
DEPARTAMENTO DE CIENCIA Y TECNOLOGÍA AGROFORESTAL

# Evaluación del efecto del proceso de sexado sobre espermatozoides de ciervo rojo ibérico y estudio de herramientas complementarias para su mejora

# Evaluation of the sex-sorting process effect on Iberian red deer sperm and assessment of complementary tools for its improvement

**Por**

**Luis Anel López**

**TESIS DOCTORAL**

**Albacete, 2015**

D. José Julián Garde López-Brea, con DNI 50172450-C Catedrático del Departamento de Departamento de Ciencia y Tecnología Agroforestal y Genética, de la Universidad de Castilla-La Mancha hace constar:

Que la Tesis Doctoral titulada: "**Evaluación del efecto del proceso de sexado sobre espermatozoides de ciervo rojo ibérico y estudio de herramientas complementarias para su mejora**", ha sido realizada por D. Luis Anel López, con DNI 09808711-Q, Licenciado en Veterinaria, bajo mi dirección y que tras su revisión, considero que tiene la debida calidad para su presentación y defensa, así como para optar a la mención internacional.

Albacete, 10 de Noviembre de 2015

D. José Julián Garde López-Brea

*Esta tesis ha sido realizada en el laboratorio de Biología de la Reproducción del grupo de investigación SaBio, perteneciente al Instituto de Investigación en Recursos Cinegéticos IREC (UCLM-CSIC-JCCM). El autor ha disfrutado de una ayuda de formación de personal investigador de la Junta de Castilla y La Mancha (PRE123/2010) y los experimentos han sido financiados por el Ministerio de Economía y Competitividad por medio del proyecto del Plan Nacional AGL2010-21487. Además, el doctorando ha contado con financiación de la UCLM para realizar una estancia de 3 meses en el SLU de Uppsala (Suecia), lo que ha permitido cumplir con los requisitos para acceder a la mención de doctorado internacional.*

**AGRADECIMIETOS**

A mi director de tesis, Julián Garde por TODO. Por sus consejos, sus broncas y su preocupación, su tiempo y su trabajo. Porque gracias a el tuve la oportunidad de iniciarme en el mundo de la investigación y porque gran parte de lo que soy hoy, es gracias a el

A Pepi, Rocío y Manuel Ramón por todo su apoyo, su tiempo, su trabajo y su gran labor a la hora de ayudarme en la elaboración de los experimentos y la redacción de los papers que componen la presente tesis doctoral

A mis compañeros de Albacete; Enrique, Pilar, María y Alfonso por su trabajo durante estos años y muy especialmente a Olga y Alejandro, por todo lo que me habéis enseñado, y por el tiempo que hemos pasado juntos… viajes, laboratorio, playa, pádel…. Que no todo iba a ser trabajar!

To professor Jane Morrell. For her concern and her great help during my stay in Sweden

A José Antonio y demás compañeros de la finca Las Lomas, por todo su trabajo, disposición, empeño y dedicación

Al equipo de reproducción de la facultad de veterinaria de Murcia; Emilio, Jordi, Juan María por su apoyo, por los buenos consejos y momentos vividos juntos. Y muy especialmente a Inma y su equipo de currantes del sorter por todas sus horas y horas de trabajo con la “maquina infernal”

A Merce y a Paulino, gracias por todo lo que me habéis enseñado, por vuestro cariño, trabajo, tiempo y esfuerzo

A mis compañeros de León; Patri, Carmen, Susana, María, Patricia Manrique, Elena y Felipe. Gracias por todo vuestro tiempo y vuestro trabajo, por el cariño que me habéis dado y por todo lo que me habéis enseñado

A Santi, Prieto y Marañón por su cariño y su colaboración en Cabarceno

A mis amigas de la carrera: Aroa, Patri, Marta, Moni, Virginia y Lu. Por todos los buenos momentos que hemos pasado juntos, y porque aunque ahora estemos desperdigados y no podamos vernos a diario, cada vez que nos juntamos es como si no hubiera pasado el tiempo

A mis “Muchachos”: Oscarón, Dito, Jorgito, Vitor y Pelo. Gracias por haber estado siempre ahí, por todos los momentos que hemos vivido juntos y porque se que siempre podre contar con vosotros

A mi amigo Dieguito por los buenos momentos y por meterme el veneno en el cuerpo con mi hobby y mi pasión, la pesca

A Félix y Mariaje por su cariño y por todos los buenos momentos juntos.

A mis Profesores de Ingles; Laura, Leti, Jaquie y Simon por hacer de mi ingles algo mas o menos presentable

A mi familia de Albacete: Julián, Mini, Carlos, Minie y Verdel. Por su cariño y por hacerme sentir como uno mas en su familia

A todos mis Familiares. Mis abuel@s; Aurelio, Araceli, Fernando y Chelo, mis ti@s; Carlos y Domi, Gloria y Maxi, Gelo, Joaquín y María Jesús, Enrique y Giovana, y Fernando. A mis prim@s; Carli, Sandra, Judith, Rodri, María, Ruth, Lucia y Marta. Por todo su cariño, su apoyo y su preocupación durante toda mi vida

A mis padrinos Joaquín y Domi, por vuestro cariño y por haberme hecho sentir una parte importante de vuestra vida

A mi Familia: a mi padre, a mi madre, a Tochín y a Ras. Porque hoy soy lo que soy gracias a vosotros. Por vuestro apoyo, vuestro amor incondicional y por procurarme siempre lo mejor aunque yo a veces no sepa verlo. Sin vosotros esto no habría sido posible. Os quiero!

A mi Marietina. No hay palabras de agradecimiento que describan todo lo que me has dado. Gracias por haber estado siempre ahí apoyándome y empujándome, especialmente en los momentos duros. Gracias por todas las buenas cosas que hemos vivido juntos y las que nos quedan por vivir, y sobre todo, gracias por regalarme cada día tu mejor sonrisa

A mi familia

A María

Aquel que tiene un “porqué” para vivir, se puede enfrentar a todos los “cómos”

Friedrich Nietzsche

# TABLA DE CONTENIDOS

# RESUMEN

En el primer capitulo de la presente tesis doctoral hemos estudiado el efecto del proceso de sexado (mediante un sistema de citometría de flujo SX MoFlo, DakoCytomation Inc. Fort Collins, CO, USA) en muestras espermáticas de ciervo rojo. Las muestras fueron obtenidas de 10 machos adultos en época reproductiva mediante electroeyaculación Se evaluaron varios parámetros de calidad espermática *in-vitro* entre muestras sometidas al proceso de sexado pero no separadas (BSS), muestras espermáticas separadas en pureza Y (YSS), muestras separadas en pureza X (XSS) y muestras espermáticas convencionales no sometidas al proceso de sorting (NS). Todos los grupos experimentales se sometieron a un proceso de congelación en vapores de nitrógeno. Tras la descongelación, las muestras YSS no mostraron diferencias significativas ($P<0.05$) para la motilidad total respecto al BSS ni al grupo NS, en cambio las muestras XSS mostraron un descenso significativo de la misma respecto a los otros 3 grupos experimentales. La motilidad espermática disminuyo para los 4 grupos experimentales tras someter las muestras a una incubación de 2 h a 37 ºC. Además, el porcentaje de espermatozoides apoptóticos tras la incubación fue mas alto para las muestras XSS que para el resto. El porcentaje de espermatozoides con alto daño en la cromatina (%DFI) fue similar entre los 4 grupos experimentales tras la descongelación e incrementó tras la incubación para todos los grupos menos para las muestras YSS. Debido a la ausencia de grandes diferencias en los parámetros de calidad analizados *in-vitro* se planteo una segunda parte donde se realizaron inseminaciones artificiales en ciervas con muestras YSS y BSS. Una semana antes del parto las hembras fueron estabuladas individualmente y la identificación del sexo se llevo a cabo sobre las crías paridas. El porcentaje de machos paridos de las muestras YSS fue del 93%. La fertilidad fue significativamente superior para el grupo BSS (51%) respecto al YSS (43%).

Los objetivos del segundo capitulo de la presente tesis doctoral fueron evaluar la susceptibilidad al estrés oxidativo de las muestras espermáticas sometidas a un proceso de sex sorting, y evaluar el efecto de la adición de 2 sustancias antioxidantes [glutatión reducido (GSH) y trolox (TRX)] en dichas muestras espermáticas. Se utilizaron muestras espermáticas de 3 machos adultos en época reproductiva obtenidas por electroeyaculación. De cada

muestra, la mitad del eyaculado fue procesado como semen convencional NS y la otra mitad fue sometida a un proceso de sorting BSS. Posteriormente ambos grupos fueron congelados. Tras la descongelación se realizo una evaluación de parámetros espermáticos como la motilidad, viabilidad o fragmentación de la cromatina. La susceptibilidad al estrés oxidativo se midió mediante la adición de $H_2O_2$ a 2 concentraciones diferentes($H_2O_2$ 0 mM = H000; $H_2O_2$ 50 mM = H050; $H_2O_2$ 100 mM = H100) en el medio de congelación y se incubo 2 h a 37 ºC. Tras la descongelación la motilidad espermática mostro mejores valores ($P<0.05$) para las muestras convencionales NS (59%±3.3) respecto muestras BSS (36.9%±5.8). Ademas, el porcentaje de espermatozoides con marcadