UNIVERSIDAD DE CORDOBA (ESPAÑA)

PROGRAMA DE DOCTORADO DE BIOCIENCIAS Y CIENCIAS AGROALIMENTARIAS

APORTACIONES AL CONOCIMIENTO REPRODUCTIVO EN EL CABALLO PURA RAZA ESPAÑOL: fertilidad, vitrificación de esperma y monitorización de la gestación

CONTRIBUTIONS TO THE REPRODUCTIVE KNOWLEDGE IN SPANISH PUREBRED HORSES: fertility, sperm vitrification and pregnancy monitorization

Directores:

Estrella I. Agüera Buendía y Carlos C. Pérez Marín

Autor:
Fernando Requena Domenech

Fecha de depósito tesis en Idep: 27 de febrero de 2018

TITULO: *Aportaciones al conocimiento reproductivo en el caballo pura raza español: fertilidad, vitrificación de esperma y monitorización de la gestación*

AUTOR: *Fernando Requena Domenech*

Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

https://www.uco.es/ucopress/index.php/es/
ucopress@uco.es

Tesis Doctoral

APORTACIONES AL CONOCIMIENTO REPRODUCTIVO EN EL CABALLO PURA RAZA ESPAÑOL: fertilidad, vitrificación de esperma y monitorización de la gestación

Memoria de Tesis Doctoral presentada por el Licenciado en Veterinaria

D. Fernando Requena Domenech

para optar al Grado de Doctor por la Universidad de Córdoba

VºBº Los Directores

Prof. Dra. Estrella Agüera Buendía **Prof. Dr. Carlos C. Pérez Marín**

Córdoba, a 27 de febrero de 2018

Fernando Requena Domenech

TÍTULO DE LA TESIS: APORTACIONES AL CONOCIMIENTO REPRODUCTIVO EN EL CABALLO PURA RAZA ESPAÑOL: fertilidad, vitrificación de esperma y monitorización de la gestación.

DOCTORANDO/A: FERNANDO REQUENA DOMENECH

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

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El doctorando D. Fernando Requena Domenech ha llevado a cabo a lo largo de su vida profesional una trayectoria impecable, centrada en la reproducción equina y en dar respuesta a los problemas de fertilidad del Pura Raza Español que encuentra a diario en su trabajo. Desde el año 2012 ha trabajado en la confección de su Tesis Doctoral dentro del Programa de Doctorado de la Universidad de Córdoba correspondiente al área de Biociencias y Ciencias Agroalimentarias, la cual se ha llevado a cabo sobre tres artículos científicos publicados y apoyada en numerosas aportaciones a Congresos Nacionales e Internaciones en forma de comunicaciones y presentación de posters.

En este tiempo, el doctorando ha demostrado su inquietud por la investigación y la presentación de resultados en foros de alta calidad, además de esforzarse en que las publicaciones aparecieran en revistas de alto índice de impacto, lo que ha permitido, que la Tesis que de todo esto se ha derivado, haya podido presentarse por compendio de artículos. Por ello informamos que la Tesis titulada “APORTACIONES AL CONOCIMIENTO REPRODUCTIVO EN EL CABALLO PURA RAZA ESPAÑOL: fertilidad, vitrificación de esperma y monitorización de la gestación” de la que es autor el Licenciado en Veterinaria Don FERNANDO REQUENA DOMENECH y que ha sido realizada bajo nuestra dirección y asesoramiento en los Departamentos de Biología Celular, Fisiología e Inmunología y Medicina y Cirugía Animal de la Universidad de Córdoba, reúne requisitos científicos necesarios para ser defendida como "Tesis por

Compendio de artículos" ante el tribunal correspondiente con el fin de obtener el Grado de Doctor en Veterinaria por la Universidad de Córdoba.

Así pues, autorizamos la presentación y defensa de esta Tesis Doctoral.

Córdoba, 27 de febrero de 2018

Firma del/de los director/es

Fdo.: Estrella I. Agüera Buendía
Directora de la Tesis

Fdo.: Carlos C. Pérez Marín
Coodirector de la Tesis

“Si buscas resultados distintos no hagas siempre lo mismo”

Albert Einstein

AGRADECIMIENTOS

¡Por fin la termine! Ya era hora de terminar esta etapa de doctorado que comenzó hace años. Aunque casi todo lo que me he propuesto lo he conseguido, este desafío del doctorado ha sido duro por la dificultad que me ha supuesto compaginar la tesis con un trabajo profesional tan exigente y minucioso como es la reproducción equina. Pero gracias a varias personas y a que la Reproducción me apasiona, he alcanzado el objetivo, ser Doctor en Veterinaria.

Tengo que agradecer este logro, en primer lugar, a mis directores de tesis, Estrella Agüera (Yeyi) y Carlos Pérez. Qué decir de Yeyi, una gran persona, gran profesional, minuciosa, ordenada y con la que puedes contar desde las 6:00 de la mañana sin problema. Una veterinaria que viene de una gran dinastía de veterinarios y que sabe lo que es trabajar fuera de la Universidad. Me ha enseñado y me sigue enseñando el funcionamiento y trasfondo de la Universidad. Fue ella la primera persona del departamento que me enseñó el laboratorio en una visita que realicé al Campus Rabanales cuando aún estaba en Bachillerato. Me ha insistido siempre en la tesis, que escribiera todos los días un poco, que al final se termina. Gracias por todo Yeyi. Y qué decir de Carlos, un crack de la reproducción, fue él quien me dio las bases de reproducción y con el que realice mi primer lavado uterino en yegua cuando era estudiante de licenciatura. Siempre con ideas nuevas y con ganas de superarse, es un placer trabajar contigo. Gracias por todo Carlos. Sin ambos directores, este trabajo no habría sido posible. Gracias de nuevo.

Quiero dar las gracias a todos los profesores del Departamento de Fisiología. También al Profesor Librado Carrasco, gran docente, colega veterinario y amigo.

Agradecer también a mi familia que de una u otra forma han hecho que pueda llegar hasta aquí. En especial a mi madre, que siempre me apoyó en todo y la que más me ha ayudado en los momentos difíciles. Gracias mamá. A mis abuelos, sobre todo a mi abuelo Curro (D.E.P.), ilustre ganadero, quien me inculcó de pequeño el valor del trabajo constante y responsable, con quién aprendí a manejar el ganado, con quien aprendí lo que era el celo del potro en la yegua. Gracias abuelo. También quiero recordar a mi abuelo José (D.E.P.), siempre atento y orgulloso de mis logros académicos. En definitiva, de todos los que me han ayudado y se han alegrado por mí.

Gracias a María José, por ayudarme y apoyarme siempre. Por estar ahí en los momentos malos y siempre que hacía falta para cumplir los objetivos del trabajo diario.

También quiero dar las gracias al personal de la Yeguada Cárdenas, en especial a Don Miguel Ángel Cárdenas Osuna (D.E.P.), quien apostó por mí, un joven ilusionado y dispuesto a darlo todo. Siempre puso a mi disposición toda clase de recursos para hacer de su Yeguada la mejor del mundo. Gracias al inmenso número de animales que puso a mi disposición, pude investigar y analizar nuevos tratamientos para mejorar la fertilidad equina. Gracias Don Miguel. A su esposa, Doña María del Carmen Jiménez-Alfaro y Salas, gracias por confiar en mi la reproducción de sus ejemplares para

continuar el legado de la Estirpe Cárdenas. Gracias a Manolo Corzo, experto en la gestión de documentación, informática y siempre veloz en resolver problemas. También dar las gracias a Manuel Priego Gómez, el “yegüero”, por quedarse después de su horario de trabajo para ecografiar e inseminar yeguas, por ayudarme siempre en los días en que todo el trabajo se junta y no hay tiempo para nada.

Gracias a todos mis clientes por confiar en mí, sobre todo a Doña Rosario Rodríguez de Torres y Doña Rocío Monsalves.

Gracias a Ander, un fenómeno en el laboratorio, aplicado, responsable y siempre dispuesto a trabajar.

Gracias a varios amigos que de una u otra forma me han ayudado, Jorge, Ana, Fran y Elena.

Muchas gracias a todos.

APORTACIONES AL CONOCIMIENTO REPRODUCTIVO EN EL CABALLO PURA RAZA ESPAÑOL: fertilidad, vitrificación de esperma y monitorización de la gestación.

Memoria de Tesis Doctoral presentada por Fernando Requena Domenech, Licenciado en Veterinaria, para optar al grado de DOCTOR EN VETERINARIA

TESIS DOCTORAL COMO COMPENDIO DE PUBLICACIONES

1. **Publicaciones en revistas incluidas en el Journal Citation Report (JCR).**

ARTÍCULO 1

- Pérez-Marín CC, **Requena FD**, Arando A, Requena L, Requena F, Agüera EI. Short-term tolerance of equine spermatozoa to various abiotic factors. Reprod Dom Anim. 2018; 00:1–11. https://doi.org/10.1111/rda.13142

ARTÍCULO 2

- C.C. Pérez-Marín, **F.D. Requena**, A. Arando, S. Ortiz-Villalón, F. Requena, E.I. Agüera. *Effect of trehalose- and sucrose-based extenders on equine sperm quality after vitrification: Preliminary results.* Cryobiology, 2018; 80:62-69. https://doi.org/10.1016/j.cryobiol.2017.12.002

ARTÍCULO 3

- **Fernando D. Requena**, Estrella I. Agüera, Francisco Requena, Carlos C. Pérez-Marín. *Transrectal ultrasonographic measurements of the combined thickness of the uterus and placenta in Spanish Purebred mares.* Animal Reproduction, 2017; 14 (Suppl.1): 1278-1284. DOI: 10.21451/1984-3143-AR0029.

2. **Comunicaciones en congresos nacionales e internacionales.**

- **Requena F.D.**, Agüera E.I. *Determinación de la eficiencia reproductiva en la yegua Pura Raza Española*. I Congreso Científico de Investigadores en Formación en Agroalimentación y II Congreso Científico de Investigadores en Formación de la Universidad de Córdoba Córdoba, 8 y 9 de mayo de 2012. Universidad de Córdoba.

- F. Requena, **F.D. Requena,** J Miró, P. Tovar, B. Escribano, E.I. Agüera. *Incidence of intra-uterine fluid on pregnancy rates post insemination in P.R.E. mares*. 11º Congreso Internacional de la Asociación Española de Reproducción Animal. Córdoba, 13-16 de junio de 2012. Reprod Dom Anim 47 (Suppl. 3), 114; doi: 10.1111/j.1439-0531.2012.02044.x

- **F.D. Requena**, F. Requena, C.C. Pérez-Marín, B. Escribano, P. Tovar, E.I. Agüera. *Uterine edema and pregnancy rates in Andalusian and Hispano-Arabian mares: a comparative study*. 11º Congreso Internacional de la Asociación Española de Reproducción Animal. Córdoba, 13-16 de junio de 2012. Reprod Dom Anim 47 (Suppl. 3), 110; doi: 10.1111/j.1439-0531.2012.02044.x

- **F.D. Requena**, E.I. Agüera, J. Miró, B. Escribano, P. Tovar, F. Requena. *Mejora de la eficiencia reproductiva en la yegua Pura Raza Española a través del diagnóstico de placentitis por ultrasonografía*. 13º Congreso Internacional de Medicina y Cirugía Equina. Sevilla, 23 y 24 de noviembre de 2012.

- **F.D. Requena,** F. Requena, B. Escribano, P. Tovar, E.I. Agüera. *Diagnóstico de placentitis por ultrasonografía en la yegua Pura Raza Española: un método no invasivo para mejorar la eficiencia reproductiva.* II Congreso Científico de Investigadores en Formación en Agroalimentación y III Congreso Científico de Investigadores en Formación de la Universidad de Córdoba. Fecha 9 y 10 de abril de 2013. Universidad de Córdoba.

- **Requena F.D.,** Agüera E.I., Miró J., Escribano B., Tovar P., Pérez-Marín C.C., Requena F. *Measures of the combined thickness of the uterus and placenta in pregnants Spanish Purebred mares*. Comunicación Oral. 12º Congreso Internacional de la Asociación Española de Reproducción Animal. Alicante, 16-18 octubre de 2014. Reprod Dom Anim 49 (Suppl. 4): 99; DOI: 10.1111/rda.12402.

- **F.D. Requena,** F. Requena, C.C. Pérez-Marín, L. Requena, M.J. Cantillo, J. Miró, E.I. Agüera. *Medida de espesor conjunto útero-placenta como bioindicador de salud fetal y neonatal*. 16º Congreso Internacional de Medicina y Cirugía Equina. Sevilla, 20 y 21 de noviembre de 2015.

- **F.D. Requena**, E.I. Agüera, C.C. Pérez-Marín, F. Requena. *Comparison between N- Acetylcisteine and Dimethyl sulfoxide treatments in Spanish Purebred mares with persistent endometritis*. 14º Congreso Internacional de la Asociación Española de Reproducción Animal. Barcelona 9-11 de noviembre de 2017. Reprod Dom Anim 52 (Suppl. 4): 84; DOI: 10.1111/rda.13065.

3. Artículo científico derivado de esta tesis doctoral aún no publicados.

ARTÍCULO 4

- **F.D. Requena**, E.I. Agüera, C.C. Pérez-Marín. Reproductive efficiency of Spanish Purebred mare in Spain.

ÍNDICE

1. INTRODUCCIÓN

1.1. EL PURA RAZA ESPAÑOL Y LA FERTILIDAD EN LA ESPECIE EQUINA

El caballo desde su domesticación hasta nuestros días, ha pasado de ser una herramienta de trabajo a jugar un papel fundamental en el ocio, a través de los espectáculos y competiciones deportivas ecuestres (Leckie, 2001; Pritchard y cols., 2005). Por eso es fácil comprender que la magnitud e importancia del sector equino va de la mano de sus logros en competiciones, tanto deportivas como morfológicas (Koenen y cols., 2004). En junio del 2013, la Real Federación Hípica Española publicó el "Estudio del Impacto del sector ecuestre en España", realizado por Daemon Quest by Deloitte, donde el sector ecuestre tiene un impacto económico de 5.304 millones de euros, lo que representa un 0.51% del Producto Interior Bruto. De éstos, un 64% corresponde al gasto en actividades en que incurren los agentes involucrados, es decir, criadores, propietarios, jinetes/amazonas, club, hipódromos y veterinarios. Y es el Pura Raza Español (PRE) la raza equina más importante en España, encontrándose actualmente en auge y con grandes admiradores en todo el mundo.

Según el Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente (MAPAMA), las bases para el impulso del caballo PRE fueron obra del Rey Felipe II, quien ordenó la creación de las caballerizas reales de Córdoba donde agrupó los mejores sementales y yeguas de las provincias que bordeaban el Guadalquivir, que por aquel entonces eran las más prolíficas en la cría de caballos. A fecha de 31 de diciembre de 2017, el censo total del caballo PRE asciende a un total de 239813 ejemplares, de los cuales, 190804 se encuentran en España y 49009 en 62 países distintos, tanto de la Unión Europea como en Países Terceros. El caballo PRE representa el 30% de toda la cabaña equina de España y el 85% del total de razas puras. En España, la Comunidad Autónoma de Andalucía es la que mayor censo posee (79814 ejemplares). En las Tablas 1 y 2 se resumen las características productivas y los datos censales del caballo PRE.

Características	
Tendencia evolutiva de la población:	EXPANSIÓN
Fiabilidad:	MUY FIABLE
Tendencia evolutiva de hembras reproductoras:	EXPANSIÓN
Número de hembras que han parido en pureza en el último año:	8.794
Tendencia evolutiva del número de ganaderías activas:	EXPANSIÓN
Tamaño medio de las ganaderías activas:	7,35
Nº nacimientos al año:	8.872
Nº de análisis de marcadores genéticos para filiación realizados en el año:	10.435
Nº controles de filiación realizados en el año de referencia:	10.140
Nº total de animales activos en LG y con análisis de marcadores genéticos realizados:	239.813
Nº total de Machos inscritos durante el año en el registro de nacimientos de la sección principal del libro genealógico:	5.069
Nº total de Hembras inscritas durante el año en el registro de nacimientos de la sección principal del libro genealógico:	5.071
Hembras que han pasado al Registro Definitivo durante el último año:	3.096
Distribución geográfica:	75% DE LA POBLACIÓN EN UN RADIO >50 KM
Banco de Germoplasma:	MEDIO

Tabla 1. *Características productivas de la raza equina caballar Pura Raza Española (fuente: MAPAMA)****.***

CCAA	Total reproductores		Total animales		Total	Nº Ganaderías
	Hembras	*Machos*	*Hembras*	*Machos*		
ANDALUCÍA	25.351	15.249	42.193	37.621	79.814	10.462
ARAGÓN	690	340	1.162	1.002	2.164	290
CANTABRIA	481	207	874	657	1.531	277
CASTILLA LA MANCHA	4.156	2.631	6.943	6.782	13.725	1.412
CASTILLA LEÓN	4.709	2.652	8.104	7.636	15.740	1.698
CATALUÑA	5.671	3.178	9.961	9.124	19.085	1.503
CEUTA	0	0	0	2	2	2
COMUNITAT VALENCIANA	3.921	2.464	6.565	6.676	13.241	1.712
EXTREMADURA	4.576	2.326	7.728	6.567	14.295	1.489
GALICIA	1.637	760	2.875	2.182	5.057	1.011
ILLES BALEARS	1.223	662	2.169	1.903	4.072	584
ISLAS CANARIAS	196	179	307	390	697	290
LA RIOJA	141	71	238	237	475	86
MADRID	2.149	1.779	3.846	4.513	8.359	1.012
MELILLA	0	0	0	0	0	0
MURCIA	2.336	1.655	3.977	4.291	8.268	1.222
NAVARRA	405	204	738	676	1.414	148
PAÍS VASCO	305	135	491	451	942	138
PRINCIPADO DE ASTURIAS	556	318	1.038	885	1.923	493
Totales	58.503	34.810	99.209	91.595	190.804	23.829
Otros países						
	14.795	10.020	24.883	24.126	49.009	8.809

CCAA	Total reproductores	Total animales	Total	Nº Ganaderías
			Hembras	Machos
	Registro fundacional		0.0	0.0
	Registro auxiliar no reproductores		0.0	0.0
	Registro auxiliar reproductores		0.0	0.0
	Registro nacimientos		10088.0	14106.0
	Registro definitivo		14795.0	10020.0
	Registro de méritos		0.0	0.0

Tabla 2. *Datos censales de caballos Pura Raza Española distribuidos por Comunidades Autónomas y en otros países (fuente: MAPAMA).*

El PRE se encuentra actualmente en expansión y su uso principal es el deporte (alta escuela, doma clásica, doma vaquera y enganches) y los concursos morfofuncionales (MAPAMA, 2017). Su valor económico reside directamente en sus logros y éxitos deportivos, en su morfología, en su funcionalidad y en su pedigrí. De manera generalizada, el caballo ha sido seleccionado en base a sus éxitos deportivos y pedigrí, pero no atendiendo a su fertilidad (Nagy, 2006; Nath, 2011), lo que a veces conlleva a tener que trabajar con ejemplares de baja fertilidad (Pickett y Voss, 1999). El Diccionario de la Real Academia Española define "fertilidad" como "cualidad de fértil" y "fértil" como "dicho de un ser vivo: capaz de reproducirse". Aplicándolo al campo de la Reproducción Animal, la fertilidad hace referencia a la capacidad que un animal tiene para producir descendencia. Ésta engloba varios factores y etapas que deben ser completar tanto machos como hembras, y que le permitirán alcanzar el éxito reproductivo. Para lograr esto, el macho debe producir y eyacular esperma fértil normal. La hembra debe producir, almacenar y ovular ovocitos viables. Además, la hembra también debe disponer de un sistema reproductivo compatible con el transporte del esperma, la capacitación y la fecundación de los ovocitos, el desarrollo embrionario y fetal, y finalmente el nacimiento de neonatos sanos. Así pues, en la fertilidad existen unos determinados puntos clave que deben producirse con éxito para obtener una descendencia sana y viable capaz de perpetuar la especie y que han sido ampliamente investigados: formación de los gametos, fecundación, pre-implantación del embrión, desarrollo fetal post-implantación, nacimiento, crecimiento y desarrollo hasta la madurez sexual (Foote, 2003).

El equino se considera como una especie de pobre fertilidad si se compara con otras especies como los rumiantes. Quizás la alargada duración gestacional, su marcada estacionalidad reproductiva y su incapacidad para gestar más de un potro hayan jugado un papel importante en la baja eficiencia reproductiva que se le atribuye (Nagy, 2006; Nath, 2011). En cambio, los rumiantes pueden llegar a superar el 90% de tasa de nacidos vivos, quizás asociado a la selección basada en su fertilidad y características reproductivas que se ha venido haciendo (Engelken, 1999; Menzies, 1999). Ante este panorama al que se enfrentan los ganaderos, existe un especial interés en mejorar las estrategias de manejo de los animales, así como su entorno para conseguir un mayor éxito reproductivo, esencial para que este sistema de producción sea eficiente (Foote, 2003). Con todo lo expuesto, un objetivo fundamental en cualquier yeguada debería ser, el desarrollo de un programa reproductivo adecuado para producir potros sanos. Mediante la eficiencia reproductiva se valora el manejo de las yeguas y sementales para maximizar el número de potros nacidos en una yeguada (Nath, 2011), además de contribuir de forma decisiva en el éxito económico de la empresa ganadera (Foote, 2003).

1.2. FACTORES IMPLICADOS EN LA FERTILIDAD DE LA YEGUA: EFICIENCIA REPRODUCTIVA

Para medir o cuantificar la eficiencia reproductiva, y de acuerdo con Ginther, (1993), se han estudiado varios parámetros:

-Tasa de fertilización: Porcentaje de oocitos ovulados y fertilizados.

-Tasa de concepción: Sinónimo de la tasa de fertilización y usado frecuentemente de forma errónea para describir la tasa de gestación.

-Tasa de gestación: Porcentaje de yeguas cubiertas y que resultan preñadas en un día específico.

-Tasa de gestación por ciclo: Porcentaje de yeguas cubiertas que están preñadas en un día específico post-ovulación.

-Tasa de gestación al primer ciclo: Porcentaje de yeguas que están preñadas después del primer celo en el que han sido cubiertas.

-Tasa de gestación al final de la temporada: Porcentaje de yeguas que están preñadas al final de la temporada reproductiva.

-Tasa de partos: Porcentaje de yeguas cubiertas que producen un potro vivo.

-Tasa de pérdida embrionaria: Porcentaje de oocitos fertilizados que no sobreviven a los 40 días post-ovulación.

-Tasa de muerte embrionaria temprana: Sinónimo de tasa de pérdida embrionaria.

-Tasa de pérdida fetal: Porcentaje de gestaciones que sobreviven a los 40 días, pero no dan lugar a un potro vivo.

-Tasa de muerte fetal: Porcentaje de gestaciones que sobreviven a los 300 días, pero no dan lugar a un potro vivo.

-Tasa de abortos: Término general que atiende a una pérdida gestacional que debe definirse en relación con la etapa de gestación.

-Servicios por ciclo: Número de veces que una yegua es cubierta en un periodo de celo determinado.

-Servicios por gestación: Número de veces que una yegua es cubierta antes del diagnóstico de gestación positivo.

-Ciclos por gestación: Número de ciclos estrales en los que la yegua es cubierta antes de quedar gestante en una temporada.

De esta forma, la eficiencia reproductiva equina depende de factores relacionados con la yegua, el semental y factores externos.

Existen multitud de estudios sobre eficiencia reproductiva y fertilidad en yeguas en distintas razas como Pura Raza Árabe (Demirci, 1987; Yurdaydin y cols., 1993; Azawi, 2008; Cilek, 2009; Benhajali y cols., 2010; Warriach y cols., 2014), Pura Sangre Inglés (Hevia y cols., 1994; Morris y Allen, 2002; Hemberg y cols., 2004; Allen y cols., 2007; Bosh y cols., 2009; Nath y cols., 2010; Sharma y cols., 2010a), Standardbred (Nath y cols., 2010; Katila y cols., 2010), Caballo Trotón Finlandés (Katila y cols., 2010) o Trotón de sangre fría (Haadem y cols., 2015).

También numerosas investigaciones han estudiado como la eficiencia reproductiva de la yegua puede verse afectada por factores como la edad de la yegua (Morris y Allen, 2002; Allen y cols., 2007; Bosh y cols., 2009; Benhajali y cols., 2010; Katila y cols., 2010; Nath y cols., 2010; Sharma y cols., 2010b), el estado reproductivo de la yegua (Morris y Allen, 2002; Samper y cols., 2002; Hemberg y cols., 2004; Allen y cols., 2007; Bosh y cols., 2009; Benhajali y cols., 2010; Katila y cols., 2010; Nath y cols., 2010; Sharma y

cols., 2010b), el uso de semen fresco, refrigerado o congelado (Jasko y cols., 1992c; Loomis, 2001; Samper, 2001; Sieme y cols., 2003), el sitio de inseminación en el útero y la técnica de inseminación (cuerpo uterino, cuerno uterino, con histeroscopio o guiada transrectalmente intracornual profunda) (Sieme y cols., 2004; Katila T., 2005; Hayden y cols., 2012), la concentración y el volumen de la dosis inseminante (Pickett y Voss, 1975; Squires y cols., 1989; Bedford y Hinrichs, 1994; Morris y cols., 2000; Sieme y cols., 2004; Newcombe y cols., 2005), el número de inseminaciones o cubriciones por ciclo (Sieme y cols., 2003; Allen y cols., 2007; Nath L.C. y cols., 2010), si el celo y la ovulación es natural o inducido/a (Yurdaydin y cols., 1993; Sieme y cols., 2003; Allen y cols., 2007; Sharma y cols., 2010b; Hanlon y Firth, 2012), la estación reproductiva y el año de la estación reproductiva (Morris y Allen, 2002; Hemberg y cols., 2004; Cilek, 2009; Katila y cols., 2010; Haadem y cols., 2015), la acumulación de fluido intrauterino (Allen y cols., 2007; Sharma y cols., 2010a; Lewis y cols., 2015) o el celo del potro (Morris y Allen, 2002; Sharma y cols., 2010a; Blanchard y cols., 2012).

1.3. FACTORES IMPLICADOS EN EL SEMENTAL Y EL SEMEN (CONSERVACIÓN DE ESPERMA)

Son muchos los trabajos que se han realizado sobre el efecto del semental en la fertilidad destacando, entre otros, la edad del semental, la monta directa o inseminación, el número de montas o inseminaciones por ciclo o la tasa de gestación por temporada reproductiva (Morris y Allen, 2002; Hemberg y cols., 2004; Benhajali y cols., 2010; Nath y cols., 2010; Haadem y cols., 2015). Al valorar este efecto del semental, se está realizando indirectamente una valoración de la calidad y fertilidad del semen. Pero gracias al desarrollo y difusión mundial de la inseminación artificial (IA) en la especie equina se ha conseguido incrementar la eficiencia reproductiva en esta especie (Katila, 2005; Aurich, 2012). Luego, mediante la técnica de la IA, el procesamiento de esperma se ha convertido en una práctica habitual en reproducción equina, tanto para realizar análisis de calidad del mismo como para su conservación en refrigeración o criopreservación y posterior uso en IA (Aurich, 2012; Álvarez y cols., 2014).

Durante el procesado del semen refrigerado existen gran cantidad de puntos críticos en los que los espermatozoides pueden sufrir estrés y daños por agentes externos como son la temperatura, la luz, la centrifugación y el pipeteo. Respecto a la temperatura hay que tener en cuenta que la refrigeración del semen debe hacerse lentamente aproximadamente 0.5°C/min, llegando a un rango de temperatura de almacenamiento de entre 4-6°C, el cual se determinó como óptimo para mantener la motilidad y la fertilidad del semen (Varner y cols., 1988; Varner y cols., 1989; Moran y cols., 1992). Esto es debido a que el enfriamiento disminuye la actividad metabólica de

los espermatozoides y reduce el crecimiento y la actividad microbiana. Sin embargo, si la inseminación se lleva a cabo dentro de las 12 horas posteriores a la recogida y procesado del semen, éste puede ser almacenado a temperatura ambiente (Varner y cols. 1989; Love y cols., 2002). La fertilidad del semen refrigerado se mantiene sobre unas 24-48 horas (Jasko y cols., 1992b) y almacenado a una temperatura de 5ºC mantiene una fertilidad similar a la del semen fresco (Jasko y cols., 1992a).

El factor temperatura se ha investigado ampliamente en caballos. Hay multitud de estudios relacionados con la temperatura óptima a la que el semen refrigerado puede ser conservado manteniendo una calidad adecuada (Varner y cols., 1988; Varner y cols., 1989; Moran y cols., 1992). Otros miden el efecto del tiempo de almacenamiento junto a la temperatura (Love y cols., 2002), la temperatura junto a un determinado diluyente (INRA96) (Vidament y cols., 2012), el efecto de la temperatura y las tasas de preñez (Cuervo-Arango y cols., 2015), las tasas de enfriamiento y temperaturas de almacenamiento dentro de los distintos tipos de contenedores de transporte de semen que existen (Brinsko y cols., 2000).

Sin embargo, no hay bibliografía descrita en equinos sobre los factores luz y estrés físico (pipeteo). En otras especies podemos encontrar algunos estudios sobre cómo estos factores pueden afectan la calidad espermática. Respecto a la luz, se describe cómo la radiación con luz láser al esperma de ratón mejora su penetración (Cohen y cols., 1998) y cómo la luz láser (con distintas longitudes de onda) induce la formación de óxido nítrico en espermatozoides de bovino, jugando un papel importante en la motilidad y en la reacción del acrosoma (Ankri y cols., 2010). Respecto al estrés físico,. se describe el efecto del pipeteo (Kim y cols., 2013) y la centrifugación (Ferrer y cols., 2012) sobre la función mitocondrial y el incremento de las especies reactivas de oxígeno (ERO) de los espermatozoides (Agarwal y cols., 1994; Guthrie y Welch 2006; Aitken y cols., 2010). Así mismo, Varisli y cols. (2009) compararon el esperma de rata con el de ratón, carnero, verraco y toro sometidos a diversos experimentos, entre los que se incluía el pipeteo.

Ante la importancia que tiene el esperma equino refrigerado y la falta de evidencia acerca del efecto de varios agentes abióticos sobre éste, se planteó un estudio que derivó en la publicación del Artículo 1, en el que la hipótesis fue que el semen fresco debe ser más resistente a agentes estresantes que aquel mantenido durante 24h. a 15ºC, ya que el tiempo de refrigeración debe mermar su actividad y calidad. Y así mismo, algunos factores abióticos como la luz, el estrés físico (pipeteo) y el choque térmico podrían reducir la calidad del esperma equino fresco y refrigerado durante 24h.

Durante el proceso de criopreservación de esperma hay dos factores críticos que

determinan el éxito de esta técnica, como son la temperatura y los medios crioprotectores (CP). Las muestras de esperma son sometidas a un cambio drástico de temperatura, pasando desde temperatura ambiente a -196°C, siendo necesaria la participación de CP para proteger a los espermatozoides. Entre las alteraciones que sufre el espermatozoide durante su criopreservación destacan el shock térmico, deshidratación, toxicidad de los medios CP, formación de hielo intracelular, fluctuaciones del volumen celular o alteraciones en el equilibrio metabólico (Kadirve y cols., 2009). Dichas alteraciones están asociadas con la reducción de la motilidad y reducción del potencial mitocondrial, rupturas de la membrana plasmática y fragmentación del ADN, reduciendo así la eficiencia reproductiva tras su uso en IA (O'Connell y cols., 2002; Baumber y cols., 2003; Barbas y cols., 2009).

Según la velocidad de congelación se usan varios métodos de criopreservación de semen como la congelación convencional con descongelación lenta o rápida, la liofilización (o dry-freezing) (Olaciregui y cols., 2016) y la congelación ultra-rápida o vitrificación (Vizuete y cols., 2014, Arando y cols., 2017, Pérez-Marín y cols. 2017).

Para alcanzar resultados óptimos tras la criopreservación, los espermatozoides deben ser protegidos mediante CP, describiéndose dos tipos básicos:

- Crioprotectores permeables, como por ejemplo el glicerol o el etilenglicol, que poseen bajo peso molecular, atraviesan la membrana espermática y protegen a la célula de las lesiones producidas por la congelación a velocidades lentas (Sandoval, 2005). Todos estos compuestos deshidratan la célula penetrando en ella y protegiendo el citoplasma. Presentan diferente toxicidad dependiendo del tipo, concentración, temperatura y tiempo de exposición (Cuevas-Uribe y cols., 2011).

- Crioprotectores no permeables, como por ejemplo la sacarosa o la trehalosa, que presentan un alto peso molecular, promueven la deshidratación celular, aumentan la viscosidad y son útiles en velocidades altas de congelación (Sandoval, 2005). Estos compuestos extraen el agua libre intracelular utilizando la diferencia de presión osmótica sin penetrar a la célula (Cuevas-Uribe y cols., 2011).

La vitrificación de esperma es una técnica de criopreservación que combina una congelación ultrarrápida junto a alta viscosidad de los medios y pequeños volúmenes para así evitar la formación de cristales intracelulares de hielo (Vizuete y cols., 2014). Numerosos estudios han sido publicados recientemente sobre la vitrificación de esperma en peces (Cuevas-Uribe y cols., 2011; Kása y cols., 2016), perros (Sánchez y cols., 2011), gatos (Vizuete y cols., 2014; Buranaamnuay, 2017), conejos (Rosato y

cols., 2013), humanos (Isachenko y cols., 2003, 2004, 2012) y carneros (Jiménez-Rabadán y cols., 2015; Arando y cols., 2017), lo que demuestra el interés que actualmente despierta este campo de la espermatología. Sin embargo, uno de los aspectos negativos de esta técnica es la necesidad de emplear concentraciones muy elevadas de CP, lo cual afecta negativamente a la mayoría de las especies (Asgari y cols., 2012), llegando a ser tóxicos dependiendo del tiempo de exposición al mismo. Existen algunos estudios de vitrificación de semen en perro (Sánchez y cols., 2011) y en hombre (Isachenko y cols., 2004) con resultados óptimos, en los que se considera que, entre otras características, el tamaño y la forma de la cabeza de los espermatozoides puede estar directamente relacionado con el éxito de la vitrificación. El espermatozoide equino presenta una cabeza con morfología elíptica y pequeño tamaño (parecido al perro y al humano) (Garner, 2006), lo que supone un reducido porcentaje de contenido de agua y una mayor estabilidad frente al shock térmico, reduciéndose el posible daño a nivel del ADN. En este sentido, se puede considerar que los espermatozoides humano, equino, canino y felino, cuya cabeza es plana y pequeña con DNA más compacto, presentan mayor estabilidad.

1.4. OTROS FACTORES RELACIONADOS CON LA FERTILIDAD EN LA YEGUA: PLACENTITIS

La clínica reproductiva equina requiere la realización de una monitorización y seguimiento ecográfico de la gestación para determinar si existe alguna patología que pudiera derivar en una pérdida de la gestación o alteración postnatal, reduciendo la eficiencia reproductiva. La principal causa de aborto descrita en la yegua es la placentitis ascendente (Giles y cols., 1993). Esta patología aparece en los últimos meses de gestación, cuando las bacterias alcanzan el útero principalmente desde el tracto reproductivo inferior (Cummins y cols., 2008), causando infección en la estrella cervical y en la región corioalantoidea. Esta infección provoca ulceración y engrosamiento del corioalantoides (Platt, 1975), con una alta expresión de citoquinas pro-inflamatorias y liberación de prostaglandinas, lo que inducirá la aparición de contracciones uterinas y finalmente el aborto (Dudley y Trautman, 1994; Pollard y Mitchell, 1996; LeBlanc y cols., 2002; LeBlanc, 2004). Esta infección también puede derivar en partos prematuros, nacimiento de potros débiles o muertos, e incluso ocasionar daños futuros en las yeguas, dificultando su correcta funcionalidad reproductiva en posteriores temporadas (Troedsson y cols., 1997).

Con objeto de determinar si existe riesgo de pérdida fetal en équidos se ha empleado a partir de los 7-8 meses de gestación la determinación de perfiles endocrinos (Rossdale y cols., 1991; Stawicki y cols., 2002; Morris y cols., 2007) y la ecografía transrectal y/o transabdominal (Renaudin y cols., 1997; Bucca y cols., 2005; Carrick y cols., 2010; Löf y cols., 2010; Requena y cols., 2012; Requena y cols., 2013; Requena y cols., 2014;

Requena y cols., 2015).

Como La región de la estrella cervical es la que de forma más frecuente se altera en yeguas con placentitis ascendente, y en estos casos, la ecografía transrectal es la herramienta de elección para valorar el feto y los cambios placentarios. Por ello, se considera que las medidas del espesor conjunto útero-placenta (ECUP) podrían ser usadas para identificar yeguas con riesgo de desarrollar placentitis ascendente u otras alteraciones placentarias. Reanudin y cols. (1997), describieron por primera vez la técnica de determinación del ECUP en yeguas y publicaron unos valores normales para el periodo gestacional comprendido entre el día 180 y 330. Sin embargo, desde entonces, son pocos estudios los que han profundizado en esta técnica, destando que bajo número de animales empleados y la diversidad de razas. De forma estándar, se aceptó que la médida ECUP no debe ser superior a la edad gestacional (en meses) del feto expresada en milímetros, más uno, de forma similar a lo que se hace en humanos, aunque esta regla resulta antigua e imprecisa (Reef y cols., 1995).

Giles y col (1993) informaron que la incidencia de las pérdidas fetales en diferentes razas equinas por placentitis ascendente bacteriana (muerte fetal, abortos y potros nacidos muertos) alcanzaba un 20%, mientras que otros autores cuantifican estas pérdidas en un 10% en la raza Pura Sangre (Smith y cols., 2003). LeBlanc (2010) afirmó que la placentitis afecta aproximadamente a un 3-7% de yeguas gestantes, de acuerdo con lo publicado por Troedsson y Zent (2004) en yeguas Pura Sangre.

1.5. JUSTIFICACIÓN DE LA INVESTIGACIÓN DE LA TESIS DOCTORAL

Es fácil comprender la gran importancia y repercusión nacional e internacional que presenta esta raza equina, el Pura Raza Español, debido a su amplia distribución en 62 países distintos, tanto de la Unión Europea como en Países Terceros, y constituye el 30% de toda la cabaña equina de España y el 85% del total de razas puras. Y todo esto refleja la importancia económica que tiene en el sector equino. Y como resultado de su cada vez mayor participación en eventos deportivos (concurso morfo-funcionales y de doma clásica, entre otras), se ha hecho imprescindible la obtención de los mejores ejemplares, a menudo gracias a la implementación de las últimas técnicas de reproducción en esta raza.

Entre las numerosas preguntas que reciben los veterinarios por parte de los propietarios de caballos, a menudo quieren conocer cuál es la probabilidad de que su yegua quede gestante con el semen de un determinado semental, cuál es la calidad del semen de su semental o si es normal que a pesar de que las yeguas se hayan diagnosticado como gestantes, luego no llegan a finalizar su gestación. De esta

inquietud mostrada por los propietarios y por dar un servicio profesional cada vez mejor surge la presente Tesis Doctoral, que trata de aclarar cómo mejorar la fertilidad en el caballo PRE. Pero dado que el término "Fertilidad" abarca un amplio campo en la Reproducción Equina, hemos intentado arrojar luz en algunos de estos apartados.

Sabemos que la fertilidad puede ser una complicada ecuación en la que interviene la yegua, el semental y otros factores tales como el método de cubrición, la técnica de inseminación, el tipo de dosis seminal empleada, los tratamientos instaurados, el técnico veterinario que realiza los procedimientos, etc. Y con este panorama, nos preguntamos cómo debemos determinar la eficiencia reproductiva en el PRE para, seguidamente, llevar a cabo comparaciones con otras razas equinas**, tal y como se analiza en el ARTÍCULO 4**. Este artículo ofrece un considerable tamaño muestral y analiza numerosos factores que afectan a la fertilidad de la yegua PRE a lo largo de varias estaciones reproductivas. En este sentido, destaca un estudio realizado por Vivo y cols. (1985) y, más recientemente, otro realizado por Akourki y cols. (2017) que evalúa la actividad ovárica y la longitud de la gestación en diferentes grupos de yeguas. En el artículo que forma parte de esta Tesis se analiza la tasa de gestación en PRE y la influencia de ciertos factores. Hay que reseñar que este trabajo es fruto de la toma de datos que se han hecho a lo largo de los programas de inseminación artificial desarrollados en el Centro de Reproducción Equina Miguel Ángel Cárdenas.

En el **ARTÍCULO 1** se investigó la influencia de diversos factores sobre la calidad espermática equina tras su refrigeración. Dicho estudio resulta muy novedoso y aplicativo, ya que no existían trabajos similares en équidos.

Debido al gran interés que existe acerca de nuevos protocolos de criopreservación equina, en el **ARTÍCULO 2** se abordó por primera vez la técnica de vitrificación como herramienta para conservar esperma equino. Aunque los resultados obtenidos referentes a calidad espermática tras la vitrificación fueron pobres, este estudio abre una puerta para seguir investigando en esta línea y poder mejorar la vitrificación de semen equino hasta llegar a hacerla una técnica fiable y práctica.

Las yeguas gestantes se evaluaron para determinar la existencia de problemas placentarios, y poder así maximizar la eficiencia reproductiva en la yegua. En el **ARTÍCULO 3** se realizó la primera descripción de las medidas ECUP en yeguas PRE, que no habían sido publicadas hasta la fecha.

2. OBJETIVOS

Objetivo general

El objetivo general planteado en la presente Tesis Doctoral ha sido determinar la fertilidad que presenta el Pura Raza Español y analizar diferentes factores implicados en ésta, de modo que esto ayude para la toma de decisiones en programas de inseminación artificial con ejemplares de élite, maximizando los éxitos reproductivos y económicos de las Yeguadas y Centros de Reproducción Equina.

Objetivos específicos

✓ Objetivo 1. Determinar cómo influyen algunos factores abióticos en el procesado y conservación del semen refrigerado equino **(Artículo 1)**.

✓ Objetivo 2. Evaluar nuevos procedimientos de criopreservación espermática, como es la vitrificación, en semen equino utilizando sacarosa o trehalosa como crioprotectores **(Artículo 2).**

✓ Objetivo 3. Establecer los valores normales de medidas del espesor conjunto útero-placenta en yeguas PRE **(Artículo 3).**

✓ Objetivo 4. Determinar la eficiencia reproductiva en la yegua PRE y analizar los factores implicados **(Artículo 4).**

3. RESULTADOS

Los resultados obtenidos en la presente Tesis Doctoral se muestran en forma de artículos, tres de ellos ya aceptados y publicados, y otro pendiente de ser enviado. A continuación se citan dichos artículos:

ARTÍCULO 1

Pérez-Marín CC, **Requena FD**, Arando A, Requena L, Requena F, Agüera EI. Short-term tolerance of equine spermatozoa to various abiotic factors. Reprod Dom Anim. 2018; 00:1–11. https://doi.org/10.1111/rda.13142

ARTÍCULO 2

C.C. Pérez-Marín, **F.D. Requena**, A. Arando, S. Ortiz-Villalón, F. Requena, E.I. Agüera. *Effect of trehalose- and sucrose-based extenders on equine sperm quality after vitrification: Preliminary results.* Cryobiology, 2018; 80:62-69. https://doi.org/10.1016/j.cryobiol.2017.12.002

ARTÍCULO 3

Fernando D. Requena, Estrella I. Agüera, Francisco Requena, Carlos C. Pérez-Marín. *Transrectal ultrasonographic measurements of the combined thickness of the uterus and placenta in Spanish Purebred mares.* Animal Reproduction, 2017; 14 (Suppl.1): 1278-1284. DOI: 10.21451/1984-3143-AR0029.

ARTÍCULO 4

FD Requena, EI Agüera, CC Pérez-Marín. Reproductive efficiency of Spanish Purebred mare in Spain. En preparación.

ARTÍCULO DE INVESTIGACION 1

Short-term tolerance of equine spermatozoa to various abiotic factors

Pérez-Marín CC, **Requena FD**, Arando A, Requena L, Requena F, Agüera EI

Reprod Dom Anim. 2018; 00:1–11
https://doi.org/10.1111/rda.13142

Received: 24 October 2017 | Accepted: 22 December 2017
DOI: 10.1111/rda.13142

ORIGINAL ARTICLE

WILEY Reproduction in Domestic Animals

Short-term tolerance of equine spermatozoa to various abiotic factors

CC Pérez-Marín[1] | FD Requena[2] | A Arando[3] | L Requena[1] | F Requena[2] | El Agüera[2]

[1]Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Cordoba, Cordoba, Spain

[2]Department of Cell Biology, Physiology and Immunology, University of Cordoba, Cordoba, Spain

[3]Department of Genetics, University of Cordoba, Cordoba, Spain

Correspondence
Carlos C. Perez-Marin, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Cordoba, Cordoba, Spain.
Email: pv2pemac@uco.es

Contents

The aim of this study was to determine the effects of various abiotic factors, such as light, physical stress (pipetting) and thermal shock, on the quality of fresh and cooled equine sperm. In experiment I, four sperm aliquots were subjected to different light exposures: (i) protected control samples (CTRL), (ii) exposed to UV light at 10 cm (UV10), (iii) exposed to UV light at 20 cm (UV20) and (iv) exposed to laboratory lighting (LAB). In experiment II, four semen aliquots were subjected to repeated pipetting for 0, 10, 20 and 30 times (CTRL, P10, P20 and P30, respectively). In experiment III, four semen aliquots at 15ºC were subjected to thermal oscillations: (i) cooled control sperm at 15ºC (CTRL), (ii) oscillations of 1.9ºC/min to a temperature of 30ºC (T30), (iii) oscillations of 1.4ºC/min, with the temperature rapidly falling until reaching 1.3ºC (T0R) and (iv) oscillations of 1.1ºC/min, with the temperature slowly falling until reaching 4.2ºC (T0S). The results revealed that after 30 min, UV10 and UV20 sperm samples showed significantly ($p < .05$) lower total and progressive motility values, sperm kinematic parameters and mitochondrial potential. After 45 min of exposure, differences were highly significant ($p < .001$). No significant differences ($p > .05$) were found for pipetting or thermal oscillations. The results suggest that, even if equine sperm samples are not handled in the laboratory under optimal conditions, fresh and cooled equine spermatozoa are able to resist the impact of various abiotic stimuli without any reduction in their quality. This study analyses the effect on normospermic samples, but future research could look at the tolerance that asthenozoospermic equine samples have to these abiotic influences.

1 | INTRODUCTION

Abiotic stress is defined as the impact induced by inert factors on cells or other organisms in a determined environment. Although such factors usually exert a negative influence on cells, beneficial effects are also sometimes described (McCarthy, Baumber, Kass, & Meyers, 2010; Rurangwa, Kime, Ollevier, & Nash, 2004). Numerous assumptions have been made about the damage that spermatozoa may endure during handling and management, but few trials have been conducted to determine the true impact of factors such as light, temperature and physical stress.

One of the main precautions taken when working with spermatozoa is to guard against the negative impact of light on sperm samples. However, recent studies suggest that red light or laser light could improve sperm viability and oocyte penetration (Cohen, Lubart, Rubinstein, & Breitbart, 1998; Yeste et al., 2016). Temperature also induces alterations in the sperm function when a drastic drop occurs, and then, a controlled temperature descent is recommended when sperm preservation is carried out (Gao, Mazur, & Critser, 1997). Physical stress can also lead to injuries in sperm cells, although positive effects have been also reported (Kim, Agca, & Agca, 2013; Varisli, Uguz, Agca, & Agca, 2009).

Semen processing is a habitual practice in equine reproduction for evaluating the quality of samples and preparing them for subsequent insemination (Aurich, 2012). Numerous critical points, particularly susceptible to potential damage, can be found in semen processing, and cells can be stressed by external agents such as light, temperature, pipetting and centrifugation. It has been reported that spermatozoa have a species-specific sensitivity to various external agents and that therefore the most advisable method of sperm processing for improving cell quality may vary from species to species (Varisli et al., 2009).

The use of cooled semen offers optimal results in equine artificial insemination, and its preparation and distribution are comparatively easy. Cooled semen techniques have accordingly been developed, also motivated by the difficulties arising from the freezing of some horses' spermatozoa (Vidament et al., 1997). Cooled equine semen maintains a high fertility rate for 24–48 hr (Jasko, Martin, & Squires, 1992), which is comparable to fresh semen (Jasko, Hathaway, Schaltenbrand, Simper, & Squires, 1992). The lowering of temperature should be performed slowly, at approximately 0.5°C/min, until a temperature of 4–6°C has been reached, which is believed to be the optimal temperature for maintaining sperm motility and fertility (Moran, Jasko, Squires, & Amann, 1992; Varner, Blanchard, Love, Garcia, & Kenney, 1988; Varner, Blanchard, Meyers, & Meyers, 1989). Refrigeration of spermatozoa reduces their metabolic activity, and also the growth and activity of bacteria.

Temperature has been widely studied in horse sperm, including its effect on viable storage time (Love, Thompson, Lowry, & Varner, 2002), on extender function (Vidament et al., 2012) and on the fertility rate (Cuervo-Arango, Nivola, Väihkönen, & Katila, 2015). However, no studies have been found on the effect of light and pipetting on horse sperm. In their study of rats, Cohen et al. (1998) reported that laser irradiation induces an improvement in oocyte penetration, and similarly, Ankri et al. (2010) observed that different luminous intensities promote nitric oxide formation in bovine spermatozoa, and it is also involved in sperm motility and the acrosome reaction. Spermatozoa are exposed to physical stress from the time they are ejaculated, and their processing increases the possible cellular damage to these cells, affecting the mitochondrial function and the reactive oxygen species (ROS; Agarwal, Ikemoto, & Loughlin, 1994; Aitken, De Iuliis, Finnie, Hedges, & McLachlan, 2010; Guthrie & Welch, 2006). The most studied physical stress in spermatozoa is that provoked by centrifugation, as described by Ferrer, Lyle, Eilts, Eljarrah, and Paccamonti (2012) in equine sperm. In relation to the pipetting of sperm, Kim et al. (2013) observed that rat sperm is highly sensitive to this source of stress, which accords with studies carried out by Varisli et al. (2009) comparing spermatozoa from rats, mice, rams, boars and bulls.

The main motivations for this study were the importance of the use of cooled semen in the management of horses and the relative lack of information about the ways in which various abiotic factors affect equine spermatozoa. It was hypothesized that fresh equine semen would prove to be more resistant to stressing agents than sperm cooled at 15°C for 24 hr, because their quality could be expected to diminish over time. Thus, the aim of this study was to determine the effect of such abiotic factors as light, physical stress (by pipetting) and thermal oscillations on fresh and cooled equine sperm.

2 | MATERIAL AND METHODS

This study was carried out according to the guidelines of the Spanish legislation on the protection of animals used in experiments (Real Decreto 841/2011) and of the Ethical Committee of University of Cordoba (Spain).

2.1 | Sperm collection and processing

A total of seven ejaculates per donor were collected from four Spanish-breed stallions, aged between 6 and 14 years, which were clinically healthy, with good semen quality and proven fertility. Previous assessment of sperm freezability showed good results in all the stallions. All collections were carried out in an European Union-approved centre for equine semen collection and storage. For the purposes of semen collection, the penis was cleaned with warm water and then dried. The stallions mounted a phantom, and semen was obtained in an artificial vagina (Missouri model, Minitub Ibérica S.L., Tarragona, Spain) at 45–50°C, equipped with a cellulose filter to separate the gel fraction.

After collection, the semen volume and concentration were determined (SpermaCueTM, Minitub Ibérica S.L., Tarragona, Spain). The ejaculates from four stallions were pooled, and Equiplus extender (Minitub Iberica, Tarragona, Spain) was added at a ratio of 1:2. The diluted semen was then centrifuged at 650 *g* for 10 min, and the pellet was resuspended to reach a final concentration of 25×10^6 spz/ml. Two 40 ml aliquots were prepared and sent to the laboratory in a journey lasting 1.5 hr into Neopor boxes containing (for cold sperm samples) or not containing (for fresh sperm samples) cool packs. The experiments were carried out upon the reception of the sperm samples (i.e., approximately 2 hr after collection) and 24 hr later. Then, cold sperm samples were maintained at 15°C into a temperature-controlled system (cell incubator SH-0205, Welson, Korea).

2.2 | Experimental design

Three experiments were planned for the evaluation of sperm tolerance to abiotic factors (Figure 1).

2.2.1 | Experiment 1: effect of light on sperm quality

Four aliquots containing 1 ml of sperm were maintained at room temperature and prepared as follows:

(a) Light-protected samples (CTRL): the samples were loaded in Eppendorf tubes, which were covered in aluminium and exposed to ultraviolet light (UV) at a distance of 10 cm.
(b) UV10: the samples were exposed to UV light at a distance of 10 cm.
(c) UV20: samples were exposed to UV light at a distance of 20 cm.
(d) LAB: samples were exposed to fluorescent (white) light at a distance of 10 cm.

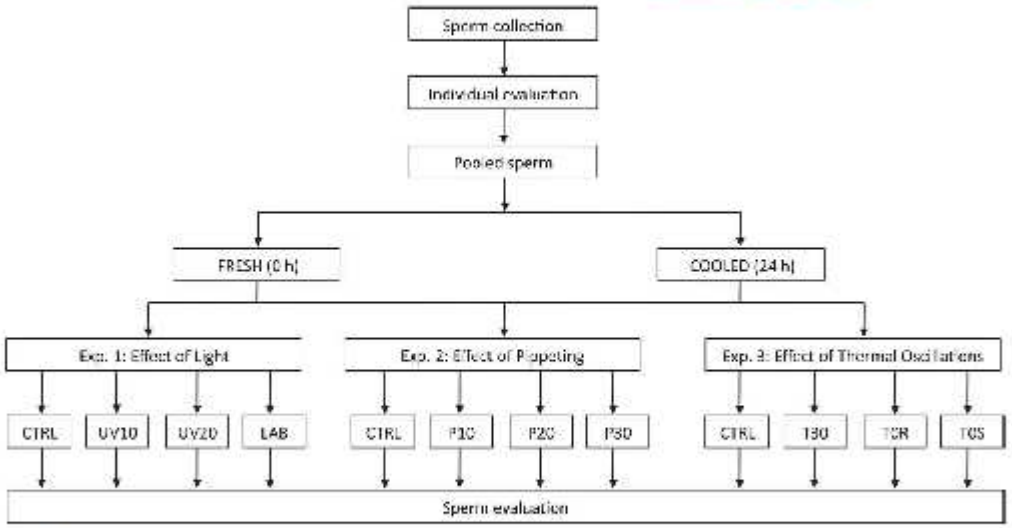

FIGURE 1 Flow diagram representing the experimental design

UV light was obtained from a laminar flow cabin (Telstar, Woerden, The Netherlands) with the following characteristics: UV-irradiated 15.9 w, 0.34 A, 54 v, radiation UV-C 4.9 w.

The experiments were conducted upon reception of the sperm samples and 24 hr later. Sperm motility assessment was carried out at 0-, 15-, 30-, 45- and 60-min intervals, sperm morphology and DNA fragmentation were evaluated at minutes 0 and 60, and finally sperm viability, acrosome integrity and mitochondrial potential were measured at minutes 0, 30 and 60.

2.2.2 | Experiment 2: effect of pipetting of sperm quality

Four aliquots were subjected to different physical stresses:

(a) Control: samples were not manipulated.
(b) P10: samples were pipetted 10 times.
(c) P20: samples were pipetted 20 times.
(d) P30: samples were pipetted 30 times.

For inducing physical stress, samples of 1 ml were collected by an automatic laboratory pipette (1,000 µl capacity) and expelled in the same tube. Sperm assessment was carried out in a way similar to that described in experiment 1.

2.2.3 | Experiment 3: effect of thermal oscillations on sperm quality

The sperm samples were subjected to changes of temperature, and sperm quality was determined at 0 and 40 min. Figure 2 represents the temperature variations the sperm samples were subjected to. Sperm sample (1 ml) was placed in Eppendorf tubes, and four groups were compared:

(a) CTRL: samples maintained at 15°C for 40 min.
(b) T30: samples at 15°C were immersed in a water bath at 30°C (having previously been loaded into a Falcon tube to avoid thermal shock) for 10 min (temperature reached 30°C at a rate of 1.9°C/min). Subsequently, they were again placed into a Neopor box at 15°C, and this sequence was repeated. In brief, the sequence of incubation temperature was 15°C → 30°C (10 min), 15°C (10 min), 30°C (10 min), 15°C (10 min).
(c) T0R: the temperature of sperm samples was reduced to 1.3°C by rapid cooling (at 1.4°C/min) when they were directly placed in contact with crushed ice for 10 min. The sequence was 15°C → 0°C (10 min), 15°C (10 min), 0°C (10 min), 15°C (10 min).
(d) T0S: samples were loaded into a Falcon tube and placed in contact with crushed ice for 10 min; the samples temperature was reduced to 4.2°C by cooling at 1.1°C/min. In this case, the sequence was 15°C → 0°C (10 min), 15°C (10 min), 0°C (10 min), 15°C (10 min).

2.3 | Sperm motility assessment

A computerized analysis system (Integrated Semen Analyser System, Proiser, Valencia, Spain) was used for determining total motility (TM, %) and progressive motility (PM, %) of the sperm samples. A volume of 5 µl was placed on a slide and covered with 20 × 20 mm coverslip. Four fields and a crushed ice minimum of 500 spermatozoa were randomly captured at 10× contrast phase. A total of 25 images per second were acquired, selecting particles with an area between 10

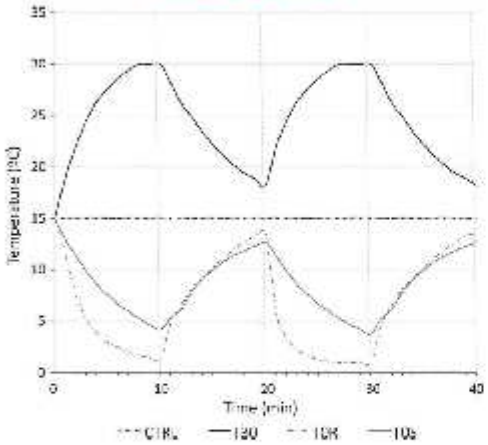

FIGURE 2 Experimental design for studying thermal oscillations in equine sperm samples

and 70 μm², and sperm were considered linearly motile when they deviated <45% from a straight line.

2.4 | Sperm morphology assessment

For the evaluation of sperm morphological abnormalities, Hemacolor staining (Merck, Darmstadt, Germany) was used. Ten microlitres of semen sample was extended along a slide and then stained in accordance with the manufacturer's instructions. The percentage of sperm abnormalities was evaluated by counting 200 sperm cells under 100× oil immersion objective (Olympus, Tokyo, Japan).

2.5 | Flow cytometer measurement

A FACScalibur flow cytometer (Becton Dickinson Immunochemistry, San Jose, CA, USA) was used for analysing sperm viability, acrosome integrity, mitochondrial membrane potential and DNA integrity, as described below. An argon blue laser (488 nm) was used to excite all dyes. Green fluorescence from SYBR-14, fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA), lipophilic cation JC-1 and acridine orange (AO) was read with an FL1 photodetector (530/30 band-pass filter). Red fluorescence of propidium iodide (PI) and orange fluorescence of JC-1 were read with an FL2 photodetector (585/42-nm band-pass filter), and red fluorescence of AO with an FL3 photodetector (630 nm long-pass filter). Approximately 10,000 events of a gated population were counted per sample.

2.5.1 | Sperm viability assessment

Sperm viability was assessed using a LIVE/DEAD® sperm viability kit (Molecular Probes Europe, Leiden, The Netherlands). A volume of 100 μl of diluted sperm was mixed with 150 μl cytometer buffer (Becton Dickinson Inmunochemistry, San Jose, CA, USA) at a final concentration of 3×10^6 sperm/ml. This was then incubated in darkness for 15 min at room temperature with 2.5 μl of SYBR-14 (20 nmol/L final concentration) and 5 μl of PI (10 μmol/L). After incubation, the proportion of live/dead sperm cells was measured: sperm stained with SYBR-14 (green fluorescence) were deemed to be alive, while the sperm stained with PI (red fluorescence) were considered to be dead. Mathematical corrections were used for the estimation of the percentage of events that were not sperm cells, as suggested by Petrunkina, Waberski, Bollwein, and Sieme (2010).

2.5.2 | Acrosome integrity assessment

Acrosome integrity was assessed using FITC-PNA and PI. A total of 100 μl of sperm sample was mixed with 5 μl FITC-PNA (100 μg/ml in DMSO) and 5 μl PI (6 μmol/L final concentration) and incubated at room temperature in darkness for 5 min. Then, 400 μl cytometer buffer was added to the sample, and the result was analysed. PI-negative and FITC-PNA-negative cells were deemed to be sperm with intact acrosome. Also, events that were not sperm cells were mathematically corrected (Petrunkina et al., 2010).

2.5.3 | Sperm mitochondrial membrane potential assessment

Lipophilic cation JC-1 (Molecular Probes, USA) was used for determining mitochondrial potential of spermatozoa. Diluted sperm (100 μl) was mixed with cytometer buffer (150 μl) at a final concentration of 10×10^6 sperm/ml. This was then incubated in darkness for 40 min at 37°C with 25 μl of JC1 (15 μmol/L at final concentration). When spermatozoa have low membrane potential, JC-1 has a monomeric status, and a green emission (FL1) can be detected. After oxidation, aggregates are formed and JC1 is transformed to multimeric status. An orange emission can then be detected (FL2), which indicates that spermatozoa have high membrane potential.

2.5.4 | Sperm DNA assessment

For DNA assessment, the SCSA technique was used. Samples were diluted in 200 μl TNE buffer at a final concentration of 2×10^6 spz/ml. Samples were plunged into liquid nitrogen (LN_2), warmed at laboratory temperature and plunged again into LN_2, finally to be stored at −80°C pending analysis. For analysis, samples (200 μl) were thawed and 400 μl of acid solution was added to induce denaturation of the DNA. After 30 s, 1.2 ml of AO solution was added (6 μg/ml) and incubated in darkness for 3 min. AO staining fluoresces in the green band when combined with the intact double DNA helix (FL1 photodetector) and in the red band when combined with denatured DNA (FL3 photodetector). A total of 5,000 events were counted per sample.

2.6 | Statistical analysis

SPSS 15.0 software (Chicago, IL, USA) was used for the statistical analysis. Data normality was tested using the Kolmogorov–Smirnov

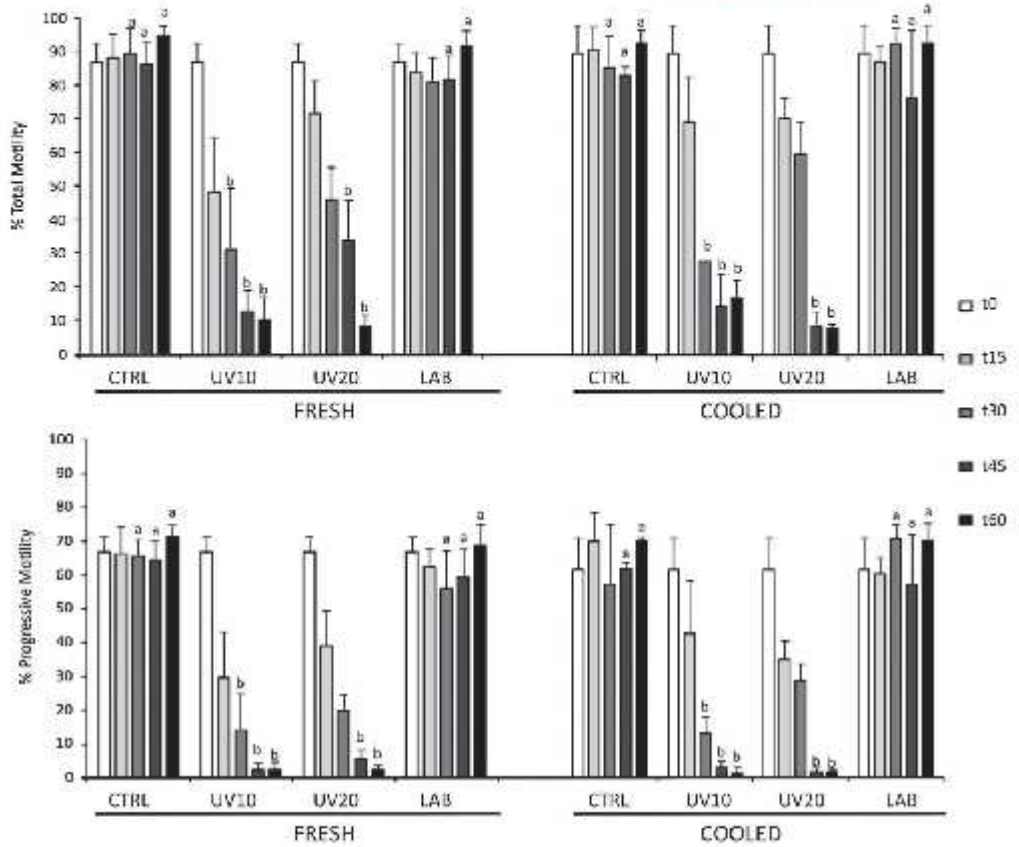

FIGURE 3 Total and progressive sperm motility percentage in fresh (0 hr) and cooled (24 hr) samples exposed to different light sources. Values were determined every 15 min for 1 hr. Different letters (a, b) within type of samples (0 and 24 hr) indicate significant differences ($p < .05$) between light source (CTRL, UV10, UV20 and LAB) at determined times

test. Because the data exhibited a non-normal distribution, arcsine and log transformation was carried out for percentages and continuous data, respectively. One-way ANOVA was used for comparing the groups and storage time. When significant differences were observed ($p < .05$), Bonferroni's correction was carried out. Data are shown as mean ± SEM.

3 | RESULTS

3.1 | Effect of light on sperm quality

Fresh samples analysed after collection and subjected to a variety of light emissions showed significantly lower ($p < .05$) total and progressive motility after 30 min of UV light exposure (Figure 3). In samples cooled for 24 hr, UV-exposed groups showed a significant ($p < .05$) motility reduction after 30 min (Figure 3).

The morphological assessment of sperm did not reveal any significant differences ($p > .05$) between groups (Table 1).

No differences ($p > .05$) were detected between cooled sperm samples at 0 and 24 hr when comparing the same light treatment (Table 1 and Figure 3).

The percentage of high mitochondrial potential was significantly lower ($p < .05$) in samples exposed to UV light. However, fresh samples showed differences at 30 min after UV light exposure, while in samples maintained for 24 hr at 15°C differences appear later, at 60 min (Table 1).

The other sperm parameters analysed did not exhibit any significant differences ($p > .05$).

3.2 | Effect of pipetting on sperm quality

Sperm motility was not affected ($p > .05$) by physical stress induced by repeated pipetting of samples, nor was it influenced by the storage

TABLE 1 Percentage of different sperm quality indicators, expressed as mean ± SEM, in fresh and cooled samples subjected to different light exposure over the time (from 0 to 60 min)

	Fresh			Cooled		
	0 min	30 min	60 min	0 min	30 min	60 min
% Viability						
INITIAL	81.95 ± 6.64			80.11 ± 9.11		
CTRL		74.46 ± 6.42	77.13 ± 5.88		76.3 ± 7.49	78.63 ± 6.17
UV10		73.62 ± 8.98	72.8 ± 7.81		73.86 ± 6.75	54.93 ± 42.08
UV20		74.05 ± 6.1	76.72 ± 4.62		72.45 ± 9.49	77.83 ± 7.39
LAB		74.56 ± 7.14	77.61 ± 6.05		76.91 ± 6.36	78.2 ± 7.84
% Acrosome integrity						
INITIAL	64.67 ± 3.69			70.07 ± 6.94		
CTRL		60.58 ± 6.06	65.66 ± 1.87		64.29 ± 5.25	66.58 ± 6.43
UV10		59.54 ± 8.6	59.26 ± 3.64		63.52 ± 4.44	66.98 ± 7.45
UV20		60.48 ± 6.32	59.79 ± 4.63		65.58 ± 5.65	64.56 ± 7.29
LAB		61.92 ± 6.29	61.37 ± 5.09		64.52 ± 5.75	66.71 ± 6.31
% Low mitochondrial potential						
INITIAL	16.7 ± 6.43			16.54 ± 7.41		
CTRL		25.14 ± 6.84	24.5 ± 7.17		25.21 ± 8.4	22.42 ± 4.17
UV10		61.88 ± 24.33	81.3 ± 14.6		45.81 ± 7.64	68.06 ± 11.15
UV20		43.58 ± 7.72	68.99 ± 11.47		35.17 ± 2.25	56.57 ± 13.78
LAB		26.05 ± 5.74	21.01 ± 5.6		24.42 ± 8.07	21.02 ± 4.3
% High mitochondrial potential						
INITIAL	80.74 ± 7.8			80.79 ± 8.13		
CTRL		72.13 ± 8.38[a]	70.11 ± 10.89[a]		71.28 ± 10.65	75 ± 4.13[a]
UV10		35.24 ± 24.83[b]	15.93 ± 14.92[b]		49.22 ± 8.9	29.56 ± 10.93[b]
UV20		52.88 ± 7.6	26.85 ± 11.01[b]		60.26 ± 1.71	40.37 ± 12.77[b]
LAB		70.66 ± 7.88[a]	76.13 ± 7.35[a]		73 ± 10.03	77.19 ± 4.35[a]
% DNA fragmentation						
INITIAL	9.26 ± 0.65			9.14 ± 0.23		
CTRL			9.12 ± 0.58			9.56 ± 0.96
UV10			10.66 ± 1.04			11.51 ± 1.89
UV20			8.33 ± 0.37			10.01 ± 0.24
LAB			10.44 ± 0.57			11.87 ± 1.26
% Normal morphology						
INITIAL	77.5 ± 5.9			68.3 ± 5.7		
CTRL			75.5 ± 5.8			70.3 ± 7.3
UV10			71.3 ± 6.3			69 ± 5.5
UV20			75.3 ± 3.2			68.3 ± 5
LAB			80.2 ± 5.5			63.7 ± 5.3

INITIAL indicates the sperm quality values at the beginning of the experiment. Different letters (a, b) indicate significant differences ($p < .05$) within column for each sperm parameter analysed.

time (Figure 4). Morphological sperm characteristics did not vary significantly ($p > .05$) when comparing the experimental groups or between storage times (Table 2).

The other sperm parameters (sperm viability, acrosome integrity, mitochondrial potential and DNA fragmentation) were not significantly affected ($p > .05$) by pipetting (Table 2).

3.3 | Effect of thermal oscillations on sperm quality

No significant differences ($p > .05$) were observed for any of the sperm characteristics evaluated when the samples were subjected to thermal variations, and storage time did not affect this result either (Table 3 and Figure 5). When the temperature dropped after contact

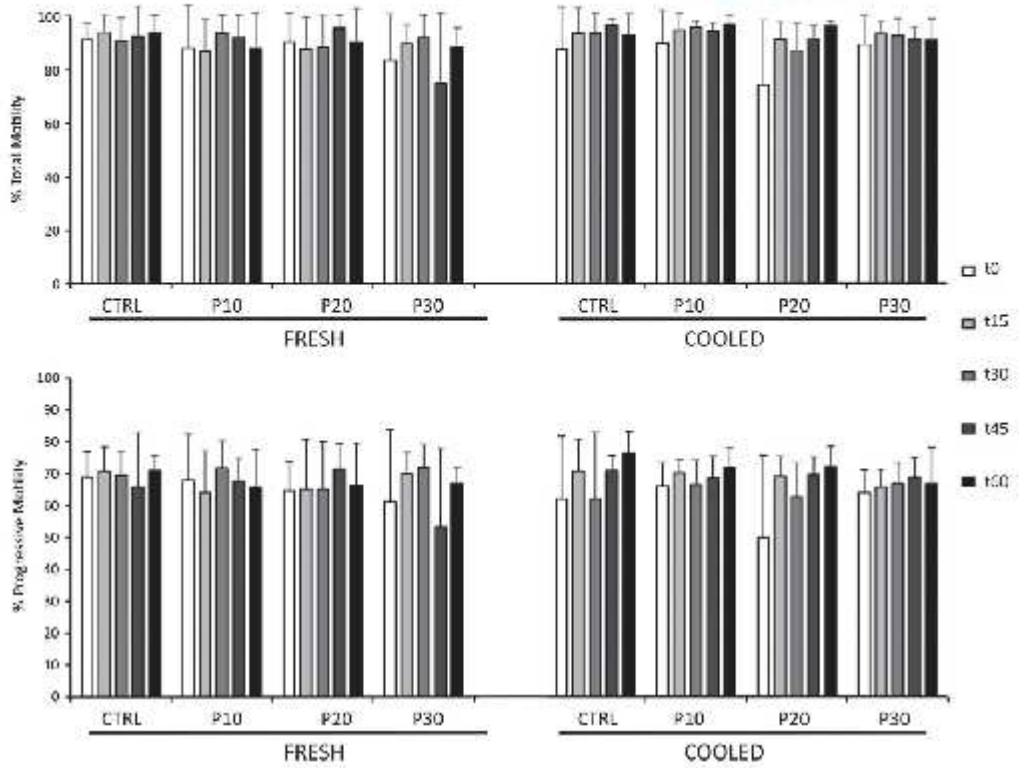

FIGURE 4 Effect over the time (t0–t60) of mechanical stress by 10 (P10), 20 (P20) or 30 (P30) times pipetting in fresh (0 hr) and cooled (24 hr) samples on total and progressive sperm motility

with crushed ice (to approximately 1–4°C), the sperm samples showed slightly lower ($p < .05$) motility (Table 3), viability, acrosome integrity and mitochondrial potential (Figure 5).

4 | DISCUSSION

The present study demonstrates that fresh equine sperm is highly resistant to stress induced by abiotic factors such as light intensity, physical damage by pipetting and thermal variations; equine spermatozoa also maintain their tolerance of the aforementioned stressors during 24-hr cold storage.

It has been reported that light-exposed semen exhibits an increase in ROS production that provokes a diminution in sperm quality (Shahar et al., 2011). However, recent studies suggest that stimulating spermatozoa with light offers beneficial effects in terms of oocyte penetration, increasing fertility and prolificacy (Yeste et al., 2016). UV light has sterilizing power and it has been proposed as a means of avoiding or reducing bacteria in sperm samples, to restrict the use of antimicrobials.

The results of the present study suggest that spermatozoa are not negatively affected by a continuous exposure to UV light during the first 30 min, but they later suffer a drastic negative impact on their motility and mitochondria. However, visible light exposure did not reduce sperm quality, despite continuous exposure for 60 min. When spermatozoa are exposed to a light source, part of the energy reaches the cells as light, but another part is transmitted as heat, and depending on the wavelength, higher or lower degrees of penetration will result. Experiment I demonstrated that sperm samples located further away from a UV light source were less damaged than others placed nearby. These results suggest that spermatozoa could be exposed to a UV light source for 30 min without suffering deleterious effects, taking advantage of its sterilizing power as well as other little-understood effects associated with an increase in the penetrating capacity of spermatozoa.

It has been demonstrated that rat sperm is highly sensitive to physical stress (Kim et al., 2013) and that great care is therefore needed during its processing. The repeated (fourfold) pipetting of mice and rat sperm reduces their viability by half (Varisli et al., 2009). By contrast, studies carried out in boar, bulls and rams showed that

TABLE 2 Percentage of different sperm quality indicators, expressed as mean ± SEM, in fresh and cooled samples subjected 10, 20 or 30 times pipetting over the time (from 0 to 60 min). DNA fragmentation and sperm morphological assessments were carried out at time 0 and 60 min, but not at 30 min

	Fresh			Cooled		
	0 min	30 min	60 min	0 min	30 min	60 min
% Viability						
CTRL	77.58 ± 6.31	77.23 ± 5.31	77.95 ± 6.54	79.49 ± 8.17	81.66 ± 6.5	76.22 ± 5.04
P10	73.58 ± 6.60	74.73 ± 6.26	73.9 ± 7.55	75.6 ± 10.62	74.87 ± 9.15	71.46 ± 8.14
P20	71.17 ± 8.98	73.9 ± 8.08	72.63 ± 7.40	73.22 ± 11.21	71.62 ± 12.49	69.34 ± 9.57
P30	66.89 ± 12.28	71.78 ± 11.62	68.38 ± 10.94	68.06 ± 15.32	67.32 ± 12.56	62.5 ± 11.17
% Acrosome integrity						
CTRL	67.58 ± 5.29	65.15 ± 3.93	63.36 ± 5.27	72.67 ± 5.31	66.55 ± 5.82	64.27 ± 8.11
P10	65.05 ± 5.61	66.07 ± 8.04	64.05 ± 7.52	70.23 ± 7.43	63.74 ± 9.97	63.95 ± 5.59
P20	60.74 ± 5.73	61.83 ± 7.94	63.92 ± 5.60	67.68 ± 7.43	58.87 ± 10.98	59.44 ± 5.06
P30	54.68 ± 9.91	51.16 ± 10.51	52.24 ± 8.10	58.21 ± 9.56	59.38 ± 9.47	54.71 ± 6.78
% Low mitochondrial potential						
CTRL	22.72 ± 7.17	20.18 ± 3.99	22.54 ± 5.09	17.35 ± 7.24	19.77 ± 9.32	19.52 ± 6.29
P10	23.24 ± 5.96	24.05 ± 9.21	26.26 ± 8.16	22.50 ± 9.93	21.64 ± 5.12	22.69 ± 9.41
P20	23.03 ± 5.05	24.91 ± 4.88	24.75 ± 5.65	27.68 ± 6.51	25.15 ± 7.87	30.80 ± 8.42
P30	28.22 ± 5.16	30.41 ± 9.45	31.15 ± 8.78	30.10 ± 9.32	29.57 ± 9.20	33.13 ± 11.32
% High mitochondrial potential						
CTRL	74.63 ± 7.73	76.55 ± 5.11	74.47 ± 6.66	81.05 ± 7.36	78.51 ± 3.69	78.68 ± 6.26
P10	73.39 ± 7.74	71.99 ± 10.96	70.74 ± 9.28	75.57 ± 10.34	75.31 ± 6.95	74.01 ± 8.71
P20	72.93 ± 4.60	70.75 ± 7.32	72.05 ± 7.01	69.72 ± 6.44	70.21 ± 12.03	66.71 ± 8.36
P30	68.92 ± 5.37	64.68 ± 11.01	66.12 ± 10.48	68.02 ± 9.34	65.78 ± 13.21	63.7 ± 10.02
% DNA fragmentation						
CTRL	9.60 ± 1.02	–	9.32 ± 2.01	7.81 ± 1.66	–	10.61 ± 0.67
P10	9.86 ± 2.46	–	9.31 ± 1.70	8.36 ± 0.76	–	11.59 ± 1.03
P20	10.51 ± 2.00	–	8.44 ± 2.75	7.85 ± 1.36	–	10.11 ± 0.90
P30	12.09 ± 4.51	–	10.17 ± 2.58	7.37 ± 1.06	–	10.77 ± 1.56
% Normal morphology						
CTRL	64.33 ± 9.07	–	61.67 ± 12.86	71.21 ± 10.39	–	66.33 ± 8.62
P10	69.33 ± 7.64	–	64.05 ± 14.05	70.33 ± 7.77	–	64.33 ± 7.23
P20	65.33 ± 8.51	–	65.13 ± 13.00	66.33 ± 12.50	–	66.14 ± 7.81
P30	70.67 ± 11.68	–	70.67 ± 10.07	66.33 ± 17.01	–	63.67 ± 13.50

sperm motility is not affected by pipetting (Varisli et al., 2009). The current study demonstrates that horse sperm is also resistant to pipetting stress. Unlike pipetting, other physical stressors involved in centrifugation did not affect rat sperm motility, which leads the present authors to suggest that horse sperm will not be affected by centrifugation, as defined by Ferrer et al. (2012). Pipetting and centrifugation have been found to affect mitochondrial function in the rat (Kim et al., 2013). However, the mitochondrial function of pipetted horse spermatozoa in the present study was not affected by physical stress, supporting the suggestion that horse spermatozoa are more tolerant of physical stress than rat spermatozoa. It has been suggested that the long length of the tail or flagellum of rodent spermatozoa could be linked to their high sensitivity to physical agents (Gao et al., 1997; Varisli et al., 2009). While various studies affirm that mechanical stress increases ROS in the spermatozoa (Agarwal et al., 1994; Aitken et al., 2010; McCarthy et al., 2010), Kim et al. (2013) recently observed that rat spermatozoa showed a reduction in ROS, which suggests that this stressor may activate the antioxidant defence mechanisms of spermatozoa. Mitochondria are the main organelles involved in the intracellular formation of ROS and abiotic stress could reduce the presence of spermatozoa with high mitochondrial potential, which are necessary to produce mitochondrial ATP for providing motility to the spermatozoa (Guthrie & Welch, 2006).

Many studies describe the effect of temperature on spermatozoa, but no impact of thermal oscillations on sperm physiology has been found. In the present study, horse spermatozoa were cooled at

TABLE 3 Percentage of different sperm quality indicators, expressed as mean ± *SEM*, in fresh and cooled samples subjected to different temperature oscillations over the time (from 0 to 40 min)

	Fresh		Cooled	
	0 min	40 min	0 min	40 min
% Viability				
INITIAL	79.84 ± 5.56		80.71 ± 6.63	
CTRL		82.62 ± 6.74		79.32 ± 4.78
T30		80.38 ± 4.97		77.47 ± 7.47
TOR		70.40 ± 0.05		77.29 ± 6.41
TOL		72.03 ± 8.42		79.17 ± 8.16
% Acrosome integrity				
INITIAL	71.18 ± 7.58		68.22 ± 5.34	
CTRL		70.15 ± 6.73		71.95 ± 7.67
T30		70.97 ± 7.28		69.27 ± 5.09
TOR		61.42 ± 0.42		68.88 ± 7.18
TOL		61.24 ± 8.26		69.87 ± 6.73
% Low mitochondrial potential				
INITIAL	17.4 ± 5.6		17.95 ± 7.13	
CTRL		17.92 ± 8.13		19.88 ± 4.17
T30		18.97 ± 7.04		22.98 ± 8.42
TOR		27.63 ± 1.77		22.26 ± 9.32
TOL		26.62 ± 5.72		21.11 ± 5.17
% High mitochondrial potential				
INITIAL	80.84 ± 6.03		79.88 ± 8.18	
CTRL		80.32 ± 8.39		78.1 ± 4.69
T30		79.27 ± 7.06		74.23 ± 9.23
TOR		69.97 ± 1.42		74.51 ± 10.46
TOL		70.66 ± 6.15		74.91 ± 4.82
% DNA fragmentation				
INITIAL	7.76 ± 0.27		9.14 ± 2.14	
CTRL		8.23 ± 0.49		7.48 ± 1.64
T30		7.46 ± 1.49		7.40 ± 0.76
TOR		9.04 ± 1.80		9.16 ± 3.43
TOL		8.20 ± 0.88		9.57 ± 3.51
% Normal morphology				
INITIAL	70.3 ± 9.7		65.3 ± 8.9	
CTRL		69.3 ± 8.7		64.7 ± 11.3
T30		71.7 ± 9.0		63.7 ± 12.9
TOR		70.3 ± 9.3		58.0 ± 13.2
TOL		72.5 ± 9.5		61.0 ± 11.3

INITIAL indicates the sperm quality value at the beginning of the experiment.

15°C and were subsequently either cooled by contact with crushed ice (approximately 1 or 4°C) or warmed into water bath at 30°C before finally being returned to their initial temperature (15°C). The results demonstrate the high tolerance of equine spermatozoa to abrupt changes of temperature when they are diluted with non-penetrating cryoprotectants such as skimmed milk and sugars. Although no significant differences were detected between different temperature oscillations, it was noted that a drastic reduction in temperature (near to 0°C) induced slightly greater cell damage than an increase in temperature (near to 30°C). This observation indicates that the current systems used for transporting equine samples, such as Neopor boxes, are safe for the preservation of spermatozoa. The semen used in this study exhibited good quality, and it can be concluded that its quality was not affected by temperature changes associated with sample management, as can occur in a warm climate or during cold seasons. A potentially fruitful study would be to carry out a similar experiment

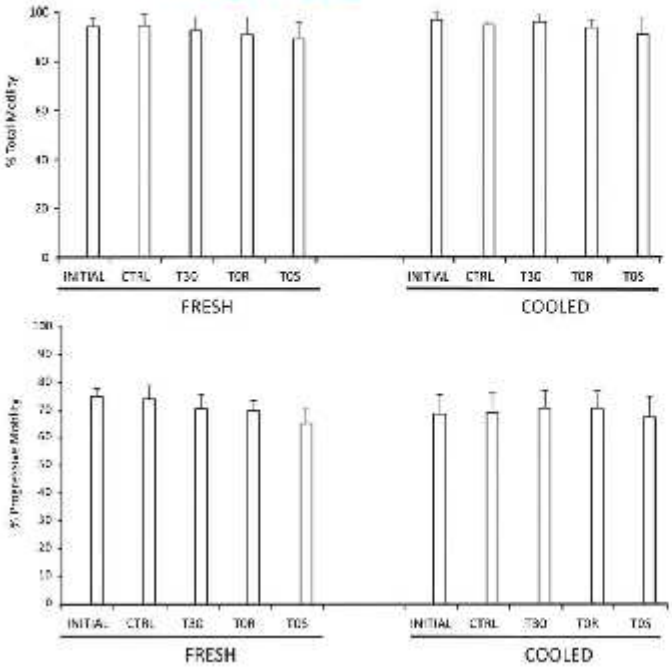

FIGURE 5 Total and progressive sperm motility percentage in fresh (0 hr) and cooled (24 hr) samples during the exposure to different temperature oscillations. INITIAL corresponds to values obtained at time 0, and CTRL, T30, T0R and T0S inform about the values obtained 40 min later, after different temperature treatments

using low-quality sperm, in order to elucidate whether abiotic stress has a negative impact on the quality of these spermatozoa, and subsequently their fertilizing capacity.

5 | CONCLUSIONS

The results demonstrate that equine sperm have a high tolerance of mechanical (induced by pipetting) and thermal (induced by temperature oscillations during conservation) stress, and they corroborate the idea that it is possible to maintain high sperm quality during long-distance shipping, in spite of the way such samples are handled.

In terms of the impact of light, equine sperm was very resistant to normal stimuli, such as occurs in routine laboratory work, but sperm samples showed diminution of quality when they were exposed to UV light for a minimum of 30 min.

ACKNOWLEDGEMENTS

The authors are grateful to Miguel Angel Cárdenas (R.I.P.) and his staff for allowing them to do this research in his stud.

CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

Fernando D. Requena and Francisco Requena were involved in the semen collection and management. Dr. Pérez-Marín and Dra. Agüera prepared the experimental design and was involved in statistical analysis. Ander Arando and Lucia Requena analysed the sperm samples by CASA and flow cytometer. Dr. Pérez-Marín and Ander Arando prepared and discussed the manuscript. In any case, all the authors were equally involved in the interpretation of the results and in manuscript preparation.

ORCID

CC Pérez-Marín http://orcid.org/0000-0002-9574-383X

REFERENCES

Agarwal, A., Ikemoto, I., & Loughlin, K. R. (1994). Effect of sperm washing on levels of reactive oxygen species in semen. *Archives of Andrology*, *33*, 157–162. https://doi.org/10.3109/01485019408987819

Aitken, R. J., De Iuliis, G. N., Finnie, J. M., Hedges, A., & McLachlan, R. I. (2010). Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: Development of diagnostic criteria. *Human Reproduction*, *25*, 2415–2426. https://doi.org/10.1093/humrep/deq214

Ankri, R., Friedman, H., Savion, N., Kotev-Emeth, S., Breitbart, H., & Lubart, R. (2010). Visible light induces nitric oxide (NO) formation in sperm and endothelial cells. *Lasers in Surgery and Medicine*, 42, 348–352.

Aurich, J. E. (2012). Artificial insemination in horses - More than a century of practice and research. *Journal of Equine Veterinary Science*, *32*, 458–463. https://doi.org/10.1016/j.jevs.2012.06.011

Cohen, N., Lubart, R., Rubinstein, S., & Breitbart, H. (1998). Light irradiation of mouse spermatozoa: Stimulation of in vitro fertilization and calcium signals. *Photochemistry and Photobiology*, *68*, 407–413. https://doi.org/10.1111/j.1751-1097.1998.tb09700.x

Cuervo-Arango, J., Nivola, K., Väilhkönen, L., & Katila, T. (2015). The effect of storage temperature of stallion semen on pregnancy rates. *Journal of Equine Veterinary Science*, *35*, 611–616. https://doi.org/10.1016/j.jevs.2015.06.003

Ferrer, M. S., Lyle, S. K., Eiltz, B. E., Eljarrah, A. H., & Paccamonti, D. L. (2012). Factors affecting sperm recovery rates and survival after centrifugation of equine semen. *Theriogenology*, *78*, 1814–1823. https://doi.org/10.1016/j.theriogenology.2012.07.011

Gao, D., Mazur, P., & Critser, J. K. (1997). Fundamental cryobiology of mammalian spermatozoa. In A. Karow, & J. K. Critser (Eds.), *Reproductive tissue banking: Scientific principles* (pp. 263–328). San Diego, CA: Academic Press. https://doi.org/10.1016/B978-012399770-8/50007-1

Guthrie, H. D., & Welch, G. R. (2006). Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. *Journal of Animal Science*, *84*, 2089–2100. https://doi.org/10.2527/jas.2005-766

Jasko, D. J., Hathaway, J. A., Schaltenbrand, V. L., Simper, W. D., & Squires, E. L. (1992). Effect of seminal plasma and egg yolk on motion characteristics of cooled stallion spermatozoa. *Theriogenology*, *37*, 1241–1252. https://doi.org/10.1016/0093-691X(92)90180-Y

Jasko, D. J., Martin, J. M., & Squires, E. L. (1992). Effects of insemination volume and concentration of spermatozoa on embryo recovery in mares. *Theriogenology*, *37*, 1233–1239. https://doi.org/10.1016/0093-691X(92)90179-U

Kim, S., Agca, C., & Agca, Y. (2013). Effects of various physical stress factors on mitochondrial function and reactive oxygen species in rat spermatozoa. *Reproduction, Fertility and Development*, *25*, 1051–1064. https://doi.org/10.1071/RD12212

Love, C. C., Thompson, J. A., Lowry, V. K., & Varner, D. D. (2002). Effect of storage time and temperature on stallion sperm DNA and fertility. *Theriogenology*, *57*, 1135–1142. https://doi.org/10.1016/S0093-691X(01)00689-6

McCarthy, M. J., Baumber, J., Kass, P. H., & Meyers, S. A. (2010). Osmotic stress induces oxidative cell damage to rhesus macaque spermatozoa. *Biology of Reproduction*, *82*, 644–651. https://doi.org/10.1095/biolreprod.109.080507

Moran, D. M., Jasko, D. J., Squires, E. L., & Amann, R. P. (1992). Determination of temperature and cool rate induced cold shock in stallion spermatozoa. *Theriogenology*, *38*, 999–1012. https://doi.org/10.1016/0093-691X(92)90114-7

Petrunkina, A. M., Waberski, D., Bollwein, H., & Sieme, H. (2010). Identifying non-sperm particles during flow cytometric physiological assessment: A simple approach. *Theriogenology*, *73*, 995–1000. https://doi.org/10.1016/j.theriogenology.2009.12.006

Rurangwa, E., Kime, D. E., Ollevier, F., & Nash, J. P. (2004). The measurements of sperm motility and factors affecting sperm quality in cultured fish. *Aquaculture*, *234*, 1–28. https://doi.org/10.1016/j.aquaculture.2003.12.006

Shahar, S., Wiser, A., Ickowicz, D., Lubart, R., Shulman, A., & Breitbart, H. (2011). Light-mediated activation reveals a key role for protein kinase A and sarcoma protein kinase in the development of sperm hyperactivated motility. *Human Reproduction*, *26*, 2274–2282. https://doi.org/10.1093/humrep/der232

Varisli, O., Uguz, C., Agca, C., & Agca, Y. (2009). Effects of various physical stress factors on rat sperm motility, acrosome and plasma membrane integrity. *Journal of Andrology*, *30*, 75–86.

Varner, D. D., Blanchard, T. L., Love, C. L., Garcia, M. C., & Kenney, R. M. (1988). Effects of cooling rate and storage temperature on equine spermatozoal motility parameters. *Theriogenology*, *29*, 1043–1054. https://doi.org/10.1016/S0093-691X(88)80028-1

Varner, D. D., Blanchard, T. L., Meyers, P. J., & Meyers, S. A. (1989). Fertilizing capacity of equine spermatozoa stored for 24 h at 5° or 20°C. *Theriogenology*, *32*, 515–525. https://doi.org/10.1016/0093-691X(89)90273-2

Vidament, M., Dupere, A. M., Julienne, P., Evain, A., Noue, P., & Palmer, E. (1997). Equine frozen semen: Freezability and fertility field results. *Theriogenology*, *48*, 907–917. https://doi.org/10.1016/S0093-691X(97)00319-1

Vidament, M., Magistrini, M., Le Foll, Y., Levillain, N., Yvon, J. M., Duchamp, G., & Blesbois, E. (2012). Temperature from 4 to 14°C are suitable for preserving the fertilizing capacity of stallion semen stored for 22 h or more in INRA96 extender. *Theriogenology*, *78*, 297–307. https://doi.org/10.1016/j.theriogenology.2012.01.018

Yeste, M., Codony, F., Estrada, E., Lleonart, M., Balasch, S., Peña, A., … Rodriguez-Gil, J. E. (2016). Specific LED-based red light photostimulation procedures improve overall sperm function and reproductive performance of boar ejaculates. *Scientific Report*, *6*, 22569. https://doi.org/10.1038/srep22569

How to cite this article: Pérez-Marín CC, Requena FD, Arando A, Requena L, Requena F, Agüera EI. Short-term tolerance of equine spermatozoa to various abiotic factors. *Reprod Dom Anim*. 2018;00:1–11. https://doi.org/10.1111/rda.13142

ARTÍCULO DE INVESTIGACION 2

Effect of trehalose- and sucrose-based extenders on equine sperm quality after vitrification: Preliminary results

C.C. Pérez-Marín, **F.D. Requena**, A. Arando, S. Ortiz-Villalón, F. Requena, E.I. Agüera

Cryobiology, 2018; 80:62-69
https://doi.org/10.1016/j.cryobiol.2017.12.002

Cryobiology 80 (2018) 62–69

Contents lists available at ScienceDirect

Cryobiology

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

Effect of trehalose- and sucrose-based extenders on equine sperm quality after vitrification: Preliminary results

C.C. Pérez-Marín[a,*], F.D. Requena[b], A. Arando[c], S. Ortiz-Villalón[a], F. Requena[b], E.I. Agüera[b]

[a] Department of Animal Medicine and Surgery, University of Cordoba, Cordoba 14014, Spain
[b] Department of Cell Biology, Physiology and Immunology, University of Cordoba, 14014 Cordoba, Spain
[c] Department of Genetics, University of Cordoba, Cordoba 14014, Spain

ARTICLE INFO

Keywords:
Disaccharides
Stallion
Spermatozoa
Vitrified

ABSTRACT

There has been a lack of research into equine sperm vitrification to date, but studies of other species suggest it may have significant potential. To evaluate the impact of various cryoprotectant agents (CPA) and vitrification on equine sperm quality, a controlled study was carried out. A total of 12 ejaculates were subjected to exposure to CPA and vitrification. Sperm was diluted in a range of CPA: fresh, control (BSA), sucrose (0.15M, 0.3M and 0.5M), trehalose (0.15M, 0.3M and 0.5M) and the combination of sucrose and trehalose (M1: 0.15M sucrose + 0.5M trehalose; M2: 0.5M sucrose + 0.15M trehalose). Sperm motility, viability, acrosome integrity and DNA fragmentation were assessed at the time of CPA exposure and after vitrification. The exposure of spermatozoa to various concentrations of sucrose and/or trehalose significantly reduced sperm motility, with lower concentrations resulting in higher sperm motility. Sperm viability and DNA fragmentation did not vary after exposure to CPA, but acrosome integrity fell significantly when spermatozoa were exposed to CPA with high osmolality. When spermatozoa were vitrified, motility values were significantly higher than those obtained during the exposure. Low concentrations of sucrose (0.15M and 0.3M) and trehalose (0.15M) showed the best progressive sperm motility. The vitrification-warmed procedure significantly reduced sperm viability and acrosome integrity, but DNA did not vary with any of CPA used. Equine sperm vitrification demonstrates a low capacity for preserving sperm motility, and extenders containing trehalose or sucrose at lower concentrations are associated with a better protective effect on sperm motility. After vitrification, acrosome and plasma membranes were severely impaired, while the DNA structure was maintained. Equine spermatozoa partially recover the motility after vitrification, but there is a need for further studies into the preservation of sperm membranes.

1. Introduction

In recent years, with the widespread use of assisted reproductive techniques (ARTs) in animal breeding industry, new sperm cryopreservation methods have been developed such as freeze-drying (lyophilisation) [27] and vitrification [4].

In sperm vitrification, which consists of the direct immersion of spermatozoa in liquid nitrogen, the combination of ultra-rapid cooling rates, the high viscosity of the medium and small sample sizes tend to prevent the formation of intracellular ice crystals [38]. Such crystals induce the sperm cryodamage that it is associated with reduced sperm motility and mitochondrial membrane potential [25], irreversible plasma membrane damage [7], and DNA fragmentation [8], which are liable to reduce the fertilising potential of the spermatozoa.

The addition of cryoprotectant agents (CPA) to the medium has the potential to improve sperm preservation. Sucrose and trehalose are the most commonly used disaccharides for protecting sperm from cold temperatures, because they have the capacity to balance extracellular and intracellular osmotic pressure during freezing and improve the glass transition temperature, thereby preventing ice formation [28]. In addition, trehalose changes the membrane fluidity of sperm, incorporating itself into the membranes' phospholipids bilayer, yielding more stable membranes during the freezing procedure [2]. However, toxic effects have been linked to CPA, depending on their concentration and the exposure time [4,32], and some studies suggest that the protective effect of CPA depends on the storage temperature, the molecular weight and the buffer used as extender [34]. In this context, a previous study carried out on ovine sperm vitrification [4] demonstrated that the refrigeration of sperm at 5 °C offers an improvement in vitrified sperm quality when sucrose is used.

The few studies of sperm vitrification that have been published in different species [4,18,19,31,32,33] report diverse results. In light of

* Corresponding author. Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Campus de Rabanales, Ctra. Madrid-Cadiz km 396, 14014 Cordoba, Spain.
E-mail address: pv2pemac@uco.es (C.C. Pérez-Marín).

https://doi.org/10.1016/j.cryobiol.2017.12.002
Received 18 October 2017; Received in revised form 4 December 2017; Accepted 4 December 2017
Available online 08 December 2017

the successful results obtained in dog [33] and human studies [17], and the comparable head dimensions of equine spermatozoa, it was hypothesised that vitrification could prove fruitful for equine sperm cryopreservation. This study aims to analyse the influence of various concentrations of sucrose, trehalose and the combination of the two sugars on equine sperm quality after vitrification.

2. Materials and methods

2.1. Sperm collection and processing

A total of 12 ejaculates were collected during breeding season from two Spanish-breed stallions, aged 7 and 14 respectively. The animals were clinically healthy, and semen was collected twice a week. Previous assessment of sperm freezability showed good results in both of the stallions used in this study. For the purposes of semen collection, the penis was cleaned, the stallions were allowed to mount a phantom and the semen was obtained by artificial vagina (Missouri model, Minitube Iberica, Tarragona, Spain) at 45-50 °C, equipped with a cellulose filter to separate the gel fraction. After collection, semen volume and sperm concentration (SpermaCue, Minitube Iberica, Tarragona, Spain) was determined. The ejaculates from both stallions were pooled to minimize individual variation, and 1:2 diluted with INRA 96® (IMV-Technologies, L'Aigle, France). Then, diluted semen was centrifuged at 650 g for 10 min and the pellet was re-suspended to reach a final concentration of 100×10^6 sperm/ml. An aliquot of 20 ml was prepared and carried out to the laboratory in a Neopor box (Minitube Iberica, Tarragona, Spain) at 4 °C.

Management of the stallions was conducted in accordance with the relevant European Union regulations (2010/63/EU), as transposed to Spanish law (RD 53/2013).

2.2. Experimental design

Fig. 1 illustrates the experimental design. Firstly, in order to carry out a toxicity test of CPA, sperm samples were exposed to various concentrations of sucrose, trehalose and a combination of both CPA, and their effects on the sperm quality were evaluated immediately after dilution (T0) and 5 min later (T5). Secondly, for assessing the protective effect of CPA during vitrification, sperm samples were diluted with the mentioned CPA and immediately vitrified; for the sperm quality assessment, samples were warmed in warming medium (WM). The experiments were repeated six times.

Table 1
Composition of different media used, diluted in INRA 96 extender to 100 ml (S = sucrose, T = trehalose, M1 = mixture of 0.15M sucrose + 0.5M trehalose; M2 = 0.5 M sucrose + 0.15 M trehalose; WM = warming medium).

Media*	BSA (g)	Sucrose (g)	Trehalose (g)	Osmolality (mOsm/kg)
Control	2	–	–	321
0.15M S	2	10.27	–	649
0.3M S	2	20.54	–	934
0.5M S	2	34.23	–	1158
0.15M T	2	–	11.35	690
0.3M T	2	–	22.70	1005
0.5M T	2	–	37.83	1211
M1	2	10.27	37.83	1427
M2	2	34.23	11.35	1382
WM	1	–	–	319

* Sperm samples were 1:2 diluted with different diluents to obtain the final concentration.

2.3. Media preparation

The control medium was based on INRA 96® extender supplemented with bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) (final concentration 1%), while the other CPA media were prepared with the same extender supplemented by varying concentrations of sucrose (Panreac, Barcelona, Spain) (final concentration 0.15M, 0.3M, 0.5 M), α(+)-trehalose dihydrate (Acros Organics, Geel, Belgium) (final concentration 0.15 M, 0.3 M, 0.5 M) and the combination of both (M1: 0.15 M sucrose + 0.5 M trehalose; M2: 0.5 M sucrose + 0.15 M trehalose). Sperm was diluted 1:2 in the nine different prepared media. Table 1 shows the composition of the various media used for this study. Media osmolality was measured using a vapour pressure osmometer (Vapro 5520, Wescor Inc., Logan, Utah, USA).

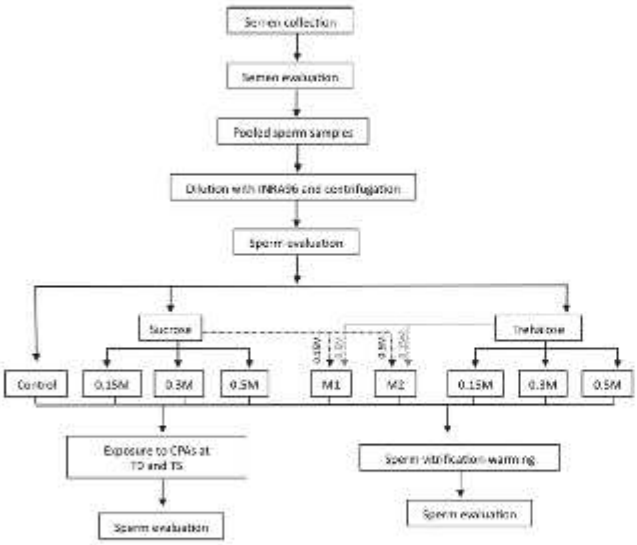

Fig. 1. Schema of the experimental design for CPA exposure and vitrification-warming procedure.

2.4. Vitrification and warming procedures

For vitrification, 25 μl of sperm aliquot were 1:2 diluted in nine different CPA media. A final drop of 50 μl (50×10^6 sperm/ml) was immediately plunged into liquid nitrogen (LN_2). Until their analysis, the sperm pellets were stored in cryotubes containing 300 μl of LN_2. For warming, vitrified-sperm pellets were immersed in 0.5 ml of warming medium (INRA+ 1% BSA; WM) at 65 °C for 5 s, and then maintained at 37 °C for 3 min. Samples were centrifuged at 300 g for 10 min, and the final pellet was re-suspended with 50 μl WM for the sperm assessment, adjusted to a final concentration of approximately 10×10^6 sperm/ml.

2.5. Assessment of sperm motility

A computerised analysis system was used for assessing sperm motility (Proiser, Valencia, Spain). Fresh pooled samples were diluted 1:5 to a final concentration of 10–20 $\times 10^6$ sperm/ml for evaluation. In order to determine the sperm quality during exposure to the CPA, fresh stock sperm aliquot was first 1:2 diluted in INRA 96 to reach 50×10^6 sperm/ml. The samples were then diluted in their corresponding CPA media to a final concentration of 10–20 $\times 10^6$ sperm/ml. Five μl was placed on a slide and covered with 20 × 20 mm coverslip. Four fields and a minimum of 500 spermatozoa were randomly captured at 10× contrast phase. A total of 25 images per second were acquired, selecting particles with an area of between 10 and 70 μm^2, and sperm were considered to be linearly motile when they deviated < 45% from a straight line. The analyses provided information about total motility (TM, %) and progressive motility (PM, %).

During the exposure test, sperm motility was assessed at the moment CPA was added and after 5 min incubation at 37 °C.

2.6. Assessment of sperm viability

A LIVE/DEAD® sperm viability kit (Molecular Probes Europe, Leiden, The Netherlands) was used to assess sperm viability. A volume of 100 μl of diluted sperm was mixed with 150 μl cytometer buffer (Becton Dickinson Inmunochemistry, San Jose, CA, USA) at a final concentration of 20 × 10^6sperm/ml, and then 2.5 μl of SYBR-14 (20 nM final concentration) and five μl of propidium iodine (PI) (10 μM) was added per sample. After incubation in darkness for 15 min at room temperature, the proportion of live/dead sperm cells was measured, live spermatozoa deemed to be those sperm stained with SYBR-14 (green fluorescence), while dead spermatozoa were stained with PI (red fluorescence). Samples were analysed using a flow cytometer.

2.7. Assessment of acrosome integrity

Acrosome integrity was assessed using FITC-labelled peanut agglutinin (FITC-PNA) Sigma-Aldrich, St. Louis, MO, USA) and PI (Molecular Probes Europe, Leiden, The Netherlands). A total of 100 μl of sperm sample was mixed with 5 μl FITC-PNA (100 μg/ml in DMSO) and 5 μl PI (6 μM final concentration), and incubated at room temperature in darkness for 5 min. A volume of 400 μl cytometer buffer was then added to the sample to a final sperm concentration of 12.5 × 10^6sperm/ml and analysed by flow cytometer. PI-negative and FITC-PNA negative cells were considered as sperm with intact acrosome.

2.8. Assessment of sperm chromatin structure

Samples were diluted in 200 μl TNE (0.15 M NaCl, 0.01 M Tris-HCl, 1 mM EDTA, pH 7.4) buffer to a final concentration of 2×10^6 sperm/ml [13]. They were plunged into LN_2, warmed to room temperature, and plunged again into LN_2, to be finally stored at −80 °C pending analysis. The samples were subsequently thawed and 400 μl of acid solution was added to induce denaturation of the DNA. After 30 s, 1.2 ml of acridine orange (AO) solution (Sigma-Aldrich, St. Louis, MO, USA) was added (6 μg/ml) and incubated in darkness for 3 min prior to flow cytometric analysis. When combined with the intact double DNA helix, AO was detected in the green band (FL1 photodetector), but when it was linked to denatured DNA it fluoresces in the red band (FL3 photodetector). A total of 5000 events were counted per sample and the results are presented as % of fragmented DNA.

2.9. Flow cytometric analysis

For flow cytometric analyses, a FACScalibur flow cytometer (Becton Dickinson Inmunochemistry, San Jose, CA, USA) was used. All dyes were excited using Argon blue laser (488 nm). An FL1 photodetector (530/30 band-pass filter) was used to read green fluorescence from SYBR-14, FITC-PNA and AO. An FL2 photodetector (585/42-nm band-pass filter) was used to read red fluorescence from PI, and an FL3 photodetector (630-nm long-pass filter) used to read red fluorescence from AO. Around 10,000 events of a gated population were counted per sample, with the exception of AO where 5000 events were counted. For viability and acrosome integrity, mathematical corrections were used for the estimation of the percentage of events that were not sperm cells [29].

2.10. Statistical analysis

SPSS software (SPSS, Chicago, IL, USA) was used for statistical analysis of the data. The Shapiro-Wilk test determined that the data did not show a normal distribution. The Wilcoxon test was used to compare the effect of exposure (T0 and T5) and the vitrification/warming process within each CPA group. The Kruskal-Wallis test was used to determine the effects of the various CPA on the motility, morphology, viability, acrosome integrity and DNA fragmentation at the initial exposure and after the vitrification/warming process. Data are presented as mean ± SEM, and differences were considered as significant when $p < 0.05$.

3. Results

Fresh and control (containing 1% BSA final concentration) sperm samples showed higher total and progressive motility, but these parameters fell significantly when sucrose and/or trehalose were added to the samples (Fig. 2). Values did not significantly vary when sperm samples were maintained for 5 min in contact with the CPA (Fig. 2). Comparison of the effect of different CPA showed that the lower concentrations of sucrose (0.15M) induced higher sperm motility. Similarly, the addition of 0.15M trehalose was also associated with significantly ($p < 0.05$) higher sperm motility. Similar comparisons carried out after 5 min of sperm-CPA incubation also demonstrated that 0.15M sucrose had the best results for total motility. It was noted that sperm diluted with 1% BSA suffered a slight fall in motility, but 5 min later values increased.

Vitrification induced low motility values in comparison to fresh sperm. After vitrification/warming, total and progressive motility significantly increased in almost all the CPA used compared to values obtained during CPA exposure, except in the case of the control and 0.15M sucrose. Samples diluted with 1% BSA (labelled C group) suffered a drastic reduction of motility. The comparison between treatments (i.e. CPA) showed significantly ($p < 0.05$) lower total motility when sperm samples were diluted with control or M1 or M2 diluents. Progressive motility was significantly ($p < 0.05$) higher in sperm samples diluted in 0.15M and 0.3M sucrose and in 0.15M trehalose.

Sperm viability was not affected by exposure to CPA before vitrification, and it remained at between 76.9 and 82.9% (Fig. 3).

Sperm viability values after vitrification were significantly lower compared to initial values ($p < 0.001$). Significant differences also

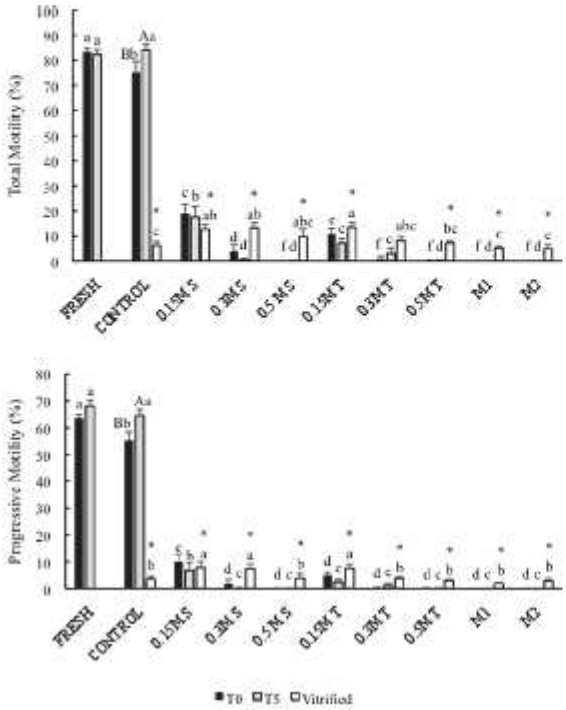

Fig. 2. Total and progressive sperm motility (%) in equine samples during the exposure (at the time of dilution and 5 min later) and the vitrification/warming with CPA based on BSA, sucrose and/or trehalose. Data are expressed as means ± SEM.

Asterisk (*) indicates significant differences ($p<0.05$) between pre- (at time 5) and post-vitrification within each cryoprotectant.
Different lowercase letters (a-f) indicate significant differences ($p<0.05$) between cryoprotectants.
Different uppercase letters (A, B) indicate significant differences ($p<0.05$) between exposure times (time 0 and time 5) within each cryoprotectants.

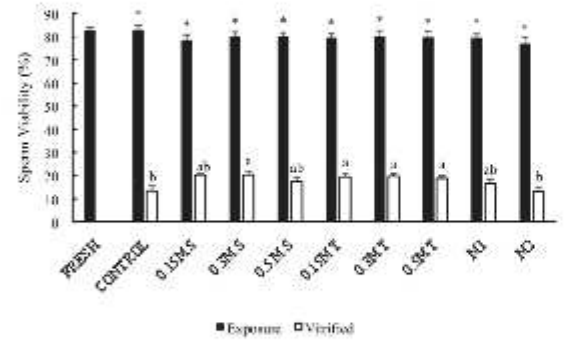

Fig. 3. Sperm viability (%) in equine samples during the exposure and the vitrification/warming with CPA based on BSA, sucrose and/or trehalose. Data are expressed as means ± SEM.

Asterisk (*) indicates significant differences ($p<0.05$) between exposure and vitrification within each cryoprotectant.
Different letters (a, b) indicate significant differences ($p<0.05$) between cryoprotectants.

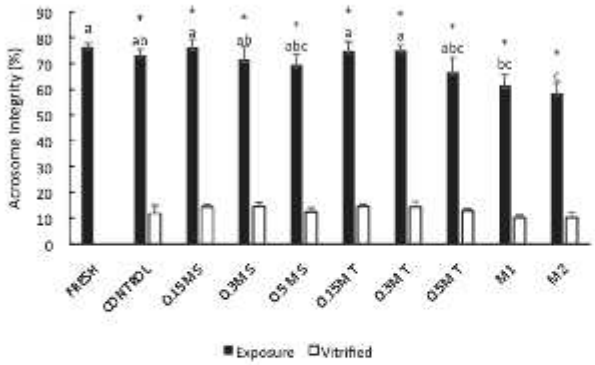

Fig. 4. Integrity of sperm acrosome (%) in equine samples during the exposure and the vitrification/warming with CPA based on BSA, sucrose and/or trehalose. Data are expressed as means ± SEM.

Asterisk (*) indicates significant differences ($p<0.05$) between exposure and vitrification within each cryoprotectant.
Different letters (a, b) indicate significant differences ($p<0.05$) between cryoprotectants.

emerged when comparing the various CPA groups ($p < 0.001$); the control and M2 groups showed significantly ($p < 0.05$) lower values (Fig. 3).

Acrosome integrity was significantly ($p < 0.02$) reduced in sperm samples diluted with the highest molarity CPA, i.e. the mixture of sucrose and trehalose.

When vitrification was carried out, acrosome integrity was also significantly reduced ($p < 0.001$) in all the CPA groups. However, comparing CPA after vitrification did not reveal any differences and a low percentage of spermatozoa (10–15%) maintained their acrosome integrity (Fig. 4).

Fresh and CPA-exposed spermatozoa did not show differences for DNA fragmentation between treatments (Fig. 5). And similarly, values obtained after vitrification did not showed differences between CPA (Fig. 5). However, significant differences ($p < 0.05$) were observed between spermatozoa exposed and vitrified with 0.15 M and 0.3 M sucrose, and in M2 diluent.

4. Discussion

Difficulties in obtaining good results after freezing-thawing procedures have been described in equine sperm, and interest is increasing about new extenders, selective methods for separating superior sperm populations and modifications to freezing-thawing procedures as a means of improving these results. While other studies describe new cryopreservation procedures, such as dry-freezing for the preservation of equine sperm [26], no experiments have hitherto been conducted on equine sperm vitrification. And although vitrification has been successfully implemented for human sperm cryopreservation [17], few studies have focused on other species.

The present study demonstrates the negative impact of vitrification on equine spermatozoa diluted in various concentrations of sucrose and/or trehalose, and did not find notable differences between the two disaccharides. However, it was surprising to observe that vitrified-warmed sperm samples (using the same CPA) showed higher motility values than sperm samples only exposed to CPA, as described in Fig. 6. The mentioned figure shows the impact of high osmolality on the equine sperm motility, as reported by others [5,30], and also demonstrates that importance of these CPA for maintaining motility during vitrification (although values are too low to use in artificial insemination).

Many of the sperm quality variations observed in this study may be

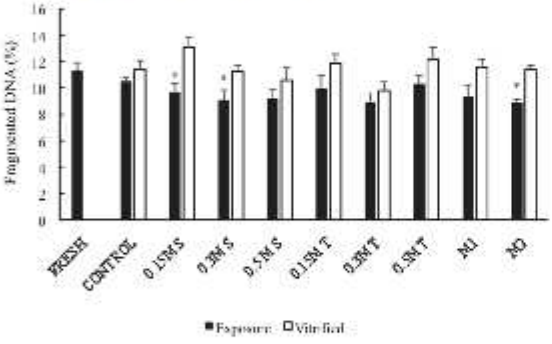

Fig. 5. Sperm DNA integrity (%) in equine samples during the exposure and the vitrification/warming with CPA based on BSA, sucrose and/or trehalose. Data are expressed as means ± SEM.

Asterisk (*) indicates significant differences ($p<0.05$) between exposure and vitrification within each cryoprotectant.

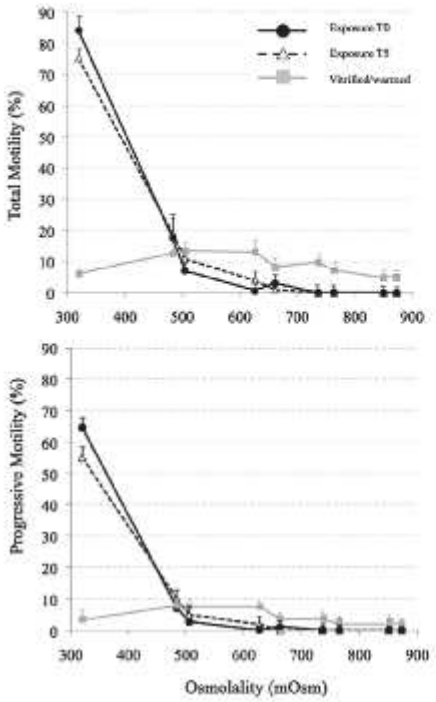

Fig. 6. Effect of different osmotic conditions on total and progressive motility of equine spermatozoa. Spermatozoa were diluted 1:2 in media based on different sugars. Theoretical osmolalities were calculated as an average of sperm samples and CPA-extenders. Effect of type of sugar was not having into account for representing these data. For exposure at T0 and T5, samples were maintained at the same osmotic conditions in the moment of the dilution and 5 min later, respectively. For vitrified/warmed samples, spermatozoa were mixed with CPA-diluents and immediately vitrified; at warming, samples were returned to isosmotic conditions before sperm quality assessment.

associated with the osmotic tolerance of equine spermatozoa. The media used for equine sperm vitrification displayed substantial differences in their osmotic pressure, which could cause the variations observed in sperm quality. The use of high CPA concentrations for sperm vitrification makes it advisable to evaluate the samples at precisely the moment of coming into contact and after 5 min of exposure [10]. In the present study, equine sperm motility after exposure to different CPA did not show significant differences either at the moment of first contact or 5 min later, except when BSA was the only addition to the sample. Significantly lower sperm motility was observed during the evaluation carried out before vitrification, i.e. when sperm were exposed to CPA. A rapid osmotic shock occurs at the moment that CPA are added to the sperm samples, and the addition of disaccharides transforms the medium into a more viscous substance, suppressing the flagellar movement of spermatozoa [39]. The dilution of these samples enables sperm motility to recover, thereby accounting for the results obtained in the present study. It has been reported that equine sperm motility decreases rapidly under hyperosmolal conditions [5,30]. However, such conditions also induce oxidative damage to sperm, with increased generation of superoxide anions, although plasma membrane integrity is not affected until 600 mOsm [6]. And the return to isosmolal conditions during thawing or warming is also critical for spermatozoa, which recover their initial volume. It has also been reported that a decrease of mitochondrial activity and viability are observed in equine spermatozoa during the thawing process [30], which underscores the importance of this step for sperm survival and functioning. It has been pointed out that the rate of warming constitutes a critical aspect of sperm vitrification, more so than the cooling rate, and slow warming at 37 °C strongly reduces sperm motility, sperm viability and acrosome integrity compared to rapid warming at 60 °C [32].

The impact of CPA and vitrification observed in the present study of sperm viability suggests that the plasmalemma (or plasma membrane) is damaged not only by intracellular ice formation, but also by the composition of the CPA inducing physical impacts on the cells. In the literature, various studies concur that the optimum concentration of sugars for sperm cryopreservation differs between species, and it is also affected by the type of extender [12]. It has been reported that both disaccharides offer similar effects on sperm cryopreservation [20], and extenders such as INRA possess additional sugars capable of acting synergically with the disaccharides used as CPA [11]. Sucrose and trehalose are disaccharides that cannot penetrate the cells and are used to improve glass formation at a certain critical cooling rate [9,36]. It has been reported that trehalose inserts itself into the membrane phospholipids bilayer, which makes it more stable during freezing [1]. The antioxidant effect of trehalose has been linked to its ability to protect membranes during cryopreservation, since it increases the level of glutathione and reduces the level of lipid peroxide [3]. It is possible that it promotes the protection of the functional integrity of acrosome and mitochondria and improvement of post-thaw sperm motility [20]. In a recent study [35], human sperm motility was increased after vitrification using 0.1 M trehalose, but no comparative experiments were carried out with similar sucrose concentrations. Different disaccharides were combined in this trial, but their osmolality was higher than in single-sugar media. It is known that combining CPA could harness the synergic action of each individual CPA component, and reduce toxicity compared to single CPA in high concentrations.

Acrosome plays an important role in the fertilisation process and injuries have the capacity to impede the success of spermatozoa. In the present study, the exposure of sperm to low-osmolality CPA did not affect acrosome integrity, but vitrification induced a great negative effect on the acrosome membranes. No protective effect of sucrose or trehalose was noted on sperm acrosome after vitrification, and the same holds for spermatozoa plasma membranes. This may be linked to an alteration in membrane proteins located in the phospholipid regions that occurs during the lipid phase transition in cooling [23]. This observation constitutes a major impediment to using vitrified sperm for artificial insemination, and underscores the case for acrosome and plasma membrane protective strategies being adopted to improve sperm vitrification; other such strategies may include the addition of detergents (sodium dodecyl sulphate), the introduction of a prior refrigeration period [4] and ultra-rapid protocols [37].

The addition of low concentrations of BSA to semen extender produces a slight increment of osmolality in the medium and it has also been reported that it offers higher spermatozoa survival rates, although the underlying mechanism has not been elucidated [24]. In the present study, a control diluent containing 1% BSA was compared with others based on different concentrations of non-penetrating CPA, namely sucrose and/or trehalose. It was hypothesised that BSA-based diluent would maintain sperm quality during the exposure test but, due to the absence of CPA, sperm motility would not be maintained. Surprisingly, a low percentage of spermatozoa maintained their motility, despite the fact that CPA had not been added to the medium. In order to explain why equine spermatozoa could tolerance vitrification under the aforementioned conditions, it should be borne in mind that BSA is a macromolecule that is absorbed into the surface of spermatozoa plasma membranes, where it plays a protective role, and it can also transport large amounts of free fatty acids, which offer extra energy to spermatozoa [15]. Additional antioxidant characteristics are linked to BSA, which improve sperm membrane integrity and reduce DNA fragmentation by eliminating free radicals [22]. However, various additional

compounds (such as sugars) added to the extender [11] have the potential to protect the spermatozoa. Human sperm has been successfully vitrified in the absence of CPA, demonstrating a resistance to cryo-environments that it is ascribed to their seminal plasma composition and sperm intracellular substances [16]. The shape and size of the spermatozoa has been also linked to their ability to tolerate cryopreservation, with the suggestion that small blade-shaped flat heads are less sensitive to cold shock [38]. Comparison of mammalian sperm shows that human spermatozoa have the smallest dimensions, followed by equine, feline and canine spermatozoa [14]. Thus the size of equine spermatozoa might suggest that it is a good candidate for vitrification. This is the first study to focus on sperm vitrification in horses, and although results are for the time being not as fruitful as conventional freezing procedures, further studies could throw new light on ways in which it could be improved.

In human sperm, DNA integrity was not affected by vitrification or freezing [17]. This is consistent with the results obtained in equine sperm here, since DNA did not exhibit increased fragmentation after vitrification, supporting the idea that the ICSI technique could be used on such spermatozoa. In this case, disaccharides added to the vitrification extender could protect the spermatozoa, reducing the structural fluctuations and denaturation of DNA through the formation of numerous hydrogen bonds [21].

In conclusion, equine sperm vitrification demonstrates a low capacity for preserving sperm motility, and extenders containing trehalose or sucrose at lower concentrations offer better protective effects on sperm motility. Surprisingly, a small percentage of spermatozoa protected with only BSA also resisted the vitrification process. While DNA integrity was slightly reduced after vitrification, no differences between CPA were observed, suggesting that sperm chromatin could maintain its function under the conditions being studied. Acrosome and plasma membranes suffered substantial damage during vitrification, but not during CPA exposure, and protective measures for these structures should be considered in order to improve the vitrification technique.

Contributions

Fernando D. Requena and Francisco Requena were involved in the semen collection and management. Dr Pérez-Marín and Dra. Agüera prepared the experimental design and was involved in statistical analysis. Ander Arando and Sandra Ortiz-Villalón analysed the sperm samples by CASA and flow cytometer. Dr. Pérez-Marín and Ander Arando prepared and discussed the manuscript. In any case, all the authors were equally involved in the interpretation of the results and in manuscript preparation.

Conflicts of interest

The authors have no conflicts of interest with regard to these results.

Acknowledgements

This study was partially funded by the INIA-FEDER, project RZ2008-00025-00-00. Sandra Ortiz-Villalón was supported by a grant of II Plan Propio Galileo de Innovación y Transferencia de la Universidad de Córdoba.

The authors are grateful to Miguel Angel Cárdenas (R.I.P.) and his staff for allowing them to do this research in his stud.

References

[1] E.M. Almagla, T. Yorada, Trehalose-enhanced fluidity of the goat sperm membrane and its protection during freezing, Biol. Reprod. 60 (2003) 1248–1250.
[2] E.M. Almagla, T. Yorada, Effects of the supplementation of trehalose extender containing egg yolk with sodium dodecyl sulfate on the freezability of goat spermatozoa, Theriogenology 62 (2004) 809–818.
[3] E. Aisen, M. Quintana, V. Medina, H. Morello, A. Venturino, Ultrastructuroscopic and biochemical changes in ram spermatozoa cryopreserved with trehalose-based hyperosmotic extenders, Cryobiology 50 (2005) 230–240.
[4] A. Arando, A. Gonzalez, J.V. Delgado, F.A. Arrebola, C.C. Pérez-Marín, Storage temperature and sucrose concentration affect ram sperm quality after vitrification, Anim. Reprod. Sci. 181 (2017) 175–185.
[5] B.A. Ball, A. Vo, Osmotic tolerance of equine spermatozoa and effects of soluble cryoprotectants on equine sperm motility, viability, and mitochondrial membrane potential, J. Androl. 22 (2001) 1062–1069.
[6] B.A. Ball, Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse, Anim. Reprod. Sci. 107 (2008) 257–267.
[7] J.F. Barbas, R.D. Mascarenhas, Cryopreservation of domestic animal sperm cells, Cell Tissue Bank. 10 (2009) 49–63.
[8] J. Baumber, B.A. Ball, J.J. Linfor, S.A. Meyers, Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa, J. Androl. 24 (2003) 621–628.
[9] G. Berrios, R. Sánchez, Ultra rapid freezing in human spermatozoa: effect on sperm function and reactive oxygen species production, Int. J. Morphol. 29 (2011) 899–906.
[10] R. Cuevas-Uribe, H. Yang, J. Daly, M.G. Savage, R.B. Walter, T.R. Tiersch, Production of F1 offspring with vitrified sperm from a live-bearing fish, the green swordtail (Xiphophorus hellerii), Zebrafish 8 (2011) 167–180.
[11] D.A. El-Badry, A.M.A. El-Maaty, G.A. El Sisy, The effect of trehalose supplementation of INRA-82 extender on quality and fertility of cooled and frozen-thawed stallion spermatozoa, J. Equine Vet. Sci. 48 (2017) 86–92.
[12] R.I. El-Shalofawy, G.A. Sisy, W.S. El-Nattat, Effects of different concentrations of sucrose or trehalose on the post-thawing quality of cattle bull semen, Asian Pac. J. Reprod. 4 (2015) 26–31.
[13] D.P. Evenson, Z. Darzynkiewicz, M.R. Melamed, Relation of mammalian sperm chromatin heterogeneity to fertility, Science 210 (1980) 1131–1133.
[14] D.L. Garner, Flow cytometric sexing of mammalian sperm, Theriogenology 65 (2006) 943–957.
[15] M.D. Hossain, K.M.A. Tanu, K. Hasnaeen, H. Taqii, Effect of fatty acids on bear sperm motility, viability and acrosome reaction, Reprod. Med. Biol. 6 (2007) 215–219.
[16] E. Isachenko, V. Isachenko, I.I. Katkov, S. Dessole, F. Nawroth, Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success, Reprod. Biomed. Online 6 (2003) 191–200.
[17] V. Isachenko, E. Isachenko, I.I. Katkov, M. Montag, S. Dessole, F. Nawroth, H. Van Der Ven, Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: effect on motility, DNA integrity, and fertilization ability, Biol. Reprod. 71 (2004) 1167–1173.
[18] V. Isachenko, E. Isachenko, A.M. Petrunkina, R.Sánchez, Human spermatozoa vitrified in the absence of permeable cryoprotectants: birth of two healthy babies, Reprod. Fertil. Dev. 24 (2012) 323–326.
[19] P. Jiménez-Rabadán, O. García-Álvarez, A. Vidal, A. Maroto-Morales, M. Iniesta-Cuerda, M. Ramón, E. del Olmo, R. Fernández-Santos, J.J. García, A.J. Soler, Effects of vitrification on ram spermatozoa using free-egg yolk extenders, Cryobiology 71 (2015) 85–90.
[20] J. Liu, C. Tanrikut, D.L. Wright, G. Lee, M. Toner, J.D. Biggers, T.L. Toth, Cryopreservation of human spermatozoa with minimal non-permeable cryoprotectant, Cryobiology 73 (2016) 162–167.
[21] P. Loi, K. Matsukawa, G. Ptak, M. Clinton, J. Fulka, Y. Nathan, A. Arav, Freeze-dried somatic cells direct embryonic development after nuclear transfer, PLoS One 3 (2008) e2978 http://doi.org/10.1371/journal.pone.0002978.
[22] T. Matsuoka, H. Imai, H. Kohno, Y. Fukui, Effects of bovine serum albumin and trehalose in semen diluents for improvement of frozen-thawed ram spermatozoa, J. Reprod. Dev. 52 (2006) 675–683.
[23] H. Mourad, M. Morshedi, J.P. Turner, S. Oehninger, Impact of cryopreservation on spermatozoa from infertile men—implications for artificial insemination, Arch. Androl. 33 (1994) 51–57.
[24] C.F. Nang, K. Osman, S.B. Bulin, M.I. Ismail, F.H.F. Jaffar, S.F.S. Mohamad, S.F. Ibrahim, Bovine serum albumin: survival and osmolality effect in bovine spermatozoa stored above freezing point, Andrologia 44 (2012) 447–453.
[25] M. O'Connell, N. McClure, S.E. Lewis, The effects of cryopreservation on sperm morphology, motility and mitochondrial function, Hum. Reprod. 17 (2002) 704–709.
[26] M. Olaciregui, V. Luño, J.I. Martí, J. Arratyoma, L. Gil, Freeze-dried stallion spermatozoa: evaluation of two chelating agents and comparative analysis of three sperm DNA damage assays, Andrologia 48 (2016) 900–906.
[27] M. Olaciregui, L. Gil, Freeze-dried spermatozoa: a future tool? Reprod. Domest. Anim. 52 (2017) 248–254.
[28] C.-Y. Pan, S. Yu, P.-F. Zhang, B. Wang, Z.-G. Zhu, Y.-Y. Liu, W.-X. Yang, Effect of sucrose on cryopreservation of pig spermatogonial stem cells, J. Integr. Agr. 16 (2017) 1120–1126.
[29] A.M. Petrunkina, D. Waberski, H. Bollwein, H. Sieme, Identifying non-sperm particles during flow cytometric physiological assessment: a simple approach, Theriogenology 73 (2010) 995–1005.
[30] A.C. Pommer, J. Rutllant, S.A. Meyers, The role of osmotic resistance on equine spermatozoal function, Theriogenology 58 (2002) 1373–1384.
[31] J. Pradiee, M.C. Esteso, C. Castaño, A. Toledano-Díaz, A. López-Sebastián, R. Guerra, J. Santiago-Moreno, Conventional slow freezing cryopreserves mouflon spermatozoa better than vitrification, Andrologia 49 (2017) e12929 https://doi.org/10.1111/and.12629.
[32] M.P. Rosato, N. Iaffaldano, Cryopreservation of rabbit semen: comparing the effects of different cryoprotectants, cryoprotectant-free vitrification, and the use of albumin plus monoprotectants on sperm survival and fertility after standard vapor

freezing and vitrification, Theriogenology 79 (2013) 508–516.
[33] R. Sánchez, J. Risopatrón, M. Schulz, J. Villegas, V. Isachenko, R. Kreinberg, E. Isachenko, Canine sperm vitrification with sucrose: effect on sperm function, Andrologia 43 (2011) 233–241.
[34] S. Sarıözkan, M.N. Bucak, F. Cecerk, S. Ünlüsoy, A. Yay, P.B. Yuncu, S. Ören, N. Sorgucu, Y. Cinar, The effects of different sugars on motility, morphology and DNA damage during the liquid storage of rat epididymal sperm at 4°C, Cryobiology 65 (2012) 93–97.
[35] M. Schulz, J. Risopatrón, G. Matus, E. Pineda, C. Rojas, V. Isachenko, E. Isachenko, R. Sánchez, Trehalose sustains a higher post-thaw sperm motility than sucrose in vitrified human sperm, Andrologia 49 (2017) e12757.
[36] R.L. Sutton, Critical cooling rates for aqueous cryoprotectants in the presence of sugars and polysaccharides, Cryobiology 29 (1992) 585–598.
[37] G. Vicente, E. Jiménez, F.J. Agüera, C.C. Pérez-Marín, Impact of ultra-rapid freezing on the motility, morphology, viability and acrosome integrity of epididymal cat sperm diluted in sucrose-based extenders, Reprod. Domest. Anim. 49 (2014) e5–e8.
[38] P.F. Watson, J.M. Plummer, The responses of boar sperm membranes to cold shock and cooling, in: L.A. Johnson, K. Larsson (Eds.), Deep Freezing of Boar Sperm, Swedish University of Agricultural Sciences, Uppsala, Sweden, 1985, pp. 113–127.
[39] H. Wouldiers, A. Matthijs, B. Engel, Effects of trehalose and sucrose, osmolality of the freezing medium, and cooling rate on viability and intactness of bull sperm after freezing and thawing, Cryobiology 35 (1997) 93–105.

ARTÍCULO DE INVESTIGACION 3

Transrectal ultrasonographic measurements of the combined thickness of the uterus and placenta in Spanish Purebred mares

Fernando D. Requena, Estrella I. Agüera, Francisco Requena, Carlos C. Pérez-Marín

Animal Reproduction, 2017; 14 (Suppl.1): 1278-1284
DOI: 10.21451/1984-3143-AR0029

DOI: 10.21451/1984-3143-AR0029

Anim. Reprod., v.14, (Suppl.1), p.1278-1284, 2017

Transrectal ultrasonographic measurements of the combined thickness of the uterus and placenta in Spanish Purebred mares

Fernando D. Requena[1], Estrella I. Agüera[1], Francisco Requena[1], Carlos C. Pérez-Marín[2,3]

[1]Cellular Biology, Physiology and Immunology Department, Veterinary Faculty, University of Cordoba, Cordoba, Spain.
[3]Animal Medicine and Surgery Department, Veterinary Faculty, University of Cordoba, Cordoba, Spain.

Abstract

The present study was conducted to establish the normal values of the combined thickness of the uterus and placenta (CTUP) in Spanish Purebred mares during late pregnancy. A total of 107 mares were examined at 210, 240, 270 and 300 days of gestation, of which 13 had abnormal gestations (foetal death, abortion or stillbirth). In mares with healthy foals, CTUP measurements showed significant differences ($P < 0.0001$) at the various gestational ages (4.83 ± 0.59 mm, 6.12 ± 0.60 mm, 7.41 ± 0.61 mm and 10.45 ± 0.92 mm at 210, 240, 270 and 300 days, respectively). A high positive correlation was observed between CTUP and gestational age ($r = 0.923$; $P < 0.001$). In mares with abnormal gestation, CTUP measurements were significantly ($P < 0.0001$) higher than in healthy gestations at day 270 (8.89 *vs.* 7.41 mm) and day 300 (14.17 *vs.* 10.45 mm). The age of the mare, fertilization time, type of semen and gestation length did not affect the CTUP. The results obtained suggest that the cut-off point for detecting abnormal pregnancies in Spanish Purebred mares is 7.75 mm on day 270 and 12.7 mm on day 300. The authors find that the incidence of placental thickness enlargement in the late gestation of Spanish Purebred is 14%, and a transrectal ultrasonographic examination is highly advisable at 270 days of gestation. Such timely placental assessment could reveal otherwise undetected disorders, thereby enabling early and proper treatments to be administered to prevent foetal loss.

Keywords: CTUP, equine, gestation, placentitis, ultrasonography.

Introduction

In terms of population size, the Spanish Purebred (SPB) is the largest Spanish horse breed and the industry surrounding it in Spain exceeds other breeds according to the national census. There are approximately 215,000 SPB horses all over the world (Ministerio de Agricultura, Alimentación y Medio Ambiente - MAGRAMA, 2016), and they are bred in around sixty countries. The use of ultrasound to evaluate pregnancy and foetal wellbeing has been employed in horses for over 30 years (Palmer and Driancourt, 1980), and it helps to determine ascending placentitis, which is one major cause of abortion in mares (Giles *et al*., 1993). This disorder occurs late in pregnancy, when bacteria reach the uterus from the lower reproductive tract (Cummins *et al*., 2008), causing infection in the cervical star and the chorioallantois region. This infection provokes ulceration and thickening of the chorioallantois (Platt, 1975), with high expression of pro-inflammatory cytokines and release of prostaglandins, inducing uterine contraction and early delivery (Dudley and Trautman, 1994; Pollard and Mitchell, 1996; LeBlanc *et al*., 2002; LeBlanc, 2004). It may be associated with an early birth, the birth of a weak foal or abortion, and harmed mares often have difficulty in conceiving during the following breeding season (Troedsson *et al*., 1997). To determine whether there is any risk of foetal loss, endocrine profiling (Rossdale *et al*., 1991; Stawicki *et al*., 2002; Morris *et al*, 2007) and transrectal and transabdominal ultrasonography (Renaudin *et al*., 1997; Bucca *et al*., 2005; Carrick *et al*., 2010; Löf *et al*., 2010) are frequently used, and are reported during late gestation, at around 8-10 months. Bailey *et al*. (2012) also compared the ability of B-mode and Doppler ultrasonography to detect ascending placentitis in pony mares with experimentally induced disease.

As the cervical star region is frequently altered in mares with ascending placentitis, transrectal ultrasonography is an excellent tool for monitoring foetal and placental changes. It has been suggested that measurements of the combined thickness of the uterus and placenta (CTUP) could be used to identify mares' risk of developing ascending placentitis or other placental disorders. Reanudin *et al*. (1997) described the CTUP evaluation technique, and reported normal values from day 180 to day 330 of pregnancy. Few animals have been involved in descriptive studies and usually different breeds were included in the same study. While the breed factor has been considered in many studies related to gestation, few horse breeds have been studied with a view to establishing their reference CTUP values.

From a clinical/practical perspective, it has been reported that utero-placental thickness should be no thicker than the gestational age of the foetus in mm plus 1, similar to that described in humans; however, this rule is excessively ambiguous and imprecise (Reef *et al*., 1995).

In SPB pregnant mares, CTUP values are unknown and better understanding of placenta status could prevent late gestation disorders. Giles *et al*. (1993) reported that the incidence of foetus loss in different equine breeds due to ascending bacterial placentitis (foetal death, abortion and stillbirth) reached 20%, while others quantified these losses at 10% in a sample where the majority of cases were from Thoroughbred mares (Smith *et al*., 2003). LeBlanc (2010) reported that placentitis affects approximately 3-

[3]Corresponding author: pv2pemac@uco.es
Received: November 16, 2017
Accepted: December 30, 2017

7% of pregnant mares, in line with Troedsson and Zent's (2004) findings in Thoroughbred mares. But there are no references describing the incidence of foetus loss due to placentitis in SPB mares.

The aim of this study was to monitor CTUP measurements during late gestation to establish a nomogram for SPB mares and determine the cut-off point for identifying ascending placentitis and initiating timely prophylactic therapies.

Materials and Methods

Animals

A total of 107 SPB mares, ranging from 3 to 17 years old, with body weights from 520 to 650 kg and body condition score between 3.5 and 4.5 (using a 5-point scale) were involved in this study. The animals were clinically and reproductively healthy, based on good vulvar conformation (with Caslick index lower than 100), absent of abnormal uterine fluid, negative uterine cytology, and normal ovarian activity before insemination; also, no abortions were reported in these mares during the last two years. They were located at the Miguel Angel Cardenas Equine Reproduction Centre, in Ecija (Seville, Spain; 15° 15' 51'' N, 12° 54' 30'' E). They were inseminated with both cooled and frozen semen, and they were treated for gastrointestinal parasites and for equine herpes virus (EHV) 1 and 4 (Duvaxyn® EHV 1,4 im, Fort Dodge Animal Health Europe) at five, seven and nine months of gestation. The animals were fed with 3 kg of feed/day, plus hay *ad libitum* and free access to water. A total of 94 mares exhibited normal gestation and healthy foals, while 13 mares had abnormal gestations (five aborted and eight gave birth to a weak foal or suffered stillbirth). Following the approach of Morris *et al.* (2007), no foeto-placental compromise was defined as delivery of a healthy foal and no gross signs of placentitis, while foeto-placental compromise was defined as birth of a non-viable, premature or dysmature foetus, or clear signs of placentitis.

Transrectal ultrasonography

All the mares were examined at 210, 240, 270 and 300 days of gestation using ultrasonography equipment (Aquila, Esaote-Pie Medical, Maastricht, Netherlands). A 7.5-MHz linear transducer was located laterally at the cervical-placental junction. The CTUP was recorded from the ventral aspect of the uterine body (Troedsson *et al.*, 1997). The attached placenta was evaluated in the area adjoining the cervical star (Troedsson *et al.*, 1997). Three measurements were taken at each examination.

A total of 415 echographies were made and 1,245 CTUP measurements were taken.

Statistical analysis

All statistical analyses were carried out using SPSS statistics v22.0.0 (Chicago, IL, USA). Data are shown as means ±SD, and differences were considered significant when $P < 0.05$.

Ventral CTUP was scanned and the nomogram for the last months of pregnancy was built. Linear regression (forward stepwise) was carried out for CTUP, including mare age, type of semen, gestation length and gestational age. Only gestational age was introduced into the model. Then, the best-fit regression equation was determined using data from term gestations (i.e. normal gestations), obtaining a linear regression model yielding the following equation: CTUP = (-8.21) + 0.06 x gestational age (days) (R^2 = 0.853)

The receiver operating characteristic (ROC) curve provided information about the CTUP cut-off value, which offers the best predictive values for healthy gestation diagnosis at different stages of pregnancy. Sensitivity and specificity were calculated for different CTUP values. With this proposal, cut-off values were established for 95% confidence interval. Furthermore, predicted positive and negative values were calculated to determine the likelihood of the test accurately identifying the presence of abnormal gestation. Positive predictive value is defined as the proportion of animals that tested positive that were truly affected. Negative predictive value is defined as the proportion of animals that tested negative and were truly healthy.

The Mann-Whitney U test was used to determine if the age of mares and the intervals from insemination were significantly different comparing the mare population showing normal and abnormal gestations.

Results

A high positive correlation was observed between CTUP and gestational age ($r = 0.923$; $P < 0.001$) in mares that showed normal term gestations, with a larger thickness increment as gestation advances. However, variables such as mare age, the interval between parturition and fertilization, type of semen or gestational length did not affect the placental thickness. Gestation length in healthy foaling mares ranged from 308 to 360 days, while mares with abnormal gestations lasted from 280 to 359 days.

As may be seen in Table 1, the monthly CTUP measurements in mares with healthy foals exhibited significant differences ($P < 0.0001$). The CTUP measurements taken on a monthly basis showed a homogeneous mean increase of approximately 1.3 mm from 210 to 270 days. However, from 270 days onwards the mean increased almost threefold (3.0 mm/month) in comparison to the preceding period (Fig. 1). A nomogram for CTUP in SPB mares was undertaken, and data from abnormal pregnancies were subsequently used to determine the cut-off points for providing information about potential gestational defects. In the population studied, the incidence of abnormal gestation was 14%. Results showed that no significant differences appeared on days 210 and 240 (0.728 and 0.053, respectively), suggesting that the measurement of CTUP at these periods is unlikely to be able to discriminate

between healthy and non-healthy gestations. However, on days 270 and 300, i.e. nearer to the parturition, significant differences ($P < 0.0001$) were detected, and it follows that CTUP values can play a role in predicting the success of gestation. The next step was to determine which cut-off value should be used in order to obtain the best diagnostic value. The authors decided to choose the cut-off value with the highest sensitivity and lowest rate of false positive results (calculated as 1-specificity). On day 270, the best cut-off value was 7.750 mm, showing a sensitivity of 92.3% and a false positive percentage of 39.4% (Fig. 2). On day 300, results gave CTUP value of 12.7 mm and enabled a diagnosis of non-healthy pregnancy in all cases (i.e., sensitivity was 100%), with no false positives (Fig. 2).

Table 1 and Fig. 3 show that the increment of CTUP between 210 and 270 days in mares with abortions or weak foals at term was not homogeneous. The increment from day 240 to day 270 was higher (2.4 mm) than that from day 210 to day 240 (1.6 mm), and in the following month (day 300) the thickness increased further (5.3 mm). However, this group of mares showed a similar CTUP measurement and ultrasound image at 210 and 240 days of gestation to those that gave birth to healthy foals (Fig. 3a, b, c and d). Highly significant differences ($P < 0.001$) were observed when comparing the 270 and 300-day CTUP measurements between healthy and non-healthy gestations (Table 1, Fig. 3e, f, g and h). On comparing these increases, significant differences were found both in the results obtained in the period from 240 to 270 days and in that of 270 to 300 days, when comparing the two groups ($P < 0.001$).

The mares that suffered abortion or placental failure were significantly older than the mares with normal gestation (11.7 ± 4.2 *vs.* 9.1 ± 3.9; $P = 0.025$), and exhibited longer intervals between parturition and insemination (220.0 ± 183.0 *vs.* 110.1 ± 160.7 days; $P = 0.025$).

Table 1. Percentage of CTUP measurements in mares with healthy gestational periods and mares with abnormal pregnancy (with stillbirths, late abortions or weak foals) for each month of pregnancy.

Days of gestation	Type of gestation	n	Mean	SD	95% C.I.		P value
					Lower	Upper	
210	Healthy	94	4.83	0.59	4.71	4.95	0.727
	Abnormal	13	4.89	0.32	4.72	5.07	
240	Healthy	94	6.12	0.60	6.00	6.24	0.053
	Abnormal	13	6.47	0.41	6.25	6.69	
270	Healthy	94	7.41	0.61	7.28	7.53	0.0001
	Abnormal	13	8.89	1.39	8.14	9.65	
300	Healthy	94	10.45	0.92	10.26	10.63	0.0001
	Abnormal	11	14.17	1.27	13.42	14.92	

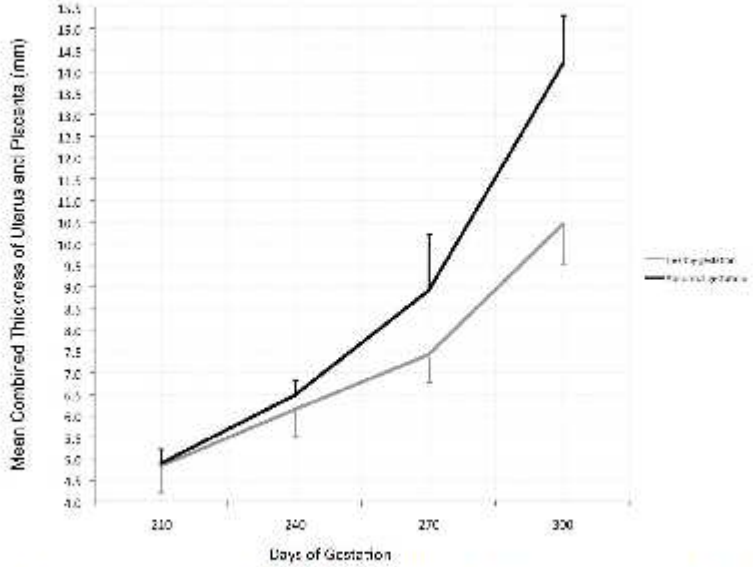

Figure 1. Monthly monitorization of CTUP records (mean ± SD) by transrectal ultrasonography in Spanish Purebred mares with healthy (solid grey line) and non-viable (solid black line) gestation.

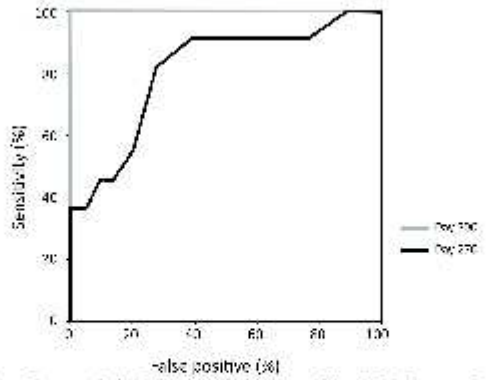

Figure 2. Receiver-operating characteristic (ROC) curves of combined thickness of uterus and placenta in SPB mares at day 270 (black line) and day 300 (grey line) of gestation.

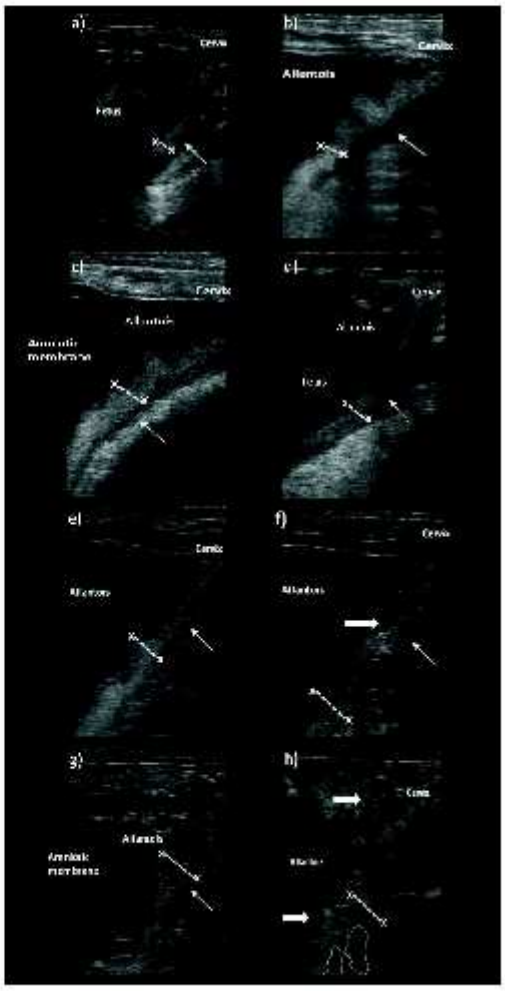

Figure 3. Measurements of combined thickness of uterus and placenta (CTUP) at various phases of gestation. The dashed white line between X refers to CTUP. The thin white arrow indicates the position of the middle branch of the uterine artery in the ventral area of uterine body. The thick white arrow indicates areas of utero-placental separation. The ultrasound images on the left correspond to CTUP measurements in mares with healthy gestation at 210 (a), 240(c), 270(e) and 300(g) days of gestation. The ultrasound images on the right correspond to mares with non-viable gestation at 210(b), 240(d), 270(f) and 300(h) days of gestation. Figure 1h shows separation from the endometrium with exudative fluid (at the bottom, areas delimited by dashed white lines), indicating disorganization of the uterine-placenta junction, mainly in the dorsal area of the placenta.

Discussion

To determine whether a given placental thickness is normal or not, normal placental thickness must be defined for a gestational age. Since this parameter may vary among different populations (Table 2), breed-specific nomograms are needed. Renaudin *et al*. (1997) defined abnormal CTUP as measurements that deviated from 95% confidence interval of previously defined normal values. However, those normal values were taken from nine mares of different breeds (Quarterhorse, Thoroughbred and American Paint Horse). Data obtained here demonstrate that CTUP measurements in pregnant mares is really effective to discriminate healthy and abnormal gestations since day 270, in accordance with the findings of Colon (2008), although placental abnormalities (perhaps associated to ascending placentitis) might occur and be evident sooner. In Criollo mares however, differences have been detected at eight months (240 days; Souza *et al*., 2010).

Table 2. CTUP measurements (mm) in normal gestations.

Breed	n	Days of pregnancy					
		180	210	240	270	300	330
Quarterhorse, Thoroughbred and American Paint Horse (Renaudin et al, 1997)	9	3.84	3.91	4.33	4.38	5.84	7.35
Arabian and ponies (Barnes et al, 2005)	20	6.3	5.0	7.7	7.9	8.4	-
Unknown breed (Bucca et al, 2005)	150	4.7	5.0	5.2	7.7	8.3	9.56
Thoroughbred (Colon 2008)	106	-	-	-	5.5	7.1	8.5
Criollo breed (Souza et al, 2010)	63	3.47	4.02	5.17	4.87	6.53	7.3
Warmblood (Coutinho et al, 2013)	15	-	-	-	-	8.8	9.7
Data obtained in the present study	107	-	4.83	6.12	7.41	10.45	-

The percentage of mares with measurements over the upper limit (based on the 95% confidence interval) was 33.9% in Criollo mares (Souza *et al*., 2010), but 3.1% (Troedsson and Zent, 2004) and 15% (Colón, 2008) in others. In the present study, non-healthy pregnancies in SPB mares represented 14% of the total, and the CTUP average was significantly higher than in healthy pregnancies from day 270. In this context, Souza *et al*. (2010) argue that many mares may be assumed to have placentitis and thus to require treatment, even though in many cases no abortion or symptoms of placentitis are observed. Bucca *et al*. (2005) observed that mares may have increased CTUP without any negative effect on gestational development.

The age of mares did not affect the CTUP in SPB mares, a finding in line with that previously described (Souza *et al*., 2010). However, it was noted that the group of mares that suffered abortion or placental failure was significantly older than mares with normal gestation and took longer to become pregnant. In the present study, the later gestations of mares exhibiting abnormal CTUP were monitored. One mare presenting abnormal CTUP measurements repeated this condition in her subsequent pregnancy, but this was not noted in others.

Gestation losses are a major problem for the equine industry, and for decades attempts have been made to address the difficulty by treating the diseases causing them. One of those cited is placentitis, with a general incidence of 3-7% (LeBlanc, 2010). In recent years ultrasonography has been recognized as providing a useful technique for estimating the state of the placenta and foetus (Troedsson and Zent, 2004). In the present study, the changes in the CTUP measurement in the last months of gestation were recorded, since this is considered to be an indicator of placentitis, as well as alterations in the gestation and/or abortions (Giles *et al*., 1993), with the aim of establishing normal values of this measurement for Spanish Purebred (SPB) mares. The ultrasonography was performed in the area of the cervical star, which is the region most affected by placentitis (Renaudin *et al*., 1997; Calderwood *et al*., 2002; Macpherson, 2005). In the present study, CTUP measurements were monitored at day 210, 240, 270 and 300 of gestation, mindful of the fact that several authors have reported that ascending placentitis causing alterations in gestation occurs over the last months of pregnancy (Troedsson and Zent, 2004; Cummins *et al*., 2008). No differences between healthy and abnormal gestations were observed until day 270, and it is therefore recommended that placental ultrasonographic inspection be initiated no later than this date.

Renaudin *et al*. (1997) obtained a non-significant increase in the monthly CTUP measurement from four to eight months of gestation, but the increase was very significant from 10 to 11 months (1.51 mm) and from 11 to 12 months of gestation (2.17 mm). Similar results in the last months were observed in the present study, with an increase in CTUP from eight to nine months of 1.23 mm, and from nine to 10 months of 3.06 mm in healthy gestations. The same trend with higher measurements was observed in abnormal gestations (2.86 and 4.705 mm from eight to nine months and from nine to ten months, respectively). This is in accordance with the findings reported by Hendriks *et al*. (2009), who found no important differences in the CTUP measurement until the fifth month of gestation, with the fastest enlargement of the placenta occurring in the eighth month. This may be attributable to foetus growth in the third phase of gestation being exponential, vascularization increasing as a consequence in order to meet the needs of the foetus. This also occurs in other species, such as jennies (Crisci *et al*., 2014). Macpherson and Bailey (2008) reported that clinical signs do not occur until the disease is in an advanced stage. The mares that suffered abortion in the present study did not present any detectable external signs. However, they did undergo an increase in their CTUP or

a separation of the endometrium membranes due to their purulent matter (hyperechoic; see Fig. 3h), which accords with other findings (Macpherson and Bailey, 2008). Likewise, in the same image an exudative fluid can be observed in the uteroplacental space (delimited by white dashes). From 210 to 240 days of gestation, both the CTUP measurements and the echography images were similar in both groups. Figure 3f shows, in addition to an increase in the CTUP measurement, an edema in the uteroplacental union in which the placental structure is distinguished as being more hypoechogenic than the uterus. However, in Fig. 3g, in spite of there being an edema both on the dorsal and ventral edge of the uterus corpus, it is apparent that this is a physiological situation in the last month of gestation. It should be noted that in this study it was estimated that 8.5% of the evaluated mares aborted, or gave birth to a weak foal, a percentage which, although based on a small sample of the population, could be representative of SPB mares in general, since it does not differ from what has been reported by other authors (Giles *et al*., 1993; Smith *et al*., 2003; Troedsson and Zent, 2004; LeBlanc, 2010).

In equine reproduction, the use of transrectal ultrasonography is a minimally invasive routine procedure. In the present study it did not cause any adverse effects on scanned pregnant mares and this is in accordance with the findings of Renaudin *et al*. (1997). Since most placentitis occurs by upward routes, the infection very often first affects the cervical star (LeBlanc, 2004; Troedsson and Zent, 2004), and for this reason the use of transabdominal ultrasonography is not advisable, since it is unable to provide scans of the area in question. The present authors found that transrectal echography may be readily evaluated, in accordance with other findings (Cummins *et al*., 2008).

These results are a preliminary attempt to determine CTUP cut-off values in Spanish mares, and indicate the importance of carrying out the transrectal evaluation of placental thickness from the ninth month of gestation. As has been highlighted by others authors (Bucca *et al*., 2005; Colón, 2008), it is important to monitor foetal status throughout pregnancy if signs of problems are to be detected in a timely manner. However, this was not the aim of the present study.

In conclusion, in SPB mares, a monthly transrectal ultrasonographic examination is recommended from 210 days of gestation, with the aim of assessing the thickness of the placenta by means of CTUP measurement; furthermore, this study demonstrated that differences between healthy and abnormal gestations are more evident since day 270 of gestation. A placental thickness nomogram has been defined for SPB mares during late-term gestation and around 14% of pregnant mares has been diagnosed as placental thickness enlargement.

Acknowledgements

The authors are grateful to Miguel Angel Cárdenas (R.I.P.) and his staff for allowing them to do this research in his stud.

Conflict of interest

None of the authors have any conflict of interest to declare. There has been no financial support for this work that could have influenced its outcome.

References

Bailey CS, Heitzman JM, Buchanan CN, Bare CA, Sper RB, Borst LB, Macpherson M, Archibald K, Whitacre M. 2012. B-mode and Doppler ultrasonography in pony mares with experimentally induced ascending placentitis. *Equine Vet J,* 44:88-94.

Barnes M, Fite C, Tibary A. 2005. Trans-rectal ultrasonographic evaluation of the placenta in Arabian and pony mares in mid- to-late gestation. *Theriogenology,* 64:787. (abstract).

Bucca S, Fogarty U, Collins A, Small V. 2005. Assessement of feto-placenta well-being in mare from mid-gestation to term: transrectal and transabdominal ultrasonographic features. *Theriogenology,* 64:542-557.

Calderwood M, LeBlanc MB, Paccamonti D. 2002. Route of fetal infection in a model of ascending placentitis. *Theriogenology,* 58:791-792.

Carrick JB, Begg AP, Perkins N, O'Meara D. 2010. Ultrasonographic monitoring and treatment of pregnant mares at risk for placentitis *Anim Reprod Sci,* 121:331-333.

Colón JL. 2008. Trans-rectal ultrasonographic appearance of abnormal combined utero-placental thickness in late-term gestation and its incidence during routine survey in a population of Thoroughbred mares (2005-2008). *Proc Am Assoc Equine Pract,* 54:279-285.

Coutinho da Silva MA, Canisso IF, MacPherson ML, Johnson AE, Divers TJ. 2013. Serum amyloid A concentration in healthy periparturient mares and mares with ascending placentitis. *Equine Vet J,* 45:619-624.

Crisci A, Rota A, Panzani D, Sgorbini M, Ousey JC, Camillo F. 2014. Clinical, ultrasonographic, and endocrinological studies on donkey pregnancy. *Theriogenology,* 81:275-283.

Cummins C, Carrington S, Fitzpatrick E, Duggan V. 2008. Ascending placentitis in the mare: a review. *Ir Vet J,* 61:307-313.

Dudley DJ, Trautman MS. 1994. Infection, inflammation and contractions: the role of cytokines in the pathophysiology of preterm labor. *Semin Reprod Endocrinol,* 12:263-272.

Giles RC, Donahue JM, Hong CG. 1993. Causes of abortion, stillbirth, and perinatal death in horses: 3527 cases (1986-1991). *J Am Vet Med Assoc,* 203:1170-1175.

Hendriks WK, Colenbrander B, van der Weijden GC, Stout TAE. 2009. Maternal age and parity influence ultrasonographic measurements of fetal growth in Dutch Warmblood mares. *Anim Reprod Sci,*115:110-123.

LeBlanc MM, Giguere S, Brauer K, Paccamonti DL, Horohov D, Lester GD, O'Donnell LJ, Sheerin BR, Pablo L, Rodgerson DH. 2002. Premature delivery in ascending placentitis associated with increased expression of placenta cytokines and allantoic fluid prostaglandins E2 and F2. *Theriogenology* 58:841-844.

LeBlanc MM. 2004. Ascending placentitis in mares: what we learned from an experimental model. *In*: Proceedings of the 10th Congreso Nazionala Multisala SIVE, Perugia, Italy. Ithaca, NY: IVIS.

LeBlanc MM. 2010. Ascending placentitis in the mare: an update. *Reprod Domest Anim*, 45:28-34.

Löf HK, Pires Neves A, Gregory JW, Winter GHZ, Garbade P, Wolf CA, Richter G, Jobim MIM, Gregory RM, Mattos RC. 2010. Evaluation of the combined utero-placental thickness (CUPT) and of vulvar conformation as indicators of placentitis in pregnant mares. *Anim Reprod Sci*, 121:329-330.

Macpherson ML. 2005. Treatment strategies for mares with placentitis. *Theriogenology* 64:528-534.

Macpherson ML, C.S. Bailey CS. 2008. A clinical approach to managing the mare with placentitis. *Theriogenology*, 70:435-440.

Ministerio de Agricultura, Alimentación y Medio Ambiente - MAGRAMA 2016. Available on: http://www.magrama.gob.es/es/ganaderia/temas/zootecnia/razas-ganaderas/razas/catalogo/autoctona-fomento/equino-caballar/espanola/iframe-ejemplo-arca.aspx. Accessed on January 9, 2017.

Morris S, Kelleman AA, Stawicki RH, Hansen PJ, Sheerin PC, Sheerin BR, Paccamonti DL, LeBlanc MM. 2007. Transrectal ultrasonography and plasma progestin profiles identifies feto-placental compromise in mares with experimentally induced placentitis. *Theriogenology*, 67:681-691.

Palmer E, Driancourt MA. 1980. Use of ultrasonic echography in equine gynecology. *Theriogenology*, 13:203-216.

Platt H. 1975. Infection of the horse fetus. *J Reprod Fertil Suppl*, 23:605-610.

Pollard KJ, Mitchell MD. 1996. Intrauterine infection and the effects of inflammatory mediators on prostaglandin production by myometrial cells from pregnant women. *Am J Obstet Gynecol*, 174:682-686.

Reef VB, Vaala WE, Worth LT, Spencer PA, Hammett B. 1995. Ultrasonographic evaluation of the fetus and intrauterine environment in healthy mares during late gestation. *Vet Radiol Ultrasound*, 36:533-541. **Renaudin CD, Troedsson MHT, Gillis CL, King VL, Bodena A**. 1997. Ultrasonographic evaluation of the equine placenta by transrectal and transabdominal approach in the normal pregnant mare. *Theriogenology*, 47:559-573.

Rossdale PD, Ousey JC, Cottrill CM, Chavatte P, Allen WR, McGladdery AJ. 1991. Effects of placental pathology on maternal plasma progestagen and mammary secretion calcium concentrations and on neonatal adrenocortical function in the horse. *J Reprod Fertil Suppl*, 44:579-590.

Smith KC, Blunden AS, Whitwell KE, Dunn KA, Wales AD. 2003. A survey of equine abortion, stillbirth and neonatal death in the UK from 1988 to 1997. *Equine Vet J*, 35:496-501.

Souza AM, Winter GHZ, Garbade P, Wolf, CA, Jobim MIM, Gregory RM, Mattos RC. 2010. Ultrasonography evaluation of the Criollo mare placenta. *Anim Reprod Sci*, 121:S320-321.

Stawicki RJ, Reubel H, Hansen PJ, Sheerin BR, O'Donnell LJ, Lester GD, Paccamonti DL, LeBlanc MM. 2002. Endocrinological findings in an experimental model of ascending placentitis in the mare. *Theriogenology*, 58:849-852.

Troedsson MHT, Renaudin CD, Zent WW, Steiner JV. 1997. Transrectal ultrasonography of the placenta in normal mares and in mares with pending abortion: a field study. *Proc Am Assoc Equine Pract*, 43:256-258.

Troedsson MHT, Zent WW. 2004. Clinical ultrasonographic evaluation of the equine placenta as a method to successfully identify and treat mares with placentitis. *In*: Betteridge L (ed.). *Workshop on the Equine Placenta*. Lexington, KY: Univeristy of Kentucky Agricultural Station. pp. 66-67.

ARTÍCULO DE INVESTIGACION 4

Reproductive efficiency of Spanish Purebred mare in Spain

FD Requena, EI Agüera, CC Pérez-Marín

EN PREPARACIÓN

REPRODUCTIVE EFFICIENCY OF SPANISH PUREBRED MARE IN SPAIN

INTRODUCTION

The Spanish Purebred (SP) horse industry in Spain is the first in the national census and the most important Spanish horse breed. This breed is known worldwide that breeds in over fifty countries. There are approximately 230.000 SP horses in all over the world (MAGRAMA, 2015). Traditionally SP was bred by his beauty and morphology, but since the late nineties is also used as sport horse in dressage. Therefore, the SP horse has been selected contemplating its sporting success more than its fertility rate.

Artificial insemination (AI) is the widely practiced breeding method in the most sport horse breeds because avoids health problems and costs associated with the transport of animals and moreover, AI helps to increase reproductive efficiency (Katila, 2005; Aurich, 2012). But equine species has been showed to have relatively lower reproductive efficiency compared to others domestics species (Engelken 1999; Menzies 1999). Thus, obtaining foals from stallions and elite mares has a high economic value. Therefore, horse breeders and veterinarians requires information about reproductive parameters of horses to make decisions (Katila et al., 2010).

On the one hand, there are many studies about reproductive efficiency in different breeds horses. Aged and reproductive status has been studied as the main factors involved in reproductive efficiency. Demirci (1987) studied the fertility in Purebred Arab horses, finding an 80.1% conception rate total, 2,4% of mares aborted or produced a dead foal and 77.8% produced live foals. Yurdaydin et al. (1993) reported a pregnancy rates in first cycle of 66.7%, 75% and 87% with different estrus synchronization

protocols. Cilek (2009) performed a retrospective analysis that showed a 74% pregnancy rate total, foaling rate of 69.07% and ratio of abortion and embryonic deaths of 4.93%. In Tunisia, in other retrospective analysis (Benhajali et al., 2010), the Arabian mares were classified according to three different reproductive status (maiden, barren and foaling mares) with a conception rate of 87%, 84% and a 77%, respectively. The overall conception and first-cycle conception rates were of 84% and 51%. Warriach et al. (2014) found in Pakistan, a conception rate in first mated mares of 75%, a 3% abortion rate and a 3% dead born rate. Also they established an overall conception rate of 52% for 3-7 years old mares, 48% for 8-12 years old mares, 38% for 13-17 years old mares, and 30% for 18 years old mares or older.

In Thoroughbred mares, Hevia et al. (1994) published a study where the fertility was 72.78%, the fecundity was 65.64% and the abortion rate of 6.30%. Morris and Allen (2002) announced the findings of a retrospective survey of 1393 Thoroughbred mares, where according to age mares, pregnancy rate at first cycle oscillated between 50-62.70%, mares pregnant rate at end season oscillated between 76.90-90.40% and mares produced a live foal oscillated between 73.10-86.60%. Conforming to reproductive status, pregnancy rate at first cycle was 36.30-64.90%, mares pregnant rate at end season was 81.80-92.40% and mares produced a live foal was 70.60-88.80%. Total pregnancies lost until day 35 was 10.39% and overall was 17.38%. In Sweden, Hemberg E. et al. (2004) found that first cycle conception rate was 68.80%, overall conception rate was 90.90% and overall live foal rate was 79.80%. The overall pregnancy loss rate from the first pregnancy diagnosis until foaling was 12.5%.

Allen et al. (2007) presented the results of an extensive survey comparing Flatrace and National Hunt Thoroughbred mares, with a total pregnancy rates per cycle of 63.20% and 65.30%, respectively. According to age, the pregnancy rates per oestrus were of 54.9% to 67.3%, and from 62.80% to 81.80% of the mares produced a live foal. According reproductive status, the pregnancy rates were of 58.40% to 68.30% and from 77.30% to 81.50% of the mares produced a live foal. Overall percentage pregnancies lost prepartum were 13.5% versus 16.2%. In Kentucky, Bosh et al. (2009) reported that overall day 15 pregnancy rate per cycle was of 64% and taking into account the age and reproductive status, the day 15 pregnancy rates per cycle, were of 48.10-66.30% and 59.80-66.20%, percentages pregnancies lost about 4,60-23.10% and 4,50-13.50% and a live foal rates per season about 55.2-82.9% and 76.50-86.50%, respectively. Another similar study, by Nath et al. (2010), found 70.80% and 68.80% in early pregnancy rate at first cycle and all cycles, respectively. These percentages range according to age between 53.70-73.70%, and reproductive status between 51.30-72.80%. In India, a study based in a retrospective survey over 7 years (Sharma et al., 2010b) showed according to age versus reproductive status, a percentage day 16 pregnancies per oestrus of 35.90-54.08% vs 41.74-55.03%, percentage day 16 per season of 63.64-89.83% vs 76.00-92.08%, late embryonic losses of 6.92-28.57% vs 4.30-18.42%, early fetal losses of 0.00-3.68% vs 1.59-4.30%, mares produced a live foal of 34.09-76.55% vs 52.00-78.22%.

Also, Nath et al. (2010) compared Standardbred horses with Thoroughbred horses, with similar results to those previously mentioned. There is a study in Finland, where Katila et al. (2010) performed a descriptive result about reproductive efficiency of Standardbred and Finnhorse Trotters horses, with foaling rates of 37.50-72.10% and 52.20-76.40% respectively taking into account age and reproductive status.

On the other hand, there are authors that have studied the influence of other factors on the mare fertility, such as cooled or frozen semen (Loomis, 2001; Samper, 2001; Sieme et al., 2003), number of inseminations/matings per cycle (Allen et al., 2007; Nath et al., 2010), type of oestrus, type of ovulation (Yurdaydin et al., 1993; Allen, 2007; Sharma et al., 2010b; Hanlon and Firth, 2012), breeding season and year of breeding season (Morris and Allen, 2002; Hemberg et al., 2004; Cilek, 2009; Katila et al., 2010; Haadem et al., 2015), uterine fluid accumulation (Allen et al., 2007; Sharma et al., 2010a; Lewis et al., 2015) and stallions (Morris and Allen, 2002; Hemberg et al., 2004; Benhajali et al., 2010; Nath et al., 2010; Haadem et al., 2015).

However, no such information in SP mares exists to the best of our knowledge. There is only little information concerning this breed that reports the gestation length in the mare (Valera et al. 2006), different sperm morphological abnormalities (Hidalgo et al. 2006, 2008), and seminal characteristics in stallions (Akourki et al. 2013).

The main objective of our investigation was to determine reproductive efficiency of SP mare through the pregnancy rates, embryonic and fetal losses and foaling rate. To gather this information, it was evaluated the influence of age, reproductive status, insemination with cooled or frozen semen, number of inseminations per cycle, type of oestrus, type of ovulation, breeding season, year of breeding season, presence of uterine fluid, stallions and stallion age.

MATERIALS AND METHODS

Database and management

Records inseminations of 122 SP mares for four consecutive (2012-2013, 2013-2014, 2014-2015, 2015-2016) breeding seasons (from October to next year May) from "Equine Reproduction Centre Miguel Angel Cárdenas S.L." (37°31' N, 5°7' W) in Southern Spain (Ecija, Seville) were analysed. A total of 359 cycles were studied and 29 cycles were excluded because ovulation no occurred. A total of 55 mares were only once to the Equine Reproduction Centre, and 67 were more than once and they were considered as new mare in the next breeding season. Thus, 244 different SPB mares were studied.

All mares were in individual paddocks or in groups of 2-5 mares. They were fed a daily ration based 4 kg of concentrate divided twice in a day, plus alfalfa ad libitum. All data was entered in Microsoft Office Excel 2003 ®. Data about breeding season, age, last pregnancy, insemination date, spontaneous or induced oestrus, spontaneous or induced ovulation, presence of intrauterine fluid, number of inseminations per cycle and method of insemination (with cooled or frozen semen), stallion, pregnancy diagnosis, confirmation of pregnancy, and foaling were taken for each oestrus cycle and mare.

When the mares arrived at the Centre, the uterus and ovaries were scanned by transrectal ultrasonography with 5 – 7,5 MHz multifrequency probe (Tringa Linear®, Esaote-Pie medical, The Netherlands) to assess the presence of oestrus. If mares were in luteal phase (presence CL) they received 0,075 mg D-cloprostenol im (Dalmazin®, Fatro-Ibérica, Barcelona, Spain) to induce oestrus. Mares were in anoestrus were expected to first natural oestrus or treated with oral altrenogest (0.044 mg/Kg;

Regumate®, Esteve, Barcelona, Spain) for 10 days or with intravaginal progesterone-releasing device (1.38 g; CIDR®, Zoetis, Madrid, Spain) for 8 days. The last day of both treatments, mares received 0,075 mg D-cloprostenol im (Dalmazin®, Fatro-Ibérica, Barcelona, Spain). Pregnancy was diagnosed 14 days after ovulation by uterine ultrasonography, and later confirmed on days 23, 42, 60, 180, 210, 240, 270 and 300.

In AI with cooled semen, usually ovulation induction was conducted with 3000 UI hCG IV (Veterin Corion®, Divasa-Farmavic, Barcelona, Spain) when there was at least one preovulatory follicle ≥ 35 mm diameter and ≤ 40mm. In case it was found preovulatory follicle with size > 40mm at the first ultrasonography, ovulation was not induced. Mares with induced ovulation were inseminated once (24 hours post-induction) or twice (24 and 48 hours post-induction), until ovulation was detected by transrectal ultrasonography. Mares, without induced ovulation, were scanned and inseminated each day until ovulation. Standard doses of cooled semen from tested stallion were used and semen was deposited into the uterine body.

In AI with frozen semen, usually ovulation induction was carried out with the same procedure that in AI with cooled semen. Mares were monitored ultrasonographically 24 hours post-induction and every 6 hours until ovulation was confirmed, after which the postovulatory insemination was performed. If in the ultrasonograpfy we found loss the spherical shape of the follicle and/or uterine edema, ovulation was not induced and mares were monitored ultrasonographically every 6 hours with the same previous procedure. Standard doses of frozen semen from tested stallion were used and semen was deposited into the tip of the uterine horn ipsilateral to the preovulatory follicle (deep AI) using transrectally guided technique.

When mares had presence of more than 2 cm of uterine fluid during oestrus pre or post-insemination or positive bacteries or fungi cultures, received standard intrauterine treatments with prostaglandins, uterine lavages and antibiotics (Brinsko et al., 2003b; Nikolakopoulos et al., 2000; Maischberger et al., 2008). Treatment altrenogest was administered until 100 days post-ovulation when an insufficient growth and development of the conceptus was found at the pregnancy diagnosis (Willmann et al., 2011).

Groups

The mares were grouped according to age: young (3-7 years), middle-aged (8-11 years), ageing (12-14 years) and very old (≥ 15 years) in relation to reproductive status: maiden (never mated), barren (mated without a pregnancy at the end of last breeding season), foaling (mare with a live or dead foal) and rested (not mated after the last foaling).

Statistical analysis

Pregnancy rate in first cycle (number of mares diagnosed pregnant on day 14 post-ovulation in the first cycle, divided by the number of mares inseminated in the first cycle), pregnancy rate in all cycles (number of mares diagnosed pregnant on day 14 post-ovulation in all cycles in the breeding season, divided by the number of mares inseminated in all cyces in the breeding season), pregnancy rate on day 14 pregnancies per season (number of mares detected pregnant on day 14 post-ovulation during the breeding season divided by total number of inseminated mares in the breeding season), pregnancy rate of mares per season (number of mares detected pregnant on 60 days after the end of the breeding season divide by total number of inseminated mares in the

breeding season) and foaling rate (number of mares that have foaled the next breeding season divided by total number of inseminated mares in the breeding season) were calculated for the type of semen used in the insemination, age group and reproductive status. Moreover, embryonic and fetal losses on day 15-60 (percentage of mares that lost their day 14 pregnancies between days 15 and 60 post-ovulation) were calculated to age group and reproductive status. As well, pregnancy rate was calculated in all cycles for number of inseminations per cycle, type of oestrus and ovulation, breeding season, year of breeding season, uterine fluid accumulation and effect of stallion (age groups).

All statistical analyses were performed using the predictive analytics software (PASW Statistics 18, Chicago, IL, USA) for Windows. Chi-squared tests were used to compare proportions. If the effect of variable was significant ($P<0.05$), multiple comparisons between groups were carried out and each comparison was considered significant only after making the Bonferroni adjustment.

RESULTS

Significant differences were observed comparing pregnancy rates using cooled or frozen semen. Pregnancy rates were highest with cooled semen in following cases: first cycle ($P<0.05$), all cycles ($P<0.05$), day 14 pregnancies per season ($P<0.001$), mares per season ($P<0.001$) and foaling rate ($P<0.001$). Major difference was on foaling rate, with 72.92% of mares foaled using cooled semen, while only 42.31% mares foaled with frozen semen. There were not differences of embryonic and fetal losses on days 15-60 with cooled or frozen semen ($P=0.18$) (Table 1).

Pregnancy and foaling rates were significantly influenced by the effect of age. The young mares (3-7 years) had the highest pregnancy rate on the first cycle (71.23%) and

there were very significant differences with the ageing (12-14 years) and very old (≥ 15 years), under Bonferroni adjustment at P<0.01. Similar results obtained in pregnancy rate on all cycles. Young mares had 70.21% and the next age group had a falling rates, middle-aged (52.00%), ageing (41.33%) and very old (41.11%), with highly significant differences between young mares compared with ageing and very old mares (P<0.001). Young mares had the best pregnancy rate on day 14 per season (91.66%) with very significant differences (P<0.01) compared with ageing (65.96%) and very old mares (61.66%). The middle-aged had 78.46% and there were not significant differences with the other age groups under Bonferroni adjustment. Also, young mares had the highest pregnancy rate of mares per season (86.11%), followed of middle-aged (70.77%), ageing (63.83%) and very old (46.67%). There were significant differences (P<0.05) between young mares and the oldest mares (ageing and very old mares) and between very old mares and youngest mares (young and middle-age). Similar results and the same significant differences (P<0.05) between groups were found to foaling rate. Very old mares had the highest percentage of embryonic and fetal losses on days 15-60 (24.32%) and there were significant differences (P<0.05) compared with young (6.06%) and ageing mares (3.23%), but no significant differences with middle-aged mares (9.80%) (Table 2).

As regards the effect of reproductive status on the pregnancy rates and foaling rate also there were significant differences but not in embryonic and fetal losses on days 15-60. Maiden mares had highest pregnancy rate in the first cycle (86.96%) and there were significant differences (P<0.05) compared with barren (42.68%) and foaling mares (54.21%). Similar results were calculated to pregnancy rate in all cycles but with significant differences (P<0.05) between maiden mares with the other reproductive

status groups. Maiden mares had highest pregnancy rate on day 14 per season (100%), mares per season (95.65%) and foaling rate (91.30%) and there were very significant differences (P<0.001) between maiden mares with barren and foaling mares, both as in pregnancy rate on day 14 per season and foaling rate. Only there were significant differences (P<0.05) in pregnancy rate of mares per season (Table 3).

When the effects of various factors like number of inseminations per cycle (P=0.65), type oestrus (P=0.38), type of ovulation (P=0.15), breeding season (P=0.07) and year of breeding season (P=0.10) in pregnancy rate in all cycles were analysed, there were not significant differences (Table 4). Neither there were not significant differences (P=0.43) with the effect stallion on pregnancy rate in all cycles (Table 6).

Regarding to the influence the uterine fluid accumulation on the pregnancy rate in all cycles, there was significant differences between normal mares (58.13%) and mares with uterine fluid accumulation (P<0.05), before breeding (42.35%) or post-breeding (25.00%) (Table 5).

DISCUSSION

This is the first report on the reproductive efficiency of SP mares. Our study found important differences between mares inseminated with cooled or frozen semen. Jasko et al., (1992). In our investigation, pregnancy rates and foaling rate were highest when cooled semen was used than when frozen semen was used, ranges between 54.70-81.25% and 39.13- 55.77%, respectively (Table 1). In return, other author affirms there are similar results between cooled and frozen semen (Loomis, 2001). Loomis explained that this is possible with good quality semen, selection of mares and stallions and good

mare management. With cooled semen, pregnancy rate in the first cycle in our study was very similar to that published by Demirci (1987), (60.30%), Loomis (2001), (59.40%), Sieme et al., (2004), (58.50%) and Benhajali et al., (2010), (51%). But Allen et al., (2007) and Nath et al., (2010) found higher values because they calculated the pregnancies rates with greater proportion of young mares (2-13 years old) than old mares (>13 years old), increasing the number of pregnant mares. Pregnancy rate in all cycles was similar to other authors (Morris and Allen, 2002; Sharma et al., 2010b). Although for this same rate, there are other articles with higher percentages than our it (Bosh et al., 2009; Nath et al., 2010), perhaps this is due to above reason about more young mares than old mares. Also pregnancy rate of mares per season was very similar to the found by Loomis (2001), (74.70%). In regard to pregnancy rate on day 14 per season and foaling rate, our study coincided with various reports (Demirci, 1987; Hemberg, 2004; Allen et al., 2007; Bosh et al., 2009; Cilek, 2009; Katila, 2010; Sharma et al., 2010b). Only Morris and Allen, (2002) showed higher foaling rate (82.69% mares produced a live foal) than our (72.92%), because they had major proportion of young mares.

With frozen semen, pregnancy rates in our study oscillated between 39-55%, in according to the results reported in one experiment where the authors (Sieme et al., 2004) used different techniques of insemination (48.40%). Samper et al., (1991) reported percentages in the same way. But Loomis (2001) obtained seasonal pregnancy rate higher (75.60%) than our study. Maybe our results with frozen semen were lower due to we inseminated a high proportion of mares older than 11 years (29/52) and 16 mares were inseminated only once by customer's decision. However, type of semen did not influence in embryonic and fetal losses on days 15-60. Moberg, (1975) cited

seminal treatments (mating or insemination with fresh and frozen semen) like a cause responsible for early embryonic death but Villahoza et al., (1985) did not find differences using fresh or frozen semen, 12.5% and 16.7% respectively. These values are very similar to those found in our study and may have been due to improvement in the freezing procedure with best semen quality (Loomis, 2001) and to the altrenogest treatment carried when an insufficient growth and development of the conceptus was found at the pregnancy diagnosis (Willmann et al., 2011).

Agree with several studies, we have identified age as the most important factor influencing pregnancy rates, embryonic and fetal losses and foaling rate (Morris and Allen, 2002; Allen et al., 2007; Bosh et al., 2009; Benhajali et al., 2010; Katila et al., 2010; Nath et al., 2010; Sharma et al., 2010b). We agree with all these authors, that the younger mares always had the best values on pregnancy rates, embryonic and fetal losses and foaling rate. We found in this group the higher percentage on pregnancy rate in first cycle (71.23%) compared with investigations of Morris and Alle, 2002 (62.7%), Allen et al., 2007 (67.3%), Boshet al., 2009 (66.3%) and Sharma et al., 2010b (54.08%). Only Nath et al. (2010) got better percentage than us (73.7% in Thoroughbred and 73.6% in Standardbred). This is because both the latter authors and us used intensive reproductive management with artificial insemination.

It is widely accepted that mare fertility begins to decline from 13 to 17 years old leading this category of animals to be referred to as subfertile (Ginthe, 1992; Allen et al., 2007). Our results agree with this statement, mares are 15 years old or older got the worst results, so we could consider that mares from 15 years old are subfertile. The decline in fertility with age has been attributed to chronic progressive degenerative changes in the endometrium (Ricketts and Alonso, 1991) that reduce its nutritive capacity for the

developing conceptus (Bracher et al. 1996). Also with senescence was observed poor perineal conformation, defects myometrial contractions, lymphatic drainage and uterine incompetence that increasing susceptibility to uterine infection and endometritis (Kalirajan and Rajasundaram, 2008; LeBlanc and Causey, 2009).

Regarding the effect of mare's reproductive status on fertility there are different results. In our study, the maiden mares had the best percentages on pregnancy rates and foaling rate, agree with other authors (Bosh et al., 2009; Nath et al., 2010; Sharma et al., 2010b). In accordance with us, Allen et al. (2007) showed significant differences between maiden and barren mares on pregnancy rate at first cycle. Although we obtained the highest percentages on pregnancies rates and foaling rate in maiden mares. Lewis et al. (2015) calculated best pregnancy rate in young maiden mares (≤11 years) that in old maiden mares (>11 years). However, other authors stated that the category of the mare or reproductive status affected only its overall conception rate (Samper et al., 2002; Benhajali et al., 2010). Hemberg et al. (2004) only found numerically difference between the conception rate and live foal rate between maiden/foaling mares and barren/resorbed/aborted mares. The main reason for this is the highest proportion of problem, barren and aborted mares being found among the older mare categories (Hemberg et al., 2004; Sharma et al., 2010b). In our results, major proportion youngest mares were in maiden mares group. But not correlation was found between age and reproductive status of the mare ($p > 0.05$) (Benhajali et al., 2010). Perhaps, it would be necessary to conduct a study like the one carried out by Katila et al. (2010) where they analysed the mares in different age groups and each age group in different reproductive status.

As for the effects of other factors on fertility, in our study the number of inseminations per cycle didn't show significant differences. But, Allen et al, (2007) published higher per cycle early pregnancy rates in a mares group that had the overall mean number of matings/oestrus higher. In the same line, Nath et al., (2010) obtained best percentage on pregnancy rate with 2 inseminations per cycle than with 1 insemination in Standarbred mares (71.8% *vs* 66%). Although in Thoroughbred obtained best percentages with 1 insemination. The reason for these results was the use of ovulation induction agents. When ovulation is not induced, more inseminations must be performed to get ovulation occurs close next to the time of insemination and thus to get better pregnancy rates (Allen et al., 2007; Nath et al., 2010). Moreover, Allen et al., (2007) explained that the significantly higher use of intrauterine antibiotic therapy was beneficial to successful conception. If we add to all this, the high demand for some stallions with chilled semen, high semen prices and that each insemination presents a challenge to the uterus in clearing accumulated fluid (Troedsson et al., 2001; Maischberger et al., 2008), it seems logical to perform a single insemination using ovulation inducing agents to improve pregnancy rates (Pycock, 2006).

According to our study, most previous studies indicate that there is no difference on pregnancy rates between spontaneous/induced oestrus and ovulation (Morris and Allen, 2002; Allen et al., 2007; Sharma et al., 2010b; Hanlon and Firth, 2012). Yurdaydin et al., (1993) obtained pregnancy rate at first cycle ranged between 66.7-87% with synchronization treatments (prostaglandin and human chorionic gonadotropin). Hanlon and Firth (2012) performed an experiment treating Thoroughbred mares in transitional phase with intravaginal progesterone releasing device. They found a significant increase the number of mares confirmed pregnant by the end of the breeding season, because

they advanced the first service date, effectively extended the breeding season. Then hormonal induction of ovulation only reduces the number of matings/inseminations per oestrus and matings/inseminations required per pregnancy (Allen et al., 2007).

Also in according to our study, the majority of authors have not reported a significant effect of the breeding season and the year of breeding season on pregnancy rates (Morris and Allen, 2002; Hemberg et al., 2004; Cilek, 2009; Katila et al., 2010; Haadem et al., 2015). Only Benhajali et al. (2010) have reported about a logistic regression results where they found that breeding year affected the first-cycle conception rate, although they did give none explanation about this result. Morris and Allen (2002) found higher percentages of twin ovulations in July (within breeding season) and higher percentages of twin pregnancies in February (transitional phase). Hemberg et al. (2004) affirmed that month of mating did not show significance influence on conception rates, although in the early breeding season (March) they obtained a numerically higher conception rate but they did not provide any explanation about this. Cilek (2009) found a significant effect of the breeding month and year on gestation duration, service period, foaling interval, oestrus cycle per pregnancy and number of inseminations per conception, but no influence on pregnancy rates. Katila et al. (2010) published a decrease on foaling rates for 15 consecutive years that they explained by an increasing age in the mares. Regarding the breeding season, it should be noted that there is a higher incidence of anovulatory follicles (Ginther et al., 2006) that could lead to worse pregnancy rates.

It is widely known for several years that accumulation of uterine fluid and mucus during oestrus or after breeding is associated with decreased pregnancy rates (McKinnon et al.

1988; Pycock and Newcombe 1996). Our results were in agreement with this. The pregnancy rate in all cycles without uterine fluid accumulation before or later insemination was higher more than twice than in cycles with uterine fluid accumulation post-insemination. We stablished a treatment as described by Maischberger et al. (2008) in all cycles with uterine fluid accumulation, then maybe we obtained best results that without treatment. Also, Lewis et al. (2015) had pregnancy rates significantly lower in cycles in which there was detectable prebreeding uterine fluid accumulation compared with cycles with no prebreeding fluid. Sharma et al. (2010a) showed better pregnancy rate on Day 16 per oestrus in mares that received treatment uterine but not in other reproductive performance parameters. They explained this could due to low mare numbers, several different veterinarians applying treatments and maybe the mares which received uterine treatment would have had minimal compromised uterine health which would be expected to be resolved with uterine therapy. Moreover, in other study (Allen et al., 2007) analysed different intrauterine therapies finding none resulted in a significant increase in the per cycle early pregnancy rate.

In accordance with our research, one article recent (Haadem et al., 2015), published that stallion age had no effect on fertility parameters. Even though, they found variations at first cycle pregnancy rate and foaling rate for individual stallions. Also, other authors found differences in pregnancy rates between individual stallions (Morris and Allen, 2002; Nath et al., 2010). (Benhajali et al., 2010) showed an effect stallion on the overall conception rate from 72% to 93% per stallion and an effect sire on the first cycle conception rate ranged per sire from 29% to 79%. Instead, Hemberg et al. (2004) claimed that there was no significant difference between stallions in overall conception rate, but there was a numerical difference in the live foal rate. Therefore, stallion can have an effect on fertility parameters depending sperm quality and number of mare

mating per stallion. In our case, all stallions had a proven fertility and good quality sperm. Maybe, for this reason we did not found that stallion age and individual stallion effect had no influence on fertility parameters.

In conclusion, SP mares show similar fertility results to other breed mares. Type of semen (cooled or frozen), mare age, reproductive status and uterine fluid accumulation are the most important factors that influence decisively on fertility parameters of SP mares. Best results are obtained with cooled semen, in young mares, maiden mares and mares in oestrus without uterine fluid accumulation. SP mares can considered like a very fertile horse breed.

Acknowledgements

The authors are grateful to Miguel Angel Cárdenas (R.I.P.) and his staff for allowing them to do this research in his stud.

Conflict of interest

None of the authors have any conflict of interest to declare. There has been no financial support for this work that could have influenced its outcome

REFERENCES

Allen W.R., Brown L., Wright M., Wilsher S., 2007. Reproductive efficiency of Flatrace and National Hunt Thoroughbred mares and stallions in England. Equine Veterinary Journal 39, 5, 438-445.

Akourki A, Echegaray A, Mitjana O, 2013: Seminal Characteristics in Spanish Purebred Stallions: A Retrospective Study. J Equine Vet Sci 33, 649-652.

Aurich JE, 2012: Artificial insemination in horses - More than a century of practice and research. J Equine Vet Sci **32**, 458-463.

Barbacini S., Necchi D., Zavaglia G., and Squires E.L., 2003: Retrospective study on the incidence of postinsemination uterine fluid in mares inseminated with frozen/thawed semen. Journal of Equine Veterinary Science. Volume 23, Issue 11, Pages 493–496

Benhajali H, Richard-Yris M-A, Ezzaouiab M, Charfic F, Hausberger M, 2010: Factors influencing conception rates of Arab mares in Tunisia. Anim Reprod Sci **117**, 106–110.

Blanchard T.L., Thompson J.A., Brinsko S.P., Varner D.D., Love C.C., Ramsey J., O'Meara A., 2010. Pregnancy Rates in Tranquilized Maiden Thoroughbred Mares. Journal of Equine Veterinary Science 30, 4, 187-190.

Bosh KA, Powell D, Shelton B, Zent W, 2009: Reproductive performance measures among Thoroughbred mares in central Kentucky, during the 2004 mating season. Equine Vet J **41**, 883-888.

Bracher, V., Mathias, S. and Allen, W.R. (1996) Influence of chronic degenerative endometritis (endometrosis) on placental development in the mare. Equine vet. J. 28, 180-188.

Brinsko SP, Rigby SL, Lindsey AC, Blanchard TL, Love CC, Varner DD, 2003a: Pregnancy rates in mares following hysteroscopic or transrectally-guided insemination with low sperm numbers at the utero-tubal papilla. Theriogenology **59**, 1001-1009.

Brinsko SP, Rigby SL, Varner DD, Blanchard TL, 2003b: A practical method for recognizing mares susceptible to post-breeding endometritis. In: Proceedings of the 49th Annual Convention American Association Equine Practitioners, 363–365.

Cilek S, 2009: The survey of reproductive success in Arabian Horse Breeding from 1976-2007 at Anadolu State farm in Turkey. J. Anim Vet Adv **8**, 389-396.

Cuervo-Arango J, Newcombe JR, 2009: The effect of manual removal of placenta immediately after foaling on subsequent fertility parameters in the mare. J Equine Vet Sci **29**, 771-774.

Demirci E, 1987: Fertility in Purebred Arab horses in Turkey. Anim Reprod Sci **15**, 265-271.

Engelken, TJ, 1999: Reproductive health programs for beef herds: analysis of records for assessment of reproductive performance. In: Youngquist, R.S. (Ed.), Current Therapy in Large Animal Theriogenology. W.B. Saunders Co., Philadelphia, pp. 451–456.

Ginther O.J., 1992. Maternal aspects of pregnancy. In: Ginther OJ, Ed. Reproductive biology of the mare: basic and applied aspects. 2nd Ed. Cross Plains, WI, USA: Equiservices. pp 291-344.

Ginther O.J., Gastal E.L., Gastal M.O., Beg M.A., 2006: Conversion of a viable preovulatory follicle into a haemorrhagic anovulatory follicle in mares. Animal Reproduction Science 3, 29-40.

Haadem C.S., Nødtvedt A., Farstad W., Thomassen R., 2015: A retrospective cohort study on fertility in the Norwegian Coldblooded trotter after artificial insemination with cooled, shipped versus fresh extended semen. Acta Vet Scand 57, 77.

Hanlon D.W. and Firth E.C., 2012: The reproductive performance of Thoroughbred mares treated with intravaginal progesterone at the start of the breeding season. Theriogenology 77, 952-958.

Hemberg E, Lundeheim N and Einarsson S, 2004. Reproductive Performance of Thoroughbred Mares in Sweden. Reprod Dom Anim 39, 81–85.

Hevia M.L., Quiles A.J., Fuentes F., Gonzalo C., 1994. Reproductive Performance of Thoroughbred horses in Spain. Journal of Equine Veterinary Science 14, 2, 89-92.

Hidalgo M, Rodriguez I, Dorado J, Soler C, 2006: Morphometric classification of Spanish Thoroughbred stallion semen according to sperm head size measurements. Anim Reprod Sci **94**, 26-28.

Hidalgo M, Rodriguez I, Dorado J, Soler C, 2008: Morphometric classification of Spanish Thoroughbred stallion sperm heads. Anim Reprod Sci **103**, 374–378.

Ishii M, Shimamura T, Utsumi A, Jitsukawa T, Endo M, Fukuda T, Yamanoi T, 2001: Reproductive performance and factors that decrease pregnancy rate in heavy draft horses bred at the foal heat. J Equine Vet Sci **21**, 131-136.

Jasko DJ, Moran DM, Farlin ME, Squires EL, Amann RP, Pickett BW. Pregnancy rates utilizing fresh, cooled and frozen-thawed semen. Proc 38th Ann. Conv. Am. Assoc. Eq. Pract. 1992; 649-660.

Kalirajan, R., Rajasundaram, R.C., 2008. Incidence of endometritis in relation to age and Caslick index in mares. Indian Vet. J. 85, 893–894.

Katila T, 2005: Effect of the inseminate and the site of insemination on the uterus and pregnancy rates of mares. Reprod Dom Anim **89**, 31-38.

Katila T, Reilas T, Nivola K, Peltonen T, Virtala A-M, 2010: A 15-year survey of reproductive efficiency of Standardbred and Finnhorse trotters in Finland -descriptive results. Acta Vet Scand **52**, 40.

LeBlanc MM and Causey RC, 2009: Clinical and Subclinical Endometritis in the Mare: Both Threats to Fertility. Reprod Dom Anim **44** (Suppl. 3), 10–22.

Lewis N., Morganti M., Collingwood F., Grove-White D.H., McGregor Argo C., 2015. Utilization of One-Dose Postovulation Breeding With Frozen-Thawed Semen at a Commercial Artificial Insemination Center: Pregnancy Rates and Postbreeding Uterine Fluid Accumulation in Comparison to Insemination With Chilled or Fresh Semen. Journal of Equine Veterinary Science 35, 882-887.

Loomis P.R., 2001. The equine frozen semen industry. Animal Reproduction Science 68, 191–200.

MAGRAMA, 2015. Raza Equina Caballar Española. Datos Censales. Ministerio de Agricultura, Alimentación y Medio Ambiente. (http://www.magrama.gob.es/es/ganaderia/temas/zootecnia/razasganaderas/razas/catalogo/autoctona-fomento/equino-caballar/espanola/iframe-ejemplo-arca.aspx). España.

Maischberger E, Irwin JA, Carrington SD, Duggan VE, 2008: Equine post-breeding endometritis: A review. Irish Vet J **61**, 163-168.

McKinnon AO, Squires EL, Harrison LA, Blach EL, Shideler RK, 1988: Ultrasonographic studies on the reproductive tract of mares after parturition: effect of involution and uterine fluid on pregnancy rates in mares with normal and delayed first postpartum ovulatory cycles. J Am Vet Med Assoc 192, 350–353.

Menzies, P.I. “Reproductive health management programs” In: Current Therapy in Large Animal Theriogenology, (1999): 643-649.

Moberg, R., 1975: The occurrence of early embryonic death in the mare in relation to natural service and artificial insemination with fresh or deep-frozen semen. J. Reprod. Fertil. Suppl. **23**, 537-539

Morris L. H. A. and Allen W. R, 2002: Reproductive efficiency of intensively managed Thoroughbred mares in Newmarket. Equine vet. J. 34, (1) 51-60.

Nath LC, Andersona GA, McKinnon AO, 2010: Reproductive efficiency of Thoroughbred and Standardbred horses in north-east Victoria. Aust Vet J 88, 169-175.

Nikolakopoulos E, Kindahl H, Watson ED, 2000: Oxytocin and PGF2a release in mares resistant and susceptible to persistent matinginduced endometritis. J Repro Fertil **56**, 363–372.

Pycock, J.F., 2006: How to maximize the chances of breeding successfully from the older maiden mare. Proceedings American Association of Equine Practitioners 52: 245-249.

Pycock JF, Newcombe JR, 1996: Assessment of the effect of three treatments to remove intrauterine fluid on pregnancy rate in the mare. Vet Rec 138, 320–323.

Ricketts, S.W., Alonso, S., 1991. The effect of age and parity on the development of equine chronic endometrial disease. Eq. Vet. J. 23, 189–192.

Samper J.C., 2001: Management and fertility of mares bred with frozen semen. Animal Reproduction Science 68, 219–228.

Samper, J.C., Hellander, J.C., Crabo, B.G., 1991. Relation between fertility of fresh and frozen stallion semen and its quality measured as sperm motility and with glass wool/Sephadex filters. J. Reprod. Fertil. Suppl. 44, 107–114.

Samper, J.C., Vidament, M., Katila, T., Newcombe, J., Estrada, A., Sargeant, J., 2002. Analysis of some factors associated with pregnancy rates of frozen semen: a multi-centre study. Theriogenology 58, 647–650.

Sharma S., Davies Morel M.C.G., Dhaliwal G.S., 2010a: Factors affecting the incidence of postpartum oestrus, ovarian activity and reproductive performance in Thoroughbred mares bred at foal heat under Indian subtropical conditions. Theriogenology 74, 90–99.

Sharma S., Dhaliwal GS, Dadarwal D, 2010b: Reproductive efficiency of Thoroughbred mares under Indian subtropical conditions: A retrospective survey over 7 years. Anim Reprod Sci **117,** 241–248.

Sieme H, Schäfer T, Stout TAE, Klug E, Waberski D, 2003: The effects of different insemination regimes on fertility in mares. Theriogenology **60**, 1153–1164.

Troedsson MH, Loset K, Alghamdi AM, Dahms B, Crabo BG, 2001: Interaction between equine semen and the endometrium: the inflammatory response to semen. Anim Reprod Sci 68:273–278.

Valera M, Blesa F, Santos RD, Molina A, 2006: Genetic study of gestation length in Andalusian and Arabian mares. Anim Reprod Sci **95**, 75-96.

Villahoza M.D., Squires E.L., Voss J.L., Shideler R.K., 1985: Some observations on early embryonic death in mares. Theriogenology, Volume 23, Issue 6, Pages 915–924

Warriach H. M., Memon M. A., Ahmad N., Norman S. T., Ghafar A., and Arif M., 2014: Reproductive Performance of Arabian and Thoroughbred Mares under Subtropical Conditions of Pakistan. Asian Australas. J. Anim. Sci. Vol 27, N 7, 932-936.

Willmann C., Schuler G., Hoffmann B., Parvizi N., Aurich C., 2011: Effects of age and altrenogest treatment on conceptus development and secretion of LH, progesterone and eCG in early-pregnant mares. Theriogenology 75, 421– 428.

Yurdaydin N, Tekin N, Gülyüz F, Aksu A, Klug E, 1993: Estrus synchronization and insemination results in Arabian mares in the Eskisehir stud. Dtsch Tierarztl Wochenschr **11**, 432-434.

Table 1.- Comparison of reproductive efficiency with cooled or frozen semen in Spanish Purebred mares.

	Pregnancy rate				Days 15-60 embryonic and fetal losses	Foaling rate
Type of Semen	First cycle	All cycles	Day 14 pregnancies per season	Mares per season		
Cooled	56.85%a (112/197)	54.70%a (157/287)	81.25%a (156/192)	73.96%a (142/192)	8.97%a (14/156)	72.92%a (140/192)
Frozen	39.13%b (18/46)	40.28%b (29/72)	55.77%b (29/52)	46.15%b (24/52)	17.24%a (5/29)	42.31%b (22/52)
P value	<0.05	<0.05	<0.001	<0.001	>0.05	<0.001

Values within the same column with different superscripts differ significantly, χ^2 test.

Table 2.- Effects of age on measures of reproductive efficiency in Spanish Purebred mares.

	Pregnancy rate				Days 15-60 embryonic and fetal losses	Foaling rate
Age group (years)	First cycle	All cycles	Day 14 pregnancies per season	Mares per season		
3-7	71.23%a (52/73)	70.21%a (66/94)	91.66%a (66/72)	86.11%a (62/72)	6.06%a (4/66)	84.72%a (61/72)
8-11	51.56%a,b (33/64)	52.00%a,b (52/100)	78.46%a,b (51/65)	70.77%a,b (46/65)	9.80%a,b (5/51)	69.23%a,b (45/65)
12-14	41.60%b (20/48)	41.33%b (31/75)	65.96%b (31/47)	63.83%b,c (30/47)	3.23%a (1/31)	59.57%b,c (28/47)
≥ 15	43.10%b (25/58)	41.11%b (37/90)	61.66%b (37/60)	46.67%c (28/60)	24.32%b (9/37)	46.67%c (28/60)
Bonferroni	P <0.01	P <0.001	P <0.01	P<0.05	P<0.05	P <0.05

Values within the same column with different superscripts differ significantly, χ^2 test, Bonferroni P <0.05, P <0.01, P<0.001

Table 3.- Effects of reproductive status on measures of reproductive efficiency in Spanish Purebred mares.

	Pregnancy rate				Days 15-60 embryonic and fetal losses	Foaling rate
Reproductive status	First cycle	All cycles	Day 14 pregnancies per season	Mares per season		
Maiden	86.96%a (20/23)	88.46%a (23/26)	100%a (23/23)	95.65%a (22/23)	4.35%a (1/23)	91.30%a (21/23)
Barren	42.68%b (35/82)	41.48%b (56/135)	65.48%b (55/84)	57.14%b (48/84)	12.73%a (7/55)	54.76%b (46/84)
Rested	54.84%a,b (17/31)	51.16%b (22/43)	75.86%a,b (22/29)	75.86%a,b (22/29)	0%a (0/22)	75.86%a,b (22/29)
Foaling	54.21%b (58/107)	54.84%b (85/155)	78.70%a,b (85/108)	68.52%a,b (74/108)	12.94%a (11/85)	67.59%a,b (73/108)
Bonferroni	P <0.05	P <0.05	P <0.01	P<0.05	P>0.05	P <0.01

Values within the same column with different superscripts differ significantly, χ^2 test, Bonferroni P <0,05, P <0,01.

Table 4.- Effects of different factors on the pregnancy rate in all cycles in Spanish Purebred mares.

	Number of inseminations per cycle		Type of oestrus		Type of ovulation		Breeding season		Year of breeding season			
	1	≥2	Spontaneous	Induced	Spontaneous	Induced	Within (Spring and Summer)	Outside (Fall and Winter)	2012-13	2013-14	2014-15	2015-16
Pregnancy rate in all cycles	52,80% (113/214)	50,34% (73/145)	53,09% (146/275)	47,62% (40/84)	60,71% (34/56)	50,17% (152/303)	57,75% (82/142)	47,93% (104/217)	49,44% (44/89)	45,24% (57/126)	54,93% (39/71)	63,01% (46/73)
P Value	0,65		0,38		0,15		0,07		0,10			

Table 5.- Effect of uterine fluid accumulation on the pregnancy rate in all cycles in Spanish Purebred mares.

	Uterine fluid accumulation		
	Normal	Before Breeding	Post-breeding
Pregnancy rate in all cycles	58,13%a (143/246)	42,35%b (36/85)	25,00%b (7/28)

Values within the same row with different superscripts differ significantly, χ^2 test, Bonferroni P <0,05.

Table 6.- Effect of stallion on the pregnancy rate in all cycles in Spanish Purebred mares.

Age group (years)	Number of Stallions	Pregnancy rate in all cycles
3-7	7	48,26% (83/172)
8-14	4	55,36% (62/112)
≥15	5	54,66% (41/75)
P Value	0,43	

4. DISCUSIÓN

Debido al desarrollo y difusión internacional de la IA en équidos, la manipulación y procesado del semen se ha convertido en una práctica habitual en esta especie (Katila, 2005; Aurich, 2012). Y con ello, han sido numerosas las ideas preconcebidas relativas al daño que factores abióticos como la luz, temperatura o estrés físico pueden infligir sobre la calidad de las células espermáticas.

La luz puede resultar perjudicial para el espermatozoide ya que, al incidir sobre él, favorece la liberación de especies reactivas del oxígeno (EROS) (Shahar y cols., 2011). Por esta razón, se aconseja que durante su manipulación la muestra sea protegida de la luz, evitando así la reducción de su calidad. Sin embargo, en contra de esta afirmación, investigaciones recientes demuestran que la incidencia de luz sobre el semen puede tener efectos beneficiosos, favoreciendo la penetración del oocito e incrementando así la fertilidad y la prolificidad (Yeste y cols., 2016). En referencia a la luz ultravioleta (UV), se ha descrito su capacidad esterilizante, por lo que se ha propuesto como posible herramienta para ayudar a reducir la carga bacteriana, reduciendo así reducir el uso de antibióticos, aunque antes habría que determinar cómo afecta sobre el espermatozoide.

Los resultados obtenidos en la presente Tesis Doctoral muestran que a partir de 30 minutos posteriores a la exposición a luz UV, los espermatozoides equinos sufren un contundente impacto negativo en su motilidad y en el potencial mitocondrial. En cambio, la exposición del semen a la luz convencional de laboratorio (luz blanca) durante 60 minutos no ocasiona un descenso reseñable de su calidad. Cuando tratamos de explicar cómo la luz afecta a los espermatozoides, debemos recordar que una fuente de luz incide sobre un cuerpo (espermatozoides en este caso), parte de la energía llega en forma de luz, pero otra gran parte lo hace en forma de calor, y dependiendo de la longitud de onda a la que se emite dicha luz, ésta tendrá mayor o menor penetración. En nuestro experimento apreciamos que a medida que las muestras se situaban más lejos de la fuente de luz UV, éstas resultaban menos dañadas. Esto sugiere que quizás, la luz UV aplicada durante un corto periodo de tiempo y a una distancia determinada, podría ejercer un poder esterilizante y favorable sobre los espermatozoides.

Se ha descrito que el espermatozoide de rata es muy sensible al estrés físico (Kim y cols., 2013), en cuyo caso se recomienda que sea tratado con especial cuidado durante su procesado. De hecho, el pipeteo repetido (cuádruple) del esperma de ratón y rata reducen su viabilidad a la mitad (Varisli et al., 2009). Sin embargo, estudios llevados a cabo con esperma de cerdo, toro y carnero, han demostrado que el pipeteo no afecta a su motilidad (Varisli et al., 2009). El presente estudio pone de manifiesto que el

esperma equino tiene gran resistencia al estrés por el pipeteo. Sin duda, el estrés físico puede ser inducido a través de diversas acciones, desde el pipeteo (como se ha indicado) hasta el centrifugado de la muestra, y su impacto sobre el espermatozoide también puede variar. En este sentido, Kim y cols. (2013) describieron que la motilidad del esperma de rata no se ve afectada por la centrifugación, tal y como describen en caballo Ferrer y cols. (2012).

No obstante, el pipeteo y la centrifugación han sido descritos como factores que alteran la función mitocondrial en la rata (Kim y cols., 2013). No obstante, en los estudios realizados en espermatozoides equinos no se apreció una reducción en el potencial mitocondrial tras someterlos a pipeteo. En consecuencia, se puede afirmar que el espermatozoide de caballo es más tolerante al estrés físico que el de rata, lo que puede conferirla una importante ventaja a la hora de usarse en IA, soportando mejor su procesamiento durante la refrigeración. Se ha sugerido que la longitud del flagelo o cola del espermatozoide (que es mucho mayor en roedores) puede estar asociado a esa alta sensibilidad de la rata a agentes estresantes físicos (Gao y cols., 1997; Varisli y cols., 2009). Varios estudios afirman que el estrés mecánico incrementa las ERO en el espermatozoide (Agarwal y cols., 1994; Aitken y cols., 2010; McCarthy y cols., 2010), aunque los resultados obtenidos por Kim y col (2013) en espermatozoides de rata sostienen que este tipo de estrés induce un descenso de ERO, lo que podría ser deberse a una activación de los mecanismos de defensa antioxidante del espermatozoide. La mitocondria es la principal organela involucrada en la formación intracelular de ERO. El estrés abiótico podría provocar un descenso en el número de espermatozoides con alto potencial mitocondrial, lo que supone una menor producción de ATP de la mitocondria y, por tanto, una disminución en la motilidad espermática (Guthrie and Welch 2006).

