocytes had reached outstanding maturation level than those originating from follicles whose oocytes had reached poor maturation level. There were no significant differences among oocyte maturation levels for low DNA fragmentation of granulosa cells (Table 2).

4 | DISCUSSION

In this study, equine immature oocytes originated from follicles containing granulosa cells with highly fragmented DNA reached the greatest meiotic competence. To the best of our knowledge, this is the first study in mares showing the relationship between meiotic competence after in vitro maturation and granulosa cell DNA fragmentation using the chromatin dispersion assay. These results are in agreement with previous studies where the atresia follicular was positively related to meiotic development after oocyte in vitro maturation in sheet (Moor, Lee, Dai, & Fulka, 1996) and horses (Hinrichs & Williams, 1997). Nevertheless, DNA fragmentation assessment of

granulosa cells can be recognized before even morphological features of atresia follicular appear (Liu et al., 2014). In this sense, the positive relationship between meiotic competence and apoptosis level of granulosa cells was previously showed in mares assessing DNA damage by laddering analysis (Dell'Aquila et al., 2003). These data and the results of our study constitute a strong indicator that oocyte meiotic competence is acquired in equine follicles containing granulosa cells with DNA damage. However, the DNA laddering analysis has an important limitation; it cannot include a small number of cells. In contrast, the chromatin dispersion test used in our study can assess the level of DNA fragmentation of a few cells (Barcena et al., 2015), providing relevant and reliable information about the level of damage in granulosa cells from any individual follicle. This work opens a new trend of DNA analysis of somatic cells in the horse, allowing the assessment of DNA fragmentation of a small number of cells, such as cumulus cells.

In conclusion, the DNA fragmentation analysis of equine granulosa cells can be a valuable test to identify equine oocytes showing the best meiotic competence after in vitro maturation.

ACKNOWLEDGEMENTS

The authors are indebted to HALOTECH DNA S.L. (Madrid, Spain) for providing the Ovoselect kits used in the experiments. This study was funded by MINECO ("Ministerio de Economía y Competitividad"), Spain (grant AGL2013-42726-R). B. Pereira was supported by an EJI-17-AGR-275 contract with funding provided by Conserjería de Conocimiento, Investigación y Universidad (Junta de Andalucía, Spain), and European Social Fund (ES-F_2014–2020).

CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

M Hidalgo and B Pereira contributed to all sections. I Ortiz, J Dorado and J Gosalvez contributed to the study design, data analysis and interpretation, preparation and revision of the manuscript. M Diaz-Jimenez and C Consuegra contributed to the development of 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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How to cite this article: Pereira B, Dorado J, Diaz-Jimenez M, et al. Relationship between DNA fragmentation of equine granulosa cells and oocyte meiotic competence after in vitro maturation. *Reprod Dom Anim*. 2019;54(Suppl. 4):78–81. https://doi.org/10.1111/rda.13514

CHAPTER 3

Effect of permeable cryoprotectant-free vitrification on DNA fragmentation of equine oocyte-cumulus cells

B. Pereira et al., 2019. Reproduction in Domestic Animals

Received: 1 May 2019 | Revised: 2 June 2019 | Accepted: 7 June 2019

DOI: 10.1111/rda.13491

SUPPLEMENT ARTICLE

Reproduction in Domestic Animals WILEY

Effect of permeable cryoprotectant-free vitrification on DNA fragmentation of equine oocyte–cumulus cells

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Funding information
MINECO (Ministerio de Economía y Competitividad), Spain, Grant/Award Number: AGL2013-42726-R; ceiA3 (Andalucía, Spain); II Plan Propio Galileo de Innovación y Transferencia (University of Cordoba, Spain)

Abstract

DNA fragmentation of cumulus cells could be used as an indicator of oocyte vitrification success as an indirect indicator of the quality of the oocyte. This study was designed to compare the DNA fragmentation of post-mortem equine cumulus cells before or after vitrification in the absence of permeable cryoprotectant agents. Cumulus–oocyte complexes (COCs; n = 56) were recovered from slaughterhouse ovaries and subjected to in vitro maturation (42 hr/38.2°C/5%CO_2) before (control group) or after a permeable cryoprotectant-free vitrification method using 1 M sucrose (vitrification group). After in vitro maturation, COCs were denuded, and cumulus cells were washed and stored at −80°C until thawing. Cumulus cell samples were processed with the chromatin dispersion test (Ovoselect, Halotech DNA, Spain). Low, high and total DNA fragmentation percentages of cumulus cells were recorded and compared between the two groups by Student's t test. Results were expressed as mean ± *SEM*. The vitrified group resulted in significantly higher ($p < 0.05$) percentages for low (16.81 ± 1.62 vs. 6.63 ± 0.77) and total (21.14 ± 1.84 vs. 12.76 ± 1.48) DNA fragmentation of cumulus cells. There were no significant differences between groups for high DNA fragmentation of cumulus cells. In conclusion, permeable cryoprotectant-free vitrification of equine oocytes increased the total DNA fragmentation rate of cumulus cells but protected them against high DNA fragmentation rates. Further studies are needed to examine the relationship between DNA fragmentation of cumulus cells and the developmental competence of equine oocytes.

KEYWORDS
cumulus cells, DNA damage, mare, sucrose, vitrification

1 | INTRODUCTION

Vitrification of equine oocytes has the potential to become an important tool to preserve female gametes from valuable individuals and endangered breeds (Smits, Hoogewijs, Woelders, Daels, & Van Soom, 2012). However, the overall success of equine oocyte vitrification, combining permeable and non-permeable cryoprotectants, resulted in low embryo production rates (Canesin et al., 2018; Ortiz-Escribano et al., 2018). A relatively new vitrification technique, avoiding permeable cryoprotectants, has been developed during the last years not only in sperm from different species, such as horses or donkeys (Consuegra et al., 2019; Diaz-Jimenez et al., 2018), but also in mouse oocytes and embryos (Jin & Mazur, 2015), obtaining satisfactory results.

It is well known that cumulus cells play a vital role for immature oocytes to resume meiosis and achieve full cytoplasmic maturation and subsequent embryo development (Ruppert-Lingham, Paynter, Godfrey, Fuller, & Shaw, 2003), and therefore, the damage

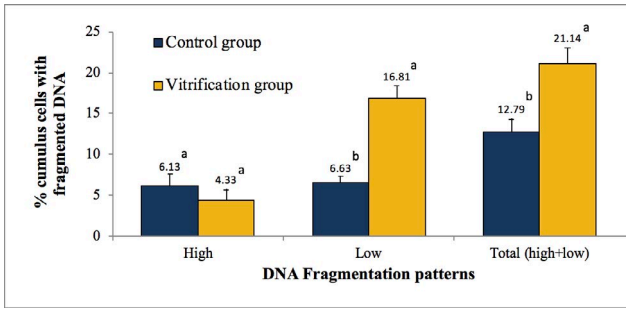

FIGURE 1 Percentages of cumulus cells with low, high and total fragmented DNA of fresh (control group) or vitrified (vitrification group) cumulus cells from equine oocytes. Different superscripts (a–b) indicate significant differences ($p < 0.05$) between control and vitrification group

of cumulus cells is critical to the oocyte developmental competence (Tanghe et al., 2003). In this sense, cumulus cells damage can be measured by DNA fragmentation (Barcena et al., 2015) and considered as a marker of oocyte stress. Chromatin dispersion test has been shown as a reliable method to assess DNA fragmentation in equine granulosa cells (Pereira et al., 2019). However, there is little information about the effect of permeable cryoprotectant-free vitrification on equine cumulus cells.

Therefore, this study was designed to compare DNA fragmentation of *post-mortem* equine cumulus cells before or after oocyte vitrification in the absence of permeable cryoprotectant agents.

2 | MATERIAL AND METHODS

All the procedures 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. Ovaries from 13 mares, from 3 to 18 years old and unknown reproductive history, were collected from a local slaughterhouse during the breeding season (February–June) and transported to the laboratory (30°C; 2–3 hr). A total of 56 cumulus–oocyte complexes (COCs; 2–7 COCs per mare) were recovered using the scraping method according to Hinrichs et al. (2005) and subjected to in vitro maturation (42 hr/38.2°C/5% CO_2 in air) immediately after collection (control group) or after a permeable cryoprotectant-free vitrification (vitrification group). Vitrification was performed at room temperature using 1 M sucrose for 2 min. Then, up to 9 COCs were placed on sterile inoculating loops, immediately plunged into liquid nitrogen and stored in tanks until warming. The warming solutions consisted of decreasing sucrose concentrations (0.5 M for 2 min–0.25 M for 5 min–0 M for unspecified time) at 38.5°C (Canesin et al., 2017). Afterwards, COCs from vitrified group were in vitro matured as described above. After maturation, all COCs were denuded, and cumulus cells were washed and stored at −80°C according to Pereira et al. (2019) until DNA fragmentation assessment. For DNA analysis, all samples were thawed, mounted on pre-treated slides and processed using a chromatin dispersion test (Ovoselect, Halotech DNA, Spain), according to the methodology

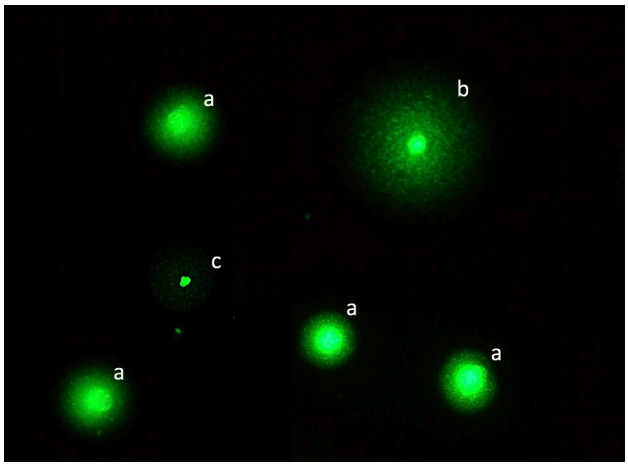

FIGURE 2 Visualization of different patterns of DNA chromatin dispersion of equine cumulus cells using the Ovoselect kit under fluorescence microscopy: (a) unfragmented DNA; (b) low fragmented DNA; and (c) high fragmented DNA

described by Barcena et al. (2015). The slides containing cumulus cells were stained with Fluogreen (Halotech DNA) and evaluated using an epifluorescence microscope. Three chromatin dispersion patterns were established: unfragmented DNA, low fragmented DNA and high fragmented DNA (Pereira et al., 2019). At least 300 cells were counted per slide, and the percentages of cumulus cells containing high, low and total (low + high) fragmented DNA were recorded. Differences in DNA fragmentation rates between groups were compared using Student's *t* test. Normality of the data distributions was checked by the Kolmogorov–Smirnov test. Arcsine transformation was applied to not normally distributed data. Results were expressed as mean ± *SEM*.

3 | RESULTS

DNA fragmentation rates of fresh and vitrified cumulus cells are shown in Figure 1. Vitrified group resulted in significantly higher ($p < 0.05$) percentages than control group for low (16.81 ± 1.62 vs. 6.63 ± 0.77) and total (21.14 ± 1.84 vs. 12.76 ± 1.48) DNA fragmentation of cumulus cells, respectively. There were no significant differences ($p > 0.05$) between groups for high DNA fragmentation values of cumulus cells (vitrified group: 4.33 ± 1.37; control group: 6.13 ± 1.46). The three patterns of DNA chromatin dispersion of equine cumulus cells using the Ovoselect kit are represented in Figure 2.

4 | DISCUSSION

According to the results obtained in this study, COC vitrification using non-permeable cryoprotectants increased the total DNA fragmentation of equine cumulus cells. To the best of our knowledge, there are no previous studies comparing DNA fragmentation of equine cumulus cells before and after vitrification of equine COCs using non-permeable cryoprotectants. These results are in

agreement with previous studies in other species where COC cryopreservation affected murine (Ruppert-Lingham et al., 2003) and ovine (Bogliolo et al., 2007) cumulus cells structure, and increased the number of dead cumulus cells (Tharasanit, Colleoni, Galli, Colenbrander, & Stout, 2009).

Permeable cryoprotectant-free vitrification method was able to protect the cumulus cells from chromatin dispersion pattern of high fragmented DNA, but not from low DNA fragmentation. These results might be explained because in immature equine COCs, outlying cumulus cells are more vulnerable to cryoinjury than those more profound in the cumulus mass (Tharasanit et al., 2009). This means that the damage on the DNA will be faster and more severe in the peripheric than in the inner cumulus cells, resulting in different patterns of DNA fragmentation on the sample (Enciso et al., 2006), depending on the situation of the cell in the COC. It may be possible that only vitrified outlying cumulus cells underwent high DNA fragmentation level (Figure 2c), which did not affect significantly the high DNA fragmentation rate of vitrified cumulus cells.

On the other hand, different cooling rates and cryoprotectants have shown no effect on DNA fragmentation of somatic cells (Pereira et al., 2019); hence, the progressive increase of nuclear DNA fragmentation of cumulus cells could be due to the lack of cryoprotective substrates and possible crystal formation under these vitrification/warming conditions.

In conclusion, permeable cryoprotectant-free vitrification of equine oocytes increased the total DNA fragmentation rate of cumulus cells but protected them against high DNA fragmentation rates. Further studies are needed to examine the DNA fragmentation of periphery versus inner cumulus cells and its relationship with the developmental competence of equine oocytes.

ACKNOWLEDGEMENTS

The authors are indebted to HALOTECH DNA S.L. (Madrid, Spain) for providing the Ovoselect kits used in the experiments. This study was funded by MINECO (Ministerio de Economía y Competitividad), Spain (grant AGL2013-42726-R). B. Pereira was supported by a postgraduate fellowships from the ceiA3 (Andalucía, Spain) and "II Plan Propio Galileo de Innovación y Transferencia" (University of Cordoba, Spain).

CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

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

DATA ACCESSIBILITY

Research data are not shared.

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How to cite this article: Pereira BC, Ortiz I, Dorado JM, et al. Effect of permeable cryoprotectant-free vitrification on DNA fragmentation of equine oocyte–cumulus cells. *Reprod Dom Anim*. 2019;54(Suppl. 3):53–56. https://doi.org/10.1111/rda.13491

CONCLUSIONS

CONCLUSIONS

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

Conclusion 1. A staining protocol with a low concentration of Hoechst 33342 is recommended to evaluate the nuclear chromatin stage of equine oocytes after *in vitro* maturation. The use of a vitrification solution in the absence of permeable cryoprotectant agents cannot be considered an alternative to vitrification solution combining permeable and non-permeable cryoprotectant agents for equine oocytes.

Conclusion 2. The use of any cryopreservation protocols combining different storage temperatures (-80ºC/-196ºC) or cryoprotectants (ethylene glycol and dimethyl sulfoxide), adequately preserved the DNA of equine granulosa cells, allowing its evaluation in further analysis. The DNA fragmentation analysis of equine granulosa cells can be a valuable test to identify equine oocytes showing the best meiotic competence after *in vitro* maturation.

Conclusion 3. Permeable cryoprotectant-free vitrification of equine oocytes increased the total DNA fragmentation rate of cumulus cells but protected them against high DNA fragmentation rates.

CONCLUSIONES

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

Conclusión 1. Se recomienda emplear una concentración baja del fluorocromo Hoechst 33342 para evaluar el estado de la cromatina nuclear de los ovocitos equinos después de ser madurados *in vitro*. El uso de una solución de vitrificación sin agentes crioprotectores permeables no puede ser considerada una alternativa a los medios de vitrificación formados por la combinación de crioprotectores permeables y no permeables.

Conclusión 2. El uso de cualquier protocolo de crioconservación combinando diferentes temperaturas (-80ºC/-196ºC) o crioprotectores (etilenglicol y dimetilsulfóxido), preservó el ADN de las células de la granulosa de équidos del mismo modo, permitiendo su evaluación en un análisis más detallado. El análisis de la fragmentación del ADN de las células de la granulosa de équidos puede ser un método valioso para identificar ovocitos equinos mostrando la mejor competencia meiótica tras ser madurados *in vitro*.

Conclusión 3. El uso de medios de vitrificación compuestos por sacarosa como único agente crioprotector, aumenta la tasa de fragmentación total del ADN de las células del cúmulo, pero las protege frente a la alta tasa de fragmentación del ADN durante la vitrificación de ovocitos equinos.

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ÍNDICES DE CALIDAD

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Primera publicación

- Título: *The effect of different vitrification staining protocols on the visibility of the nuclear maturation stage of equine oocytes.*
- Autores (p.o. de firma): *B. Pereira, I. Ortiz, J. Dorado, M. Diaz-Jimenez, C. Consuegra, S. Demyda-Peyras, M. Hidalgo*
- Revista (año, vol., pag.): *Journal of Equine Veterinary Science (2020), Vol. 90, 103021*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports, JCR (2020)*
- Área temática en la Base de Datos de referencia: *Veterinary Sciences*
- Índice de impacto de la revista en el año de publicación del Artículo: 1,583
- Lugar que ocupa/N.º de revistas del Área temática: *67/146 (Q2)*

Segunda publicación

- Título: *Evaluation of DNA damage of mare granulosa cells before and after cryopreservation using a chromatin dispersion test*
- Autores (p.o. de firma): *B. Pereira, I. Ortiz, J. Dorado, C. Consuegra, M. Diaz-Jimenez, S. Demyda-Peyras, J. Gosalvez, M. Hidalgo*
- Revista (año, vol., pag.): *Journal of Equine Veterinary Science (2019), Vol. 72, 28-30*
- 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: 1,100
- Lugar que ocupa/N.º de revistas del Área temática: *75/141 (Q3)*

Tercera publicación

- Título: *Relatioship between DNA fragmentation of equine granulosa cells and oocyte meiotic competence after in vitro maturation*
- Autores (p.o. de firma): *B. Pereira, J. Dorado, M. Diaz-Jimenez, C. Consuegra, I. Ortiz, J. Gosalvez, M. Hidalgo*
- Revista (año, vol., pag.): *Reproduction in Domestic Animals (2019), Vol. 54 (Suppl.4), 78-81*
- 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: *40/141 (Q2)*

Cuarta publicación

- Título: *Effect of permeable cryoprotectant-free vitrification on DNA fragmentation of equine oocyte-cumulus cells*
- Autores (p.o. de firma): *B. Pereira, I. Ortiz, J. Dorado, M. Diaz-Jimenez, C. Consuegra, J. Gosalvez, M. Hidalgo*
- Revista (año, vol., pag.): *Reproduction in Domestic Animals (2019), Vol. 54 (Suppl.4), 53-56*
- 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: *40/141 (Q2)*

PRODUCCIÓN CIENTÍFICA

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Otras aportaciones científicas derivadas directamente de la Tesis Doctoral:

Contribuciones a Congresos Nacionales

- "Evaluación de distintos protocolos de tinción de ovocitos equinos maduros"

Blasa Carmen Pereira Aguilar, Manuel Hidalgo Prieto, Isabel Ortiz Jaraba. I Congreso Científico de Investigadores Nóveles. Córdoba (España), 11 noviembre 2016. Tipo: Comunicación Oral.

- "Métodos de conservación de las células de la granulosa-cúmulo de ovocitos de yegua"

C. Baños, **B. Pereira**, I. Ortiz, J. Dorado, M. Diaz-Jimenez, C. Consuegra, M. Bottrel, M. Hidalgo. II Congreso de Veterinaria y Ciencia y Tecnología de los alimentos. Córdoba (España), 9 febrero 2018. Tipo: Póster.

- "Estudio de la fragmentación en el ADN de las células del cúmulo de ovocitos equinos antes y después de ser sometidos a técnicas de criopreservación"

B.Pereira, J. Dorado, M. Hidalgo. VIII Congreso Científico de Investigadores en Formación de la Universidad de Córdoba. Creando redes Doctorales. Córdoba (España), 17-18 febrero 2020. Tipo: Póster

Contribuciones a congresos Internacionales con *meeting-abstracts* publicados en Revistas Indexadas en el JCR

- **"**Conservation methods of granulosa cells from mare oocytes"

Blasa C. Pereira Aguilar, María Díaz Jimenez, Manuel Hidalgo Prieto, Cristina Baños, César Consuegra, Jesús M. Dorado Martín, María La Rubia, Isabel Ortiz Jaraba. 22nd Annual Conference of European Society of Domestic Animals Reproduction (ESDAR). Córdoba (España), 27-29 septiembre 2018.

Tipo: Póster.
Revista: Reproduction in Domestic Animals, Vol 53 (s2): 179 -180.

- "Effect of permeable cryoprotectant-free vitrification on DNA fragmentation of equine oocyte–cumulus cells"

B. Pereira, I. Ortiz, J. Dorado, M. Diaz-Jimenez, C. Consuegra, J. Gosalvez, M. Hidalgo. 23nd 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): 53 - 56.

- "Relationship between DNA fragmentation of equine granulosa cells and oocyte meiotic competence after in vitro maturation"

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

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- "Comparison of different methods to preserve equine oocyte cumulus cells for DNA assessment "

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

Revista: Reproduction in Domestic Animals

Publicaciones en revistas de divulgación

- "Evaluación de distintos protocolos de tinción de ovocitos equinos maduros". **Blasa Carmen Pereira Aguilar**, Manuel Hidalgo Prieto, Isabel Ortiz Jaraba. Revista Investigando por un Mundo Mejor. Capítulo 18: 82-86.