Aunque existen muchos estudios que describen el efecto de la temperatura sobre los espermatozoides, no se han encontrado investigaciones acerca de cómo las oscilaciones térmicas pueden alterar la fisiología normal del mismo. La presente Tesis Doctoral planteó una serie de experiencias en las que el espermatozoide refrigerado a 15ºC se llevaba a temperaturas de 30ºC o de 0ºC, para, posteriormente devolverlo a la temperatura inicial de refrigeración. Los resultados demostraron la alta tolerancia del espermatozoide equino refrigerado ante variaciones bruscas de temperatura. En este caso, los espermatozoides fueron protegidos con crioprotectores no penetrantes, aportados a través del diluyente a base de leche desnatada y azúcares. Los resultados no mostraron diferencias en ninguno de los protocolos de oscilación térmica testados, sí se apreció que el daño era ligeramente mayor cuando las células espermáticas se sometían a temperaturas de 0ºC frente a 30º C. Esta observación resalta la seguridad y aplicabilidad de los actuales sistemas de refrigeración que se emplean en esperma equino. Sin duda, en caso de que el semen provenga de sementales con alta calidad

seminal (como ocurrió en este estudio), la calidad no se verá afectada por cambios de temperatura debidos a su manipulación durante el verano o el invierno. Habría que realizar estos mismos experimentos en esperma de baja calidad, en el que quizás el estrés abiótico podría modificar negativamente la calidad espermática y hacer que las dosis seminales redujeran drásticamente su capacidad fertilizante **(Artículo 1).**

A pesar de que la congelación convencional es la técnica más usada para criopreservación de semen equino, se describen diversos inconvenientes asociados a que se induce la formación de cristales de hielo extra e intracelulares y conlleva cambios osmóticos, lo que ocasiona gran contracción celular y daños en el espermatozoide (Watson y Martin, 1975; Gao y cols., 1997). Últimamente, el interés se ha centrado en el desarrollo de nuevos diluyentes, métodos para seleccionar y separar poblaciones de espermatozoides de mejor calidad y diferentes protocolos de congelación-descongelación capaces de mejorar los resultados actuales (Pérez-Marín y cols., 2018). Otros estudios han descrito nuevos métodos de criopreservación como la congelación en seco (o liofilización) para preservar esperma equino (Olaciregui y cols., 2016), pero hasta ahora, no existían estudios acerca de cómo se comporta el espermatozoide equino tras su vitrificación. Estos estudios, abordados por primera vez en esta Tesis Doctoral, demuestran el impacto negativo que este procedimiento tiene sobre el espermatozoide equino. En nuestros experimentos comparamos el efecto de diferentes concentraciones de sacarosa y/o trehalosa sobre el espermatozoide equino y no se encontraron diferencias significativas entre ambos disacáridos. Sin embargo, sorprendió que la exposición continuada a CP provocaba un descenso radical de la motilidad y calidad espermática, mientras que cuando las muestras eran sometidas a CPs y seguidamente vitrificadas-calentadas, los valores eran superiores. En el momento en que los CP son añadidos a las muestras de esperma, tiene lugar un rápido shock osmótico, que se hace irreversible cuando el tiempo de exposición aumenta. Además, el medio se transforma en una sustancia más viscosa que conduce a la supresión del movimiento flagelar (Woelders y cols., 1997). Se apreció que la motilidad espermática disminuía a medida que aumentaban las concentraciones de CP; es decir, bajo condiciones de hiperosmolaridad se merma la motilidad del esperma equino (Ball y Vo, 2001; Pommer y cols., 2002). Estas condiciones también inducen un daño oxidativo al esperma, con un incremento en la formación de aniones superóxido, aunque la integridad de la membrana plasmática no se ve afectada hasta los 600 mOsm (Ball, 2008). La vuelta a condiciones isosmolares durante la descongelación y el calentamiento permite al espermatozoide recuperar su volumen inicial, lo que debe considerarse como un punto crítico en esta técnica. Pommer y col (2002) han informado de la disminución de la actividad mitocondrial y de la viabilidad del esperma equino durante los procesos de descongelación, lo que pone de manifiesto la gran importancia de este paso para la supervivencia y funcionalidad del espermatozoide. Se ha comprobado que el calentamiento de una muestra de esperma vitrificada es una

fase más crítica que la que transcurre durante el enfriamiento. Y se ha descrito que el calentamiento lento hasta 37°C reduce la motilidad, viabilidad e integridad del acrosoma en mayor medida que si se emplea un calentamiento rápido a 60°C (Rosato y Iaffaldano, 2013).

De los resultados derivados de este estudio también se sugiere que la membrana plasmática del espermatozoide, además de dañarse por la formación de cristales de hielo, puede sufrir impactos físicos inducidos por la composición de los CP. Existe bibliografía en la que se expone que las concentraciones óptimas de azúcares para criopreservar esperma difiere entre especies, así como la influencia del tipo de diluyente (El-Sheshtawy y cols., 2015). De esta forma, un diluyente como INRA lleva azúcares adicionales capaces de actuar sinérgicamente con los disacáridos usados como CP (El-Badry y cols., 2017). La sacarosa y la trehalosa son disacáridos que no penetran en la célula y se usan para mejorar la formación de cristales de hielo en determinadas fases críticas del proceso de enfriamiento. (Sutton, 1992; Berrios y Sánchez, 2011). La trehalosa se inserta en la doble capa fosfolipídica de las membranas haciéndolas más estables durante la congelación (Aboagla y Terada, 2003) y protegiéndolas durante la criopreservación mediante un efecto antioxidante, aumentando el nivel de glutatión y reduciendo la peroxidación lipídica (Aisen y cols., 2005). Así pues, es posible que promueva la protección de la integridad funcional del acrosoma y de las mitocondrias, mejorando la motilidad espermática después de la descongelación (Liu y cols., 2016).

El acrosoma juega un papel importante en la función del espermatozoide y, por supuesto, en la fecundación. En el **Artículo 2**, los espermatozoides sometidos a baja osmolaridad no vieron afectada negativamente su integridad acrosomal, pero el proceso de vitrificación sí indujo un tremendo efecto negativo sobre las membranas acrosómicas. Esto puede ser debido a la alteración que se produce en las proteínas de membrana que se localizan en los fosfolípidos durante la fase de transición lipídica durante el enfriamiento (Mossad y cols., 1994).

La adición de albúmina sérica bovina (BSA) al diluyente ofrece mayores tasas de supervivencia de espermatozoides, aunque no se sabe bien cuál es el mecanismo implicado (Nang y cols., 2012). Los experimentos conducidos en esperma equino demostraron que la adición única de BSA al diluyente espermático no ofreció ninguna capacidad protectora sobre los espermatozoides, lo que se reflejó en una gran pérdida de su motilidad, siendo solo unos pocos los que la conservaron. Para explicar esta tolerancia a la vitrificación de unos pocos espermatozoides en estas condiciones hay que tener en cuenta que la BSA es una macromolécula que se absorbe en la superficie de las membranas plasmáticas, donde juega un papel protector (Matsuoka y cols., 2006) y también puede transportar grandes cantidades de ácidos grasos libres que

ofrecen energía extra a los espermatozoides (Hossain y cols., 2007). El esperma humano ha sido vitrificado satisfactoriamente en ausencia de CPs, lo que se explica por la composición de su plasma seminal, su contenido intracelular (Isachenko y cols., 2003) y la forma plana y pequeño tamaño de su cabeza (Watson y Plummer, 1985). Comparando los tamaños de los espermatozoides de mamíferos, el de humano es el más pequeño, seguido por el equino, felino y canino (Garner, 2006). Estas observaciones nos hicieron pensar que el espermatozoide equino podría ser un buen candidato para la vitrificación.

El espermatozoide equino no sufrió un incremento en la fragmentación del ADN tras la vitrificación, al igual que se describió en esperma humano tras la vitrificación o congelación (Isachenko y cols., 2004). Este hecho hace sospechar que el espermatozoide equino vitrificado podría ser un perfecto candidato para su empleo en inyección intracitoplasmática (ICSI). En este caso, los disacáridos protegerían el espermatozoide, reduciendo las fluctuaciones estructurales y la desnaturalización del ADN, formando numerosos enlaces de hidrógeno (Loi y cols., 2008) **(Artículo 2).**

Enfocando el tema de la fertilidad equina hacia la eficiencia reproductiva, la evaluación de la placenta en yeguas gestantes permite determinar si existe alguna patología que conlleve la pérdida fetal, y para ello se ha descrito la determinación del espesor conjunto útero-placenta (ECUP) como indicador de viabilidad placentaria. Diversos estudios relacionan la edad gestacional con valores de ECUP en diferentes razas equinas, pudiendo notarse que existen diferencias entre ellas (Reanudin y cols., 1997; Barnes y cols., 2005; Bucca y cols., 2005; Colón 2008; Souza y cols., 2008; y Coutinho y cols., 2013). De acuerdo con Colon (2008), los resultados obtenidos ayudan a diferenciar entre una gestación sana y una anormal (probablemente debida a placentitis ascendente), desde el día 270 de gestación. No obstante, existen estudios que evidencian que las anormalidades placentarias podrían ocurrir y detectarse antes de lo indicado anteriormente (Souza y cols., 2010). Renaudin y cols., (1997) definieron una medida ECUP anormal como aquella que se desviaba por encima del intervalo de confianza del 95% obtenido de los valores medios definidos como normales. Así, Souza y cols. (2010), encontraron un 33,9% de yeguas por encima del límite superior del intervalo de confianza, y otros autores encontraron un porcentaje menor, 3,1% (Troedsson y Zent, 2004) y un 15% (Colón, 2008). En esta investigación se obtuvieron resultados similares a Colón (2008), concretamente un 14% de yeguas, cuya medida ECUP fue significativamente superior a las yeguas sanas desde el día 270 de gestación. Esto nos ilustra sobre la existencia de una patología, la placentitis, que desarrollan muchas yeguas y deben recibir un tratamiento, aunque en la mayoría de casos no se localicen síntomas externos de placentitis o abortos (Souza y cols., 2010). Bucca y cols. (2005), observaron aumentos en la medida ECUP sin repercusiones en el desarrollo gestacional.

Al igual que Souza y cols. (2010), se ha encontrado que la edad de la yegua no afecta al ECUP. Pero si advertimos que las yeguas que abortaron o presentaron alteraciones placentarias fueron las de mayor edad y en las que el intervalo parto-concepción fue más largo. No se encontraron diferencias hasta el día 270 entre gestaciones sanas y anormales, por lo que se recomienda a partir de esta fecha comenzar el seguimiento ecográfico de la gestación (Requena y cols., 2014; Requena y cols., 2015). Esto es apoyado por la publicación de Hendriks y cols. (2009), que no encontraron diferencias importantes en las medidas de ECUP hasta el quinto mes de gestación, con el crecimiento más rápido y mayor a partir del octavo mes de gestación. Hecho que se atribuye a que las necesidades del feto aumentan exponencialmente en el último tercio de gestación provocando mayor vascularización (Crisci y cols., 2014). Las yeguas que abortaron en este estudio no presentaron ningún signo externo, algo que sólo ocurre cuando la enfermedad está muy avanzada (Mcpherson y Bailey, 2008). Lo que sí se encontró fueron cambios ecográficos a partir del día 270, como aumento de la medida ECUP respecto a yeguas normales, áreas de separación útero-placenta, y presencia de fluido exudativo en zonas de desorganización de la unión endometrio y placenta. Basándonos en la muestra estudiada en nuestro artículo, se considera que el 8.5% de las yeguas PRE gestantes no producen un potro viable, lo cual no difiere con otros autores (Giles y cols., 1993; Smith y cols., 2003; Troedsson y Zent, 2004; LeBlanc, 2010) **(Artículo 3).**

En cuanto a la eficiencia reproductiva de la yegua PRE, se encontraron tasas de gestación y parto más altas con semen refrigerado que con congelado, coincidiendo con Jasko y cols. (1992c). Por el contrario, Loomis (2001) encontró resultados similares usando ambos tipos de semen, afirmando que esto es posible con semen de buena calidad, selección de los sementales y yeguas y un buen manejo reproductivo de la yegua. Las tasas de gestación conseguidas en el primer ciclo con semen congelado son similares a las publicadas por Demirci (1987), Loomis (2001), Sieme y cols., (2004) y Benhajali y cols., (2010). Otros autores (Allen y cols., 2007; Nath y cols., 2010), encontraron tasas más altas que las de esta investigación, probablemente debido a que ellos incluyeron en su estudio una mayor proporción de yeguas jóvenes (entre 2-13 años). Con respecto a las tasas de gestación total en todos los ciclos, los resultados obtenidos son similares a los de Morris y Allen (2002) y Sharma y cols., (2010b), mientras que Bosch y cols., (2009) y Nath y cols., (2010) describieron valores más altos.

Las tasas de gestación y partos obtenidas en las investigaciones realizadas son similares a los autores consultado en la bibliografía (Samper y cols., 1991; Sieme y cols., 2004). En cambio, Loomis (2001), obtuvo mejores resultados que los hallados en el trabajo realizado. Esto puede deberse a que se inseminaron una alta proporción de yeguas viejas y varias de ellas sólo se inseminaron en un celo por decisión del

propietario. Sin embargo, no se encontraron diferencias en las pérdidas embrionarias hasta el día 60 usando semen refrigerado o congelado. En esta misma línea apuntan otros estudios (Villahoza y cols., 1985; Willmann y cols., 2011).

De acuerdo con multitud de estudios (Morris y Allen, 2002; Allen y cols., 2007; Bosh y cols., 2009; Benhajali y cols., 2010; Katila y cols., 2010; Nath y cols., 2010; Sharma y cols., 2010b), se ha identificado la edad como el factor más influyente en las tasas de gestación, de pérdidas embrionarias y fetales y de parto. Se coincide con todos los autores anteriores en que las yeguas jóvenes son las que mejores porcentajes de éxito dan. Sólo Nath y cols. (2010) y este estudio tienen las tasas de gestación en el primer ciclo más altas que los anteriores trabajos, puesto que se empleó IA y un intenso manejo reproductivo.

Se acepta como norma general que las yeguas comienzan a mermar la fertilidad desde los 13 a 17 años, denominándose yeguas subfértiles (Ginther, 1992; Allen y cols., 2007). Los resultados están en concordancia con la anterior afirmación, encontrando que las yeguas de 15 años o de más edad obtienen los peores resultados. Esto puede ser explicado porque con la edad aumentan los cambios degenerativos crónicos del endometrio de forma progresiva (Ricketts y Alonso, 1991), hecho que conlleva una reducción en el aporte de nutrientes al embrión (Bracher y cols., 1996). Otros factores asociados con el aumento de la edad de la yegua son la pobre conformación perineal, defectos en las contracciones del miometrio, fallos en el drenaje linfático e incompetencia uterina que aumentan la susceptibilidad de desarrollar endometritis (Kalirajan y Rajasundaram, 2008; LeBlanc y Causey, 2009).

Respecto a la influencia del estatus reproductivo de la yegua en la fertilidad hay opiniones encontradas. Entretanto, en este estudio se apreció que las yeguas vírgenes o que se cubren por primera vez tienen los mejores porcentajes en las tasas de gestación, al igual que en otras investigaciones parecidas (Allen y cols., 2007; Bosh y cols., 2009; Nath y cols., 2010; Sharma y cols., 2010b). Sin embargo, otros trabajos informan que el estatus reproductivo sólo afecta a la tasa de concepción total (Samper y cols., 2002; Benhajali y cols., 2010). Se discrepa de los resultados descritos por Hemberg y cols. (2004) ya que no encontraron diferencias significativas entre las yeguas vírgenes y paridas con respecto a las yeguas infértiles y las que habían abortado. La razón de esto sería que ellos incluyeron una mayor proporción de yeguas problema (infértiles y que abortaron) en los grupos de yeguas de mayor edad. En las investigaciones que se ha realizado no se ha encontrado correlación entre la edad y el estatus reproductivo de la yegua (Benhajali y cols., 2010).

Valorando otros factores externos que puedes influir en la fertilidad, no se hallaron diferencias significativas en el número de inseminaciones por ciclo. Algunos autores

obtuvieron mejores resultados en yeguas que fueron cubiertas varias veces por ciclo (Allen y cols., 2007) y otros que inseminaron 2 veces por ciclo en vez de una (Nath y cols., 2010). La explicación a estos resultados es el uso o no de agentes inductores de la ovulación. Si la ovulación no es inducida, más cantidad de montas/inseminaciones serán necesarias para conseguir disminuir el tiempo entre la monta/inseminación y la ovulación y aumentar la probabilidad de concepción. Tampoco hay diferencias significativas en las tasas de gestación por celos espontáneos o inducidos (Morris y Allen, 2002; Allen y cols., 2007; Sharma y cols., 2010b; Hanlon y Firth, 2012). Pero empleando tratamientos hormonales para inducir celos, se pueden mejorar las tasas de gestación al final de la temporada reproductiva, como hicieron Hanlon y Firth (2012), que adelantaron el primer celo de la temporada, alargando el tiempo de la temporada reproductiva para tener más celos y aumentar la probabilidad de éxito.

De acuerdo a la mayoría de los autores, no hay un efecto notable de la época reproductiva y el año reproductivo en las tasas de gestación (Morris y Allen, 2002; Hemberg y cols., 2004; Cilek, 2009; Katila y cols., 2010; Haadem y cols., 2015). Aunque si se ha comprobado que la época reproductiva influye en el número de ovulaciones dobles (dentro de la época reproducitva) y en el número de gestación dobles (época de transición) (Morris y Allen, 2002). Asimismo, Cilek (2009) encontró efectos significativos del mes y año reproductivo en la duración de la gestación, periodo de servicios, intervalo entre partos, celos por gestación y número de inseminación por concepción. Atendiendo a la época reproductiva de la yegua, de días largos, hay que destacar que en otoño e invierno hay mayor incidencia de folículos anovulatorios que pueden dar peores tasas de gestación (Ginther y cols., 2006).

Desde hace muchos años, es bien conocido, que el acúmulo de fluido uterino y moco durante el celo o después de la cubrición está asociado a un descenso en las tasas de gestación (McKinnon y cols., 1988; Pycock y Newcombe 1996; Lewis y cols., 2015). De acuerdo con esto, se obtuvo que la tasa de gestación de todos los ciclos, en aquellos que no hubo acúmulo de fluido uterino antes o después de la inseminación, fue más del doble que en aquellos que hubo fluido uterino post-inseminación. Se han instaurado tratamientos, como los descritos por Maischberger y cols. (2008), en todos los ciclos que presentaron acúmulo de fluido intrauterino. Esto hace pensar que mediante tratamientos apropiados para eliminar el fluido intrauterino y/o infecciones, se podría aumentar las tasas de gestación. Así, Sharma y cols. (2010a) consiguieron mejores tasas de gestación por ciclo en el día 16 en aquellas yeguas que recibieron tratamiento.

Por último, de acuerdo con la investigación realizada, algunos autores informaron que la edad del semental no tiene efecto en los parámetros de fertilidad (Hemberg y cols., 2004; Haadem y cols., 2015). Por un lado, hay estudios que han encontrado diferencias

en las tasas de gestación en el primer ciclo y en la tasa de partos para cada semental (Morris y Allen, 2002; Nath y cols., 2010; Haadem y cols., 2015). Por otro, Benhajali y cols. (2010) mostraron que existe un efecto "semental" en la tasa de concepción total y un efecto "padre" en la tasa de concepción al primer ciclo. De esto se deduce que el semental puede tener un efecto a corto plazo en los parámetros reproductivos, dependiendo de la calidad del semen y del número de yeguas cubiertas, y un efecto a largo plazo en la transmisión de la fertilidad a sus hijas. A diferencia de otros autores, en el presente estudio se empleó IA y siempre se analizó y procesó previamente el semen, obteniendo dosis de buena calidad, por lo que es posible que esta sea la causa de que no se percibieran efectos de la edad del semental en la tasa de gestación de todos los ciclos **(Artículo 4).**

5. CONCLUSIONES

*(**DEL ARTÍCULO 1:** SHORT-TERM TOLERANCE OF EQUINE SPERMATOZOA TO VARIOUS ABIOTIC FACTORS. PÉREZ-MARÍN CC, **REQUENA FD**, ARANDO A, REQUENA L, REQUENA F, AGÜERA EI. REPROD DOM ANIM. 2018; 00:1–11)*

I. Los espermatozoides equinos tienen una alta tolerancia al estrés mecánico (inducido por el pipeteo) y al estrés térmico (inducido por oscilaciones térmicas durante la conservación), corroborando así que es posible mantener esperma de alta calidad durante envíos de larga distancia.

II. En cuanto al impacto de la luz, los espermatozoides equinos son muy resistentes a estímulos normales como sucede en la rutina de trabajo de laboratorio, pero la aplicación de luz UV durante un mínimo de 30 minutos tiene un efecto negativo en su calidad**.**

*(**DEL ARTÍCULO 2:** EFFECT OF TREHALOSE- AND SUCROSE-BASED EXTENDERS ON EQUINE SPERM QUALITY AFTER VITRIFICATION: PRELIMINARY RESULTS**.** C.C. PÉREZ-MARÍN, **F.D. REQUENA**, A. ARANDO, S. ORTIZ-VILLALÓN, F. REQUENA, E.I. AGÜERA. CRYOBIOLOGY, 2018; 80:62-69)*

III. La vitrificación de esperma equino tiene una baja capacidad para preservar la motilidad espermática, aunque los diluyentes que contienen trehalosa o sucrosa a bajas concentraciones ofrecen efectos protectores para la motilidad.

IV. La álbumina de suero bovino (BSA) parece tener por sí misma un ligero efecto protector sobre espermatozoides equinos vitrificados.

V. La integridad del ADN fue reducida ligeramente tras la vitrificación, pero no hubo diferencias cuando se emplearon CPs, lo que sugiere que la cromatina espermática podría mantener su función en estas condiciones.

VI. El acrosoma y las membranas plasmáticas sufrieron graves daños tras la vitrificación, aunque no durante la exposición a CPs, por lo que para mejorar esta técnica se precisa desarrollar medidas encaminadas a la protección de estas estructuras.

*(**DEL ARTÍCULO 3:** TRANSRECTAL ULTRASONOGRAPHIC MEASUREMENTS OF THE COMBINED THICKNESS OF THE UTERUS AND PLACENTA IN SPANISH PUREBRED MARES. **FERNANDO D. REQUENA**, ESTRELLA I. AGÜERA, FRANCISCO REQUENA, CARLOS C. PÉREZ-MARÍN. ANIMAL REPRODUCTION, 2017; 14 (SUPPL.1): 1278-1284)*

VII. En la yegua PRE, un examen ecográfico transrectal y mensual desde los 210 días de gestación ofrece ventajas para evaluar el espesor conjunto útero-placenta (ECUP) y evidenciar gestaciones sanas o anormales.

VIII. Las diferencias entre gestaciones sanas o anormales son más evidentes desde el día 270 de gestación.

IX. Un 14% de las yeguas gestantes PRE ha sido diagnosticado con aumento del espesor placentario.

*(**DEL ARTÍCULO4:** REPRODUCTIVE EFFICIENCY OF SPANISH PUREBRED MARE IN SPAIN. **FD REQUENA**, EI AGÜERACC PÉREZ-MARÍN. EN PREPARACIÓN)*

X. La yegua PRE muestra resultados similares de fertilidad a otras razas equinas. El tipo de semen (refrigerado o congelado), la edad, el estatus reproductivo y el acúmulo de fluido uterino son los factores más importantes que influyen decisivamente en los parámetros de fertilidad de la yegua PRE.

XI. La mejor fertilidad se obtiene con semen refrigerado cuando se aplica sobre yeguas jóvenes o en yeguas vírgenes que no presentan acúmulo de fluido uterino durante el estro.

6. RESUMEN

El caballo ha sido seleccionado atendiendo a sus éxitos deportivos más que a su tasa de fertilidad. Por ello, el caballo se ha mostrado por tener una eficiencia reproductiva más baja comparándolo con otras especies domésticas. Hay varios estudios sobre eficiencia reproductiva en diferentes razas equinas, pero no en el Pura Raza Español (PRE). Ésta, depende de factores relacionados con la yegua, el semental y factores externos. Para determinar la fertilidad equina se han desarrollado cuatro artículos.

En el primer artículo, se determinaron los efectos de varios factores abióticos, como la luz, el estrés físico (pipeteo) y el shock térmico en la calidad de esperma equino fresco y refrigerado. En el experimento I, cuatro alícuotas de esperma fueron sometidas a diferentes exposiciones de luz: (i) muestra control protegida (CTRL), (ii) expuesta a luz ultravioleta a 10 cm (UV10), (iii) expuesta a luz ultravioleta a 20 cm (UV20cm) y (iv) expuesta a la luz de laboratorio (LAB). En el experimento II, cuatro alícuotas de semen fueron sometidas a pipeteo repetido durante 0, 10, 20 y 30 veces (CTRL, P10, P20, P30, respectivamente). En el experimento III, cuatro alícuotas de semen a 15°C fueron sometidas a oscilaciones térmicas: (i) esperma control refrigerado a 15°C (CTRL), (ii) oscilaciones de 1.9°C/min a temperatura de 30°C (T30), (iii) oscilaciones de 1.4°C/min, con un rápido descenso de temperatura hasta alcanzar 1.3°C (T0R) y (iv) oscilaciones de 1.1°C/min, con un descenso de temperatura lento has alcanzar 4.2°C (T0S). Los resultados revelaron que después de 30min, las muestras de esperma UV10 y UV20 mostraron significativamente ($p < 0.05$) los valores más bajos de motilidad total, parámetros cinemáticos y potencial mitocondrial. Después de 45 min de exposición, las diferencias fueron altamente significantes ($p < 0.001$). No se encontraron diferencias significativas ($p > 0.05$) para el pipeteo y las oscilaciones térmicas. Los resultados sugieren que, incluso si las muestras de esperma no son manejadas en el laboratorio bajo condiciones óptimas, los espermatozoides de equino en fresco o refrigerados son capaces de resistir el impacto de varios factores abióticos sin provocar una reducción de su calidad. Este estudio analiza el efecto en muestras de esperma normales, pero en el futuro la investigación podría buscar la tolerancia que las muestras de esperma equino con astenospermia tendrían a la influencia de estos factores abióticos.

En el segundo artículo se llevó a cabo una investigación para evaluar el impacto de varios agentes crioprotectores (AC) y la vitrificación en la calidad del esperma. Un total de 12 eyaculados fueron sometidos a la exposición de CPA y a vitrificación. El esperma fue diluido en un rango de AC: fresco, control (BSA), sacarosa (0.15M, 0.3M y 0.5M), trehalosa (0.15M, 0.3M y 0.5M) y la combinación de sacarosa y trehalosa (M1: 0.15M sacarosa + 0.5M trehalosa; M2: 0.5M sacarosa + 0.15M trehalosa). La motilidad espermática, viabilidad, integridad del acrosoma y la fragmentación del ADN fueron evaluados en el momento de la exposición a los AC y después de la vitrificación. La exposición de los espermatozoides a varias concentraciones de sacarosa y/o trehalosa redujeron significativamente la motilidad espermática, aunque con bajas

concentraciones resultaron en una motilidad más alta. La viabilidad espermática y la fragmentación del ADN no variaron después de la exposición a los AC, pero la integridad del acrosoma cayó significativamente cuando los espermatozoides fueron expuestos a AC con alta osmolaridad. Cuando los espermatozoides fueron vitrificados, los valores de motilidad fueron significativamente más altos que los obtenidos durante la exposición a AC. La concentración baja de sacarosa (0.15M y 0.3M) y trehalosa (0.15M) mostraron la mejor motilidad progresiva espermática. El procedimiento de vitrificación-calentamiento redujo significativamente la viabilidad espermática y la integridad del acrosoma, pero el ADN no varía con ninguno de los AC empleados. La vitrificación del esperma equino demuestra una baja capacidad para preservar la motilidad espermática, y los diluyentes que contienen trehalosa o sacarosa a bajas concentraciones se asocian con un mejor efecto protector en la motilidad espermática. Después de la vitrificación, el acrosoma y las membranas plasmáticas se dañaron severamente, mientras la estructura del ADN se mantuvo. El espermatozoide equino recobró la motilidad después de la vitrificación, pero se precisa realizar más estudios en la preservación de las membranas espermáticas.

En el tercer artículo fueron establecidos los valores normales del espesor conjunto útero-placenta (ECUP) en yeguas Pura Raza Española (PRE) durante el último periodo de gestación. Un total de 107 yeguas fueron examinadas a los 210, 240, 270 y 300 días de gestación, de las cuales 13 tuvieron gestaciones anormales (muerte fetal, abortos o nacidos muertos). En yeguas con potros sanos, las medidas ECUP mostraron diferencias significativas ($P < 0.0001$) en varias edades gestacionales (4.83 ± 0.59 mm, 6.12 ± 0.60 mm, 7.41 ± 0.61 mm y 10.45 ± 0.92 mm at 210, 240, 270 y 300 días, respectivamente). Una alta correlación positiva se observó entre ECUP y la edad gestacional ($r = 0.923$; $P < 0.001$). En yeguas con gestación anormal las medidas ECUP fueron significativamente más altas ($P < 0.0001$) que en las gestaciones sanas en el día 270 (8.89 vs. 7.41 mm) y en el 300 (14.17 vs. 10.45 mm). La edad de la yegua, el tiempo de fecundación, el tipo de semen y la longitud de la gestación no afectan la ECUP. Los resultados obtenidos sugieren que el punto de corte para la detección anormal de las gestaciones en yeguas PRE es 7.75 mm en el día 270 y 12.7 mm en el día 300. La incidencia del aumento de ECUP en el último periodo de gestación de la yegua PRE fue del 14% y un examen ecográfico transrectal a los 270 días de gestación es muy aconsejable. La evaluación periódica placentaria podría revelar alteraciones no detectadas por los demás, permitiendo instaurar tratamiento apropiados encaminados a prevenir la pérdida fetal.

Finalmente, un estudio se llevó a cabo un estudio retrospectivo para determinar la eficiencia reproductiva de la yegua PRE. Se evaluó la influencia de la edad, el estatus reproductivo, la inseminación con semen refrigerado o congelado, el número de inseminaciones por ciclo, el tipo de celo, el tipo de ovulación, la temporada reproductiva, el año de la temporada reproductiva, la presencia de fluido uterino y la edad del semental. Se incluyeron en una base de datos las inseminaciones de 122 yeguas PRE durante 4 temporadas reproductivas consecutivas (2012-2013, 2013-2014,

2014-2015, 2015-2016). Un total de 359 ciclos fueron estudiados. Las yeguas se agruparon de acuerdo a la edad: jóvenes, mediana edad, viejas y muy viejas y de acuerdo al estatus reproductivo: vírgenes, infértiles, paridas y no cubiertas. Las tasas de gestación más altas fueron con semen refrigerado en el primer ciclo ($P < 0.05$), en todos los ciclos ($P < 0.05$), en el día 14 por temporada ($P < 0.001$) y en la tasa de partos ($P < 0.001$). Las tasas de gestación y partos fueron significativamente influenciadas por el efecto de la edad. Las yeguas jóvenes y vírgenes tuvieron las tasas de gestación más altas. No hubo diferencias significativas ($P > 0.05$) en la tasa de gestación en todos los ciclos cuando se analizó el número de inseminaciones por ciclo, tipo de celo, tipo de ovulación, temporada reproductiva, año de la temporada reproductiva y la edad del semental. Hubo diferencias significativas ($P < 0.05$) entre las yeguas que no acumularon fluido uterino (58.13%) y las que acumularon fluido intrauterino antes de la cubrición (42.35%) y después (25.00%). La yegua PRE podría ser considerada como una raza de caballo muy fértil.

7. ABSTRACT

Horse has been selected contemplating its sporting success more than its fertility rate. For this reason, horse has been showed to have relatively lower reproductive efficiency compared to others domestics species. There are many studies about reproductive efficiency in different breeds horses but not in Spanish Purebred (SP) horse. Equine reproductive efficiency depends on factors related to the mare, the stallion and external factors. To determine equine fertility four articles were developed.

In the first article, it was determined the effects of various abiotic factors, such as light, physical stress (pipetting) and thermal shock, on the quality of fresh and cooled equine sperm. In experiment I, four sperm aliquots were subjected to different light exposures: (i) protected control samples (CTRL), (ii) exposed to UV light at 10 cm (UV10), (iii) exposed to UV light at 20 cm (UV20) and (iv) exposed to laboratory lighting (LAB). In experiment II, four semen aliquots were subjected to repeated pipetting for 0, 10, 20 and 30 times (CTRL, P10, P20 and P30, respectively). In experiment III, four semen aliquots at 15°C were subjected to thermal oscillations: (i) cooled control sperm at 15°C (CTRL), (ii) oscillations of 1.9°C/min to a temperature of 30°C (T30), (iii) oscillations of 1.4°C/min, with the temperature rapidly falling until reaching 1.3°C (T0R) and (iv) oscillations of 1.1°C/min, with the temperature slowly falling until reaching 4.2°C (T0S). The results revealed that after 30 min, UV10 and UV20 sperm samples showed significantly ($p < 0.05$) lower total and progressive motility values, sperm kinematic parameters and mitochondrial potential. After 45 min of exposure, differences were highly significant ($p <0.001$). No significant differences ($p > 0.05$) were found for pipetting or thermal oscillations. The results suggest that, even if equine sperm samples are not handled in the laboratory under optimal conditions, fresh and cooled equine spermatozoa are able to resist the impact of various abiotic stimuli without any reduction in their quality. This study analyses the effect on normospermic samples, but future research could look at the tolerance that asthenozoospermic equine samples have to these abiotic influences.

In the second article was carried a research to evaluate the impact of various cryoprotectant agents (CPA) and vitrification on equine sperm quality. A total of 12 ejaculates were subjected to exposure to CPA and vitrification. Sperm was diluted in a range of CPA: fresh, control (BSA), sucrose (0.15M, 0.3M and 0.5M), trehalose (0.15M, 0.3M and 0.5M) and the combination of sucrose and trehalose (M1: 0.15M sucrose +0.5M trehalose; M2: 0.5M sucrose+0.15M trehalose). Sperm motility, viability, acrosome integrity and DNA fragmentation were assessed at the time of CPA exposure and after vitrification. The exposure of spermatozoa to various concentrations of sucrose and/or trehalose significantly reduced sperm motility, with lower

concentrations resulting in higher sperm motility. Sperm viability and DNA fragmentation did not vary after exposure to CPA, but acrosome integrity fell significantly when spermatozoa were exposed to CPA with high osmolality. When spermatozoa were vitrified, motility values were significantly higher than those obtained during the exposure. Low concentrations of sucrose (0.15M and 0.3M) and trehalose (0.15M) showed the best progressive sperm motility. The vitrification-warmed procedure significantly reduced sperm viability and acrosome integrity, but DNA did not vary with any of CPA used. Equine sperm vitrification demonstrates a low capacity for preserving sperm motility, and extenders containing trehalose or sucrose at lower concentrations are associated with a better protective effect on sperm motility. After vitrification, acrosome and plasma membranes were severely impaired, while the DNA structure was maintained. Equine spermatozoa partially recover the motility after vitrification, but there is a need for further studies into the preservation of sperm membranes.

In the third study was established the normal values of the combined thickness of the uterus and placenta (CTUP) in Spanish Purebred mares during late pregnancy. A total of 107 mares were examined at 210, 240, 270 and 300 days of gestation, of which 13 had abnormal gestations (foetal death, abortion or stillbirth). In mares with healthy foals, CTUP measurements showed significant differences ($P < 0.0001$) at the various gestational ages (4.83 ± 0.59 mm, 6.12 ± 0.60 mm, 7.41 ± 0.61 mm and 10.45 ± 0.92 mm at 210, 240, 270 and 300 days, respectively). A high positive correlation was observed between CTUP and gestational age ($r = 0.923$; $P < 0.001$). In mares with abnormal gestation, CTUP measurements were significantly ($P < 0.0001$) higher than in healthy gestations at day 270 (8.89 vs. 7.41 mm) and day 300 (14.17 vs. 10.45 mm). The age of the mare, fertilization time, type of semen and gestation length did not affect the CTUP. The results obtained suggest that the cut-off point for detecting abnormal pregnancies in Spanish Purebred mares is 7.75 mm on day 270 and 12.7 mm on day 300. The authors find that the incidence of placental thickness enlargement in the late gestation of Spanish Purebred is 14%, and a transrectal ultrasonographic examination is highly advisable at 270 days of gestation. Such timely placental assessment could reveal otherwise undetected disorders, thereby enabling early and proper treatments to be administered to prevent foetal loss.

Finally, a retrospective study was carried out to determine reproductive efficiency of Spanish Purebred (SP) mare. It was evaluated the influence of age, reproductive status, insemination with cooled or frozen semen, number of inseminations per cycle, type of oestrus, type of ovulation, breeding season, year of breeding season, presence of uterine fluid, stallions and stallion age. Records inseminations of 122 SP mares for four consecutive breeding seasons (2012-2013, 2013-2014, 2014-2015, 2015-2016). A total of 359 cycles were studied. Mares were grouped according to age: young, middle-

aged, ageing and very old and to reproductive status: maiden, barren, foaling and rested. Pregnancy rates were highest with cooled semen in the first cycle ($P < 0.05$), all cycles ($P < 0.05$), day 14 pregnancies per season ($P < 0.001$), mares per season ($P < 0.001$) and foaling rate ($P < 0.001$). Pregnancy and foaling rates were significantly influenced by the effect of age. Young and maiden mares had highest pregnancy rates. There were not significant differences ($P > 0.5$) in pregnancy rate in all cycles when number of inseminations per cycle, type oestrus, type of ovulation, breeding season, year of breeding season and stallion were analyzed. There were significant differences between normal mares (58.13%) and mares with uterine fluid accumulation ($P < 0.05$) before breeding (42.35%) or post-breeding (25.00%). SP mares could be considered like a very fertile horse breed.

8. REFERENCIAS BIBLIOGRÁFICAS

Aboagla E.M., Terada T. (2013). Trehalose-enhanced fluidity of the goat sperm membrane and its protection during freezing, Biol. Reprod. 69, 1245–1250.

Agarwal A., Ikemoto I., Loughlin K.R. (1994). Effect of sperm washing on levels of reactive oxygen species in semen. Arch. Androl. 33, 157–162.

Aisen E., Quintana M., Medina V., Morello H., Venturino A. (2005). Ultramicroscopic and biochemical changes in ram spermatozoa cryopreserved with trehalose-based hypertonic extenders, Cryobiology 50, 239–249.

Aitken R.J., De Iuliis G.N., Finnie J.M., Hedges A., McLachlan R.I. (2010). Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. Hum. Reprod. 25, 2415–2426.

Akourki A., Echegaray A., Falceto M.V., Mitjana O. (2017). Factors influencing reproductive efficiency and pregnancy in Pura Raza Española mares. Vet. Med. 62, 04, 186–191.

Allen W.R., Brown L., Wright M., Wilsher S. (2007). Reproductive efficiency of Flatrace and National Hunt Thoroughbred mares and stallions in England. Equine Vet. J. 39, 5, 438-445.

Álvarez C., Gil L., González N., Olaciregui M., Luño V. (2014). Equine sperm post-thaw evaluation after the addition of different cryoprotectants added to INRA 96 extender. Cryobiology 69, 144–148.

Ankri R., Friedman H., Savion N., Kotev-Emeth S., Breitbart H., Lubart R. (2010). Visible light induces nitric oxide (NO) formation in sperm and endothelial cells. Lasers Surg. Med. 42, 348–352.

Arando A., Gonzalez A., Delgado J.V., Arrebola F.A., Perez C.C. (2017). Storage temperature and sucrose concentrations affect ram sperm quality after vitrification. Anim. Reprod. Sci. 181, 175-185.

Asgari V., Hosseini S.M., Forouzanfar M., Hajian M., Nasr M.H. (2012). Vitrification of in vitro produced bovine embryos: Effect of embryonic block and developmental kinetics. Cryobiology 65, 278-283.

Aurich J.E. (2012). Artificial insemination in horses - More than a century of practice and research. J. Equine Vet. Sci. 32, 458-463.

Azawi O.I. (2008). Effect on fertility of human chorionic gonadotrophin and uterine lavage with oxytocin performed after mating in Arabian barren mares. Anim. Reprod. Sci. 106, 345–351.

Ball B.A. (2008). Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. Anim. Reprod. Sci. 107, 257–267.

Ball B.A., Vo A. (2001). Osmotic tolereance of equine spermatozoa and effects of soluble
cryoprotectants on equine sperm motility, viability, and mitochondrial membrane potential, J. Androl. 22, 1061–1069.

Barbas J.P., Mascarenhas R.D. (2009). Cryopreservation of domestic animal sperm cells. Cell Tissue Banking, 10, 1, 49-62.

Barnes M, Fite C, Tibary A. (2005). Trans-rectal ultrasonographic evaluation of the placenta in Arabian and pony mares in mid- to-late gestation. Theriogenology 64, 787. (abstract).

Baumber J., Ball B.A., Linfor J.J., Meyers S.A. (2003). Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J. Androl. 24, 621–628.

Bedford S.J., Hinrichs K. (1994). The effect of insemination volume on pregnancy rates of pony mares. Theriogenology 42, 579–590.

Benhajali H., Richard-Yris M.-A., Ezzaouiab M., Charfic F., Hausberger M. (2010). Factors influencing conception rates of Arab mares in Tunisia. Anim. Reprod. Sci. 117, 106–110.

Berrios O., Sánchez R. (2011). Ultra rapid freezing in human spermatozoa: effect on sperm function and reactive oxygen species production, Int. J. Morphol. 29, 899–906.

Blanchard T.L., Thompson J.A., Love C.C., Brinsko S.P., Ramsey J., O'Meara A., Varner D.D. (2012). Influence of day of postpartum breeding on pregnancy rate, pregnancy loss rate, and foaling rate in Thoroughbred mares. Theriogenology 77, 1290–1296.

Bosh K.A., Powell D., Shelton B., Zent W. (2009). Reproductive performance measures among Thoroughbred mares in central Kentucky, during the 2004 mating season. Equine Vet. J. 41, 883-888.

Bracher V., Mathias S., Allen, W.R. (1996). Influence of chronic degenerative endometritis (endometrosis) on placental development in the mare. Equine Vet. J. 28, 180-188.

Brinsko S.P., Rowan K.R., Varner D.D., Blanchard T.L. (2000). Effects of transport container and ambient storage temperature on motion characteristics of equine spermatozoa. Theriogenology 53, 1641-1655.

Bucca S., Fogarty U., Collins A., Small V. (2005). Assessement of feto-placenta well-

being in mare from mid-gestation to term: transrectal and transabdominal ultrasonographic features. Theriogenology 64, 542-557.

Buranaamnuay K. (2017). Protocols for sperm cryopreservation in the domestic cat: A review. Anim. Reprod. Sci. 183, 56-65.

Carrick J.B., Begg A.P., Perkins N., O'Meara D. (2010). Ultrasonographic monitoring and treatment of pregnant mares at risk for placentitis. Anim. Reprod. Sci. 121, 331-333.

Cilek S. (2009). The survey of reproductive success in Arabian Horse Breeding from 1976-2007 at Anadolu State farm in Turkey. J. Anim. Vet. Adv. 8, 389-396.

Cohen N., Lubart R., Rubinstein S., Breitbart H. (1998). Light irradiation of mouse spermatozoa: stimulation of in vitro fertilization and calcium signals. Photochem. Photobiol. 68, 407-413.

Colón J.L. (2008). Trans-rectal ultrasonographic appearance of abnormal combined utero-placental thickness in late-term gestation and its incidence during routine survey in a population of Thoroughbred mares (2005-2008). Proc. Am. Assoc. Equine Pract. 54, 279-285.

Coutinho da Silva M.A., Canisso I.F., MacPherson M.L., Johnson A.E., Divers T.J. (2013). Serum amyloid A concentration in healthy periparturient mares and mares with ascending placentitis. Equine Vet. J. 45, 619-624.

Crisci A., Rota A., Panzani D., Sgorbini M., Ousey J.C., Camillo F. (2014). Clinical, ultrasonographic, and endocrinological studies on donkey pregnancy. Theriogenology, 81, 275-283.

Cuevas-Uribe R., Yang H., Daly J., Savage M.G., Walter R.B., Tiersch T.R. (2011). Production of F1 offspring with vitrified sperm from a live-bearing fish, the green swordtail (Xiphophorus helleril). Zebrafish 8, 167–169.

Cuervo-Arango J., Nivola K., Väihkönen L., Katila, T. (2015). The effect of storage temperature of stallion semen on pregnancy rates. J. Equine Vet. Sci. 35, 611–616. https://doi.org/10.1016/j.jevs.2015.06.003

Cummins C., Carrington S., Fitzpatrick E., Duggan V. (2008). Ascending placentitis in the mare: a review. Ir. Vet. J. 61, 307-313.

Demirci E. (1987). Fertility in Purebred Arab horses in Turkey. Anim. Reprod. Sci .15, 265-271.

Dudley D.J., Trautman M.S. (1994). Infection, inflammation and contractions: the role of cytokines in the pathophysiology of preterm labor. Semin. Reprod. Endocrinol. 12, 263-272.

El-Badry D.A., El-Maaty A.M.A., El Sisy G.A. (2017). The effect of trehalose supplementation of INRA-82 extender on quality and fertility of cooled and frozen-thawed stallion spermatozoa. J. Equine Vet. Sci. 48, 86–92.

El-Sheshtawy R.I., Sisy G.A., El-Nattat W.S. (2015). Effects of different concentrations of sucrose or trehalose on the post-thawing quality of cattle bull semen. Asian Pac. J. Reprod. 4, 26–31.

Engelken T.J. (1999). Reproductive health programs for beef herds: analysis of records for assessment of reproductive performance. In: Youngquist, R.S. (Ed.), Current Therapy in Large Animal Theriogenology. W.B. Saunders Co., Philadelphia, 451–456.

Ferrer M.S., Lyle S.K., Eilts B.E., Eljarrah A.H., Paccamonti D.L. (2012). Factors affecting sperm recovery rates and survival after centrifugation of equine semen. Theriogenology, 78, 1814–1823. https://doi.org/10.1016/j.theriogenology.2012.07.011

Foote R.H. (2003). Fertility estimation: a review of past experience and future prospects. Anim. Reprod. Sci. 75, 119–139.

Gao D., Mazur P., Critser J.K. (1997). Fundamental cryobiology of mammalian spermatozoa. In A. Karow, & J. K. Critser (Eds.), Reproductive tissue banking: Scientific principles (pp. 263–328). San Diego, CA: Academic Press. https://doi.org/10.1016/B978-012399770-8/50007-1

Garner D.L. (2006). Flow cytometric sexing of mammalian sperm. Theriogenology 65, 943–957.

Giles R.C., Donahue J.M., Hong C.G. (1993). Causes of abortion, stillbirth, and perinatal death in horses: 3527 cases (1986-1991). J. Am. Vet. Med. Assoc. 203, 1170-1175.

Ginther O.J. (1992). Maternal aspects of pregnancy. In: Ginther OJ, Ed. Reproductive biology of the mare: basic and applied aspects. 2nd Ed. Cross Plains, WI, USA: Equiservices, 291-344.

Ginther O.J. (1993). Reproductive efficiency. In: Ginther O.J. (ed.) Reproductive Biology of the Mare: Basic and Applied Aspects, 2nd edn. Cross Plains, WI: Equiservices, 499–562.

Ginther O.J., Gastal E.L., Gastal M.O., Beg M.A. (2006) Conversion of a viable preovulatory follicle into a haemorrhagic anovulatory follicle in mares. Anim. Reprod. Sci. 3, 29-40.

Guthrie H.D., Welch G.R. (2006). Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. J. Anim. Sci. 84, 2089–2100.

Haadem C.S., Nødtvedt A., Farstad W., Thomassen R. (2015). A retrospective cohort

study on fertility in the Norwegian Coldblooded trotter after artificial insemination with cooled, shipped versus fresh extended semen. Acta Vet. Scand. 57, 77.

Hanlon D.W., Firth E.C. (2012). The reproductive performance of Thoroughbred mares treated with intravaginal progesterone at the start of the breeding season. Theriogenology 77, 952-958.

Hayden S.S., Blanchard T.L., Brinsko S.P., Varner D.D., Hinrichs K., Love C.C. (2012). Pregnancy rates in mares inseminated with 0.5 or 1 million sperm using hysteroscopic or transrectally guided deep-horn insemination techniques. Theriogenology 78, 914–920.

Hemberg E., Lundeheim N., Einarsson S. (2004). Reproductive Performance of Thoroughbred Mares in Sweden. Reprod. Dom. Anim. 39, 81–85.

Hendriks W.K., Colenbrander B., van der Weijden G.C., Stout T.A.E. (2009). Maternal age and parity influence ultrasonographic measurements of fetal growth in Dutch Warmblood mares. Anim. Reprod. Sci. 115, 110-123.

Hevia M.L., Quiles A.J., Fuentes F., Gonzalo C. (1994). Reproductive Performance of Thoroughbred horses in Spain. Journal of Equine Veterinary Science 14, (2), 89-92.

Hossain M.D., Tareq K.M.A., Hammano K., Tsujii H. (2007). Effect of fatty acids on boar sperm motility, viability and acrosome reaction. Reprod. Med. Biol. 6, 235–239.

Isachenko E., Isachenko V., Katkov I.I., Dessole S., Nawroth F. (2003). Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. Reprod. Biomed. Online 6, 191–200.

Isachenko V., Isachenko E., Katkov I.I., Montag M., Dessole S., Nawroth F., Van Der Ven H. (2004). Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: effect on motility, DNA integrity, and fertilization ability. Biol. Reprod. 71, 1167–1173.

Isachenko V., Isachenko E., Petrunkina A.M., Sanchez R. (2012). Human spermatozoa vitrified in the absence of permeable cryoprotectants: birth of two healthy babies. Reprod. Fertil. Dev. 24, 323–326.

Jasko D.J., Hathaway J.A., Schaltenbrand V.L., Simper W.D., Squires E.L. (1992a). Effect of seminal plasma and egg yolk on motion characteristics of cooled stallion spermatozoa. Theriogenology, 37: 1241–1252.

Jasko D.J., Martin J.M., Squires E.L. (1992b). Effects of insemination volume and concentration of spermatozoa on embryo recovery in mares. Theriogenology, 37: 1233–39.

Jasko D.J., Moran D.M., Farlin M.E., Squires E.L., Amann R.P., Pickett B.W. (1992c).

Pregnancy rates utilizing fresh, cooled and frozen-thawed semen. Proc 38th Ann. Conv. Am. Assoc. Eq. Pract. 649-660.

Jiménez-Rabadán P., García-Álvarez O., Vidal A., Maroto-Morales A., Iniesta-Cuerda M., Ramón M., del Olmo E., Fernández-Santos R., Garde J.J., Soler A.J. (2015). Effects of vitrification on ram spermatozoa using free-egg yolk extenders. Cryobiology 71, 85–90.

Kadirve G., Kumar S., Kumaresana A. (2009). Lipid peroxidation, mitochondrial membrane potential and DNA integrity of spermatozoa in relation to intracellular reactive oxygen species in liquid and frozen-thawed buffalo semen. Anim. Reprod. Sci. 114, 125-134.

Kalirajan R., Rajasundaram R.C. (2008). Incidence of endometritis in relation to age and Caslick index in mares. Indian Vet. J. 85, 893–894.

Kása E., Bernáth G., Kollár T., Zarski D., Lujic J., Marinovic Z., Bokor Z., Hegyi A., Urbányi B., Vílchez M.C., Morini M., Peñaranda D.S., Pérez L., Asturiano J.F., Horváth A. (2017). Development of sperm vitrification protocols for freshwater fish (Eurasian perch, Perca fluviatilis) and marine fish (European eel, Anguilla anguilla). Gen. Comp. Endocrinol. 245, 102–107.

Katila T. (2005). Effect of the inseminate and the site of insemination on the uterus and pregnancy rates of mares. Reprod. Dom. Anim. 89, 31-38.

Katila T., Reilas T., Nivola K., Peltonen T., Virtala A.-M. (2010). A 15-year survey of reproductive efficiency of Standardbred and Finnhorse trotters in Finland -descriptive results. Acta Vet. Scand. 52, 40.

Kim S., Agca C., Agca Y. (2013). Effects of various physical stress factors on mitochondrial function and reactive oxygen species in rat spermatozoa. Repro. Fert. Dev. 25, 1051–1064. https://doi.org/10.1071/RD12212

Koenen, E.P.C., Aldridge, L.I., Philipsson, J. (2004). An overview of breeding objectives for warmblood sport horses. Livest. Prod. Sci. 88, 77 – 84.

LeBlanc M.M. (2004). Ascending placentitis in mares: what we learned from an experimental model. In: Proceedings of the 10th Congreso Nazionala Multisala SIVE, Perugia, Italy. Ithaca, NY: IVIS.

LeBlanc M.M. (2010). Ascending placentitis in the mare: an update. Reprod. Domest. Anim. 45, 28-34.

LeBlanc M.M., Causey R.C. (2009). Clinical and Subclinical Endometritis in the Mare: Both Threats to Fertility. Reprod. Dom. Anim. 44, 3, 10–22.

LeBlanc M.M., Giguere S., Brauer K., Paccamonti D.L., Horohov D., Lester G.D., O´Donnell L.J., Sheerin B.R., Pablo L., Rodgerson D.H. (2002). Premature delivery in

ascending placentitis associated with increased expression of placenta cytokines and allantoic fluid prostaglandins E2 and F2. Theriogenology 58, 841-844.

Leckie, E.J. 2001. Equine Population of the UK: A Report for the International League for the Protection of Horses (ILPH). ILPH, United Kingdom.

Lewis N., Morganti M., Collingwood F., Grove-White D.H., McGregor Argo C. (2015). Utilization of One-Dose Postovulation Breeding With Frozen-Thawed Semen at a Commercial Artificial Insemination Center: Pregnancy Rates and Postbreeding Uterine Fluid Accumulation in Comparison to Insemination With Chilled or Fresh Semen. J. Equine Vet. Sci. 35, 882-887.

Liu J., Tanrikut C., Wright D.L., Lee G., Toner M., Biggers J.D., Toth T.L. (2016). Cryopreservation of human spermatozoa with minimal non-permeable cryoprotectant, Cryobiology 73, 162–167.

Löf H.K., Pires Neves A., Gregory J.W., Winter G.H.Z., Garbade P., Wolf C.A., Richter G., Jobim M.I.M., Gregory R.M., Mattos R.C. (2010). Evaluation of the combined utero-placental thickness (CUPT) and of vulvar conformation as indicators of placentitis in pregnant mares. Anim. Reprod. Sci. 121, 329-330.

Loi P., Matsukawa K., Ptak G., Clinton M., Fulka J., Nathan Y., Arav A. (2008). Freeze-dried somatic cells direct embryonic development after nuclear transfer, PLoS One 3 e2978. http://doi.org/10.1371/journal.pone.0002978.

Loomis P.R. (2001). The equine frozen semen industry. Anim. Reprod. Sci. 68, 191–200.

Love C.C., Thompson J.A., Lowry V.K., Varner D.D. (2002). Effect of storage time and temperature on stallion sperm DNA and fertility. Theriogenology 57, 1135–1142. https://doi.org/10.1016/S0093-691X(01)00689-6

MAPAMA, (2017). Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente: Raza Equina Caballar Española. Datos Censales.. http://www.mapama.gob.es/es/ganaderia/temas/zootecnia/razas-ganaderas/razas/catalogo/autoctona-fomento/equino-caballar/espanola/iframe-ejemplo-arca.aspx

Macpherson M.L., Bailey C.S. (2008). A clinical approach to managing the mare with placentitis. Theriogenology 70, 435-440.

Maischberger E., Irwin J.A., Carrington S.D., Duggan V.E. (2008). Equine post-breeding endometritis: A review. Irish Vet. J. 61, 163-168.

Matsuoka T., Imai H., Kohno H., Fukui Y. (2006). Effects of bovine serum albumin and trehalose in semen diluents for improvement of frozen-thawed ram spermatozoa. J. Reprod. Dev. 52, 675–683.

McCarthy M.J., Baumber J., Kass P.H., Meyers S.A. (2010). Osmotic stress induces oxidative cell damage to rhesus macaque spermatozoa. Biol. Reprod. 82, 644–651. https://doi.org/10.1095/biolreprod.109.080507

McKinnon A.O., Squires E.L., Harrison L.A., Blach E.L., Shideler R.K. (1988). Ultrasonographic studies on the reproductive tract of mares after parturition: effect of involution and uterine fluid on pregnancy rates in mares with normal and delayed first postpartum ovulatory cycles. J. Am. Vet. Med. Assoc. 192, 350–353.

Menzies, P.I. (1999). "Reproductive health management programs" In: Current Therapy in Large Animal Theriogenology, 643-649.

Moran D.M., Jasko D.J., Squires E.L., Amann R.P. (1992). Determination of temperature and cool rate induced cold shock in stallion spermatozoa. Theriogenology, 38, 999–1012. https://doi.org/10.1016/0093-691X(92)90114-7

Morris L.H.A., Allen W. R. (2002). Reproductive efficiency of intensively managed Thoroughbred mares in Newmarket. Equine Vet. J. 34, (1), 51-60.

Morris L.H., Hunter R.H., Allen W.R. (2000). Hysteroscopic insemination of small numbers of spermatozoa at the uterotubal junction of preovulatory mares. J. Reprod. Fertil. 118, 95–100.

Morris S., Kelleman A.A., Stawicki R.H., Hansen P.J., Sheerin P.C., Sheerin B.R., Paccamonti D.L., LeBlanc M.M. (2007). Transrectal ultrasonography and plasma progestin profiles identifies feto-placental compromise in mares with experimentally induced placentitis. Theriogenology 67, 681-691.

Mossad H., Morshedi M., Torner J.P., Oehninger S. (1994). Impact of cryopreservation on spermatozoa from infertile men– implication for artificial insemination. Arch. Androl. 33, 51–57.

Nagy R. (2006). "Effects of management on reproductive efficiency in Thoroughbreds. Thesis". University of Kentucky Master's Theses. 175. https://uknowledge.uky.edu/cgi/viewcontent.cgi?referer=https://www.google.es/&httpsredir=1&article=1177&context=gradschool_theses

Nang C.F., Osman K., Budin S.B., Ismail M.I., Jaffar F.H.F., Mohamad S.F.S., Ibrahim S.F. (2012). Bovine serum albumin: survival and osmolarity effect in bovine spermatozoa stored above freezing point, Andrologia 44, 447–453.

Nath L.C., Anderson G.A., McKinnon A.O. (2010). Reproductive efficiency of Thoroughbred and Standardbred horses in north-east Victoria. Aust. Vet. J. 88, 169-175.

Nath L.C. Reproductive efficiency. In: Mckinnon A.O., Squires E.L., Vaala W.E., Varner D.D. (2011). (eds) Equine Reproduction, second edition. Volume 2, part III: The mare.

United Kingdom, Wiley-Blackwell, Chapter 293, 2779-2789.

Newcombe J.R., Lichtwark S., Wilson M.C. (2005). Case report: The effect of sperm number, concentration, and volume of insemination dose of chilled, stored, and transported semen on pregnancy rate in standardbred mares. J. Equine Vet. Sci. 25, 525-530.

O'Connell M., McClure M.N., Lewis S.E. (2002). The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum. Reprod. 17, 3, 704-709.

Olaciregui M., Luño V., Marti J.I., Aramayona J., Gil L. (2016). Freeze-dried stallion spermatozoa: evaluation of two chelating agents and comparative analysis of three sperm DNA damage assays. Andrología 48, 900–906.

Pérez-Marín C.C., Requena F.D., Arando A., Ortiz-Villalón S., Requena F., Agüera E.I. (2018). Effect of trehalose- and sucrose-based extenders on equine sperm quality after vitrification: Preliminary results. Cryobiology 80, 62-69.

Pickett B.W., Voss, J.L. (1975). The effect of semen extenders and sperm numbers on mare fertility. J. Reprod. Fertil. Suppl. 23, 95–98.

Pickett B.W., Voss J.L. (1999). Physiology and philosophy of breeding horses. J. Equine Vet. Sci. 19, 363-365, 367-373.

Platt H. (1975). Infection of the horse fetus. J. Reprod. Fertil. Suppl. 23, 605-610.

Pollard K.J., Mitchell M.D. (1996). Intrauterine infection and the effects of inflammatory mediators on prostaglandin production by myometrial cells from pregnant women. Am. J. Obstet. Gynecol. 174, 682-686.

Pommer A.C., Rutllant J., Meyers S.A. (2002). The role of osmotic resistance on equine spermatozoal function. Theriogenology 58, 1373–1384.

Pritchard J.C., Lindberg A.C., Main D.C.J., Whay, H.R. (2005). Assessment of the welfare of working horses, mules and donkeys, using health and behavioural parameters. Prev. Vet. Med. 69, 265–283.

Pycock J.F., Newcombe J.R. (1996). Assessment of the effect of three treatments to remove intrauterine fluid on pregnancy rate in the mare. Vet. Rec. 138, 320–323.

Reef V.B., Vaala W.E., Worth L.T., Spencer P.A., Hammett B. (1995). Ultrasonographic evaluation of the fetus and intrauterine environment in healthy mares during late gestation. Vet. Radiol. Ultrasound. 36, 533-541.

Renaudin C.D., Troedsson M.H.T., Gillis C.L., King V.L., Bodena A. (1997). Ultrasonographic evaluation of the equine placenta by transrectal and transabdominal approach in the normal pregnant mare. Theriogenology 47, 559-573.

Requena F.D., Agüera E.I., Miró J., Escribano B., Tovar P., Pérez-Marín C.C., Requena F. (2014). Measures of the combined thickness of the uterus and placenta in pregnants Spanish Purebred mares. Comunicación Oral. 12º Congreso Internacional de la Asociación Española de Reproducción Animal. Alicante, 16-18 octubre de 2014. Reprod Dom Anim 49 (Suppl. 4) 99, DOI: 10.1111/rda.12402

Requena F.D., Agüera E.I., Miró J., Escribano B., Tovar P., Requena F. (2012). Mejora de la eficiencia reproductiva en la yegua Pura Raza Española a través del diagnóstico de placentitis por ultrasonografía. 13º Congreso Internacional de Medicina y Cirugía Equina. Sevilla, 23 y 24 de noviembre de 2012.

Requena F.D., Requena F., Escribano B., Tovar P., Agüera E.I. (2013). Diagnóstico de placentitis por ultrasonografía en la yegua Pura Raza Española: un método no invasivo para mejorar la eficiencia reproductiva. II Congreso Científico de Investigadores en Formación en Agroalimentación y III Congreso Científico de Investigadores en Formación de la Universidad de Córdoba. Fecha 9 y 10 de abril de 2013. Universidad de Córdoba.

Requena F.D., Requena F., Pérez-Marín C.C., Requena L., Cantillo M.J., Miró J., Agüera E.I. (2015). Medida de espesor conjunto útero-placenta como bioindicador de salud fetal y neonatal. 16º Congreso Internacional de Medicina y Cirugía Equina. Sevilla, 20 y 21 de noviembre de 2015.

Ricketts S.W., Alonso S. (1991). The effect of age and parity on the development of equine chronic endometrial disease. Eq. Vet. J. 23, 189–192.

Rosato M.P., Iaffaldano N. (2013). Cryopreservation of rabbit semen: comparing the effects of different cryoprotectants, cryoprotectant-free vitrification, and the use of albumin plus osmoprotectants on sperm survival and fertility after standard vapor freezing and vitrification. Theriogenology 79, 508–516.

Rossdale P.D., Ousey J.C., Cottrill C.M., Chavatte P., Allen W.R., McGladdery A.J. (1991). Effects of placental pathology on maternal plasma progestagen and mammary secretion calcium concentrations and on neonatal adrenocortical function in the horse. J. Reprod. Fertil. Suppl. 44, 579-590.

Samper J.C. (2001). Management and fertility of mares bred with frozen semen. Anim. Reprod. Sci. 68, 219–228.

Samper, J.C., Hellander, J.C., Crabo, B.G. (1991). Relation between fertility of fresh and frozen stallion semen and its quality measured as sperm motility and with glass wool/Sephadex filters. J. Reprod. Fertil. Suppl. 44, 107–114.

Samper J.C., Vidament M., Katila T., Newcombe J., Estrada A., Sargeant J. (2002). Analysis of some factors associated with pregnancy rates of frozen semen: a multi-centre study. Theriogenology 58, 647–650.

Sánchez R., Risopatrón J., Schulz M., Villegas J., Isachenko V., Kreinberg R., Isachenko E. (2011). Canine sperm vitrification with sucrose: effect on sperm function. Andrologia 43, 233-241.

Sandoval R.S. (2005). Criopreservación de semen ovino empleando diferentes diluctores y combinaciones de agentes crioprotectores permeantes y no permeantes. Tesis Doctoral. Lima (Perú).
http://cybertesis.unmsm.edu.pe/bitstream/handle/cybertesis/674/Sandoval_mr.pdf?sequence=1

Shahar S., Wiser A., Ickowicz D., Lubart R., Shulman A., Breitbart, H. (2011). Light-mediated activation reveals a key role for protein kinase A and sarcoma protein kinase in the development of sperm hyper-activated motility. Hum. Reprod. 26, 2274–2282. https://doi.org/10.1093/humrep/der232

Sharma S., Davies Morel M.C.G., Dhaliwal G.S. (2010a). Factors affecting the incidence of postpartum oestrus, ovarian activity and reproductive performance in Thoroughbred mares bred at foal heat under Indian subtropical conditions. Theriogenology 74, 90–99.

Sharma S., Dhaliwal G.S., Dadarwal D. (2010b). Reproductive efficiency of Thoroughbred mares under Indian subtropical conditions: A retrospective survey over 7 years. Anim. Reprod. Sci. 117, 241–248.

Sieme H., Bonk A., Hamannd H., Klugc E., Katila T. (2004). Effects of different artificial insemination techniques and sperm doses on fertility of normal mares and mares with abnormal reproductive history. Theriogenology 62, 915–928.

Sieme H., Schäfer T., Stout T.A.E., Klug E., Waberski D. (2003). The effects of different insemination regimes on fertility in mares. Theriogenology 60, 1153–1164.

Smith K.C., Blunden A.S., Whitwell K.E., Dunn K.A., Wales A.D. (2003). A survey of equine abortion, stillbirth and neonatal death in the UK from 1988 to 1997. Equine Vet. J. 35, 496-501.

Souza A.M., Winter G.H.Z., Garbade P., Wolf, C.A., Jobim M.I.M., Gregory R.M., Mattos R.C. (2010). Ultrasonography evaluation of the Criollo mare placenta. Anim. Reprod. Sci. 121, 320-321.

Squires E.L., Barnes C.K., Rowley H.S., McKinnon A.O., Pickett B.W., Shideler R.K. (1989). Effect of uterine fluid and volume of extender on fertility. In: Proc. 35th Ann. Conv. Am. Ass. Equine Practitioners, 25–30.

Stawicki R.J., Reubel H., Hansen P.J., Sheerin B.R., O'Donnell L.J., Lester G.D., Paccamonti D.L., LeBlanc M.M. (2002). Endocrinological findings in an experimental model of ascending placentitis in the mare. Theriogenology 58, 849-852.

Sutton R.L. (1992). Critical cooling rates for aqueous cryoprotectants in the presence of sugars and polysaccharides, Cryobiology 29, 585–598.

Troedsson M.H.T., Renaudin C.D., Zent W.W., Steiner J.V. (1997). Transrectal ultrasonography of the placenta in normal mares and in mares with pending abortion: a field study. Proc. Am. Assoc. Equine Pract. 43, 256-258.

Troedsson M.H.T., Zent W.W. (2004). Clinical ultrasonographic evaluation of the equine placenta as a method to successfully identify and treat mares with placentitis. In: Betteridge L (ed.). Workshop on the Equine Placenta. Lexington, KY: Univeristy of Kentucky Agricultural Station. 66-67.

Varisli O., Uguz C., Agca C., Agca Y. (2009). Effects of various physical stress factors on rat sperm motility, acrosome and plasma membrane integrity. J. Androl. 30, 75-86.

Varner D.D., Blanchard T. L., Love C.L., Garcia M.C., Kenney R.M. (1988). Effects of cooling rate and storage temperature on equine spermatozoal motility parameters. Theriogenology 29, 1043–1054. https://doi.org/10.1016/S0093-691X(88)80028-1

Varner D.D., Blanchard T.L., Meyers P.J., Meyers S.A. (1989). Fertilizing capacity of equine spermatozoa stored for 24 h at 5º or 20°C. Theriogenology 32, 515–525. https://doi.org/10.1016/0093-691X(89)90273-2

Vidament M., Magistrini M., Le Foll Y., Levillain N., Yvon J. M., Duchamp G., Blesbois E. (2012). Temperature from 4 to 14°C are suitable for preserving the fertilizing capacity of stallion semen stored for 22 h or more in INRA96 extender. Theriogenology 78, 297–307. https://doi.org/10.1016/j.theriogenology.2012.01.018

Villahoza M.D., Squires E.L., Voss J.L., Shideler R.K. (1985). Some observations on early embryonic death in mares. Theriogenology 23, 6, 915–924.

Vivo R., Santisteban R., Tovar P., Castejón F.M., 1985: Valores de fertilidad en yeguas españolas y árabes (Fertility values in Andalusian and Arabian mares). Arch. Zootec. 34, 159-167.
http://www.uco.es/organiza/servicios/publica/az/php/img/web/13_20_00_129_5.pdf

Vizuete G., Jiménez E., Agüera E.I., Pérez C.C. (2014). Impact of ultrarapid freezing on the motility, morphology, viability and acrosome integrity of epididymal cat sperm diluted in sucrose-based extenders. Reprod. Domest. Anim. 49, e5-e8.

Warriach H.M., Memon M.A., Ahmad N., Norman S.T., Ghafar A., Arif M. (2014). Reproductive Performance of Arabian and Thoroughbred Mares under Subtropical Conditions of Pakistan. Asian Australas. J. Anim. Sci. 27, (7), 932-936. doi: 10.5713/ajas.2013.13547

Watson P., Martin I. (1975). Effects of egg yolk, glycerol and the freezing rate on the

viability and acrosomal structures of frozen ram spermatozoa. Australian Journal of Biological Science 28 153-159.

Watson P.F., Plummer J.M. (1985). The responses of boar sperm membranes to cold shock and cooling, in: L.A. Johnson, K. Larsson (Eds.), Deep Freezing of Boar Sperm, Swedish University of Agricultural Sciences, Uppsala, Sweden, 1985, 113–127.

Willmann C., Schuler G., Hoffmann B., Parvizi N., Aurich C. (2011). Effects of age and altrenogest treatment on conceptus development and secretion of LH, progesterone and eCG in early-pregnant mares. Theriogenology 75, 421– 428.

Woelders H., Matthijs A., Engel B. (1997). Effects of trehalose and sucrose, osmolality of the freezing medium, and cooling rate on viability and intactness of bull sperm after freezing and thawing. Cryobiology 35, 93–105.

Yeste M., Codony F., Estrada E., Lleonart M., Balasch S., Peña A., Bonet S., Rodriguez-Gil J.E. (2016). Specific LED-based red light photo-stimulation procedures improve overall sperm function and reproductive performance of boar ejaculates. Sci. Rep. doi:10.1038/srep22569

Yurdaydin N., Tekin N., Gülyüz F., Aksu A., Klug E. (1993). Estrus synchronization and insemination results in Arabian mares in the Eskisehir stud. Dtsch. Tierarztl. Wochenschr. 11, 432-434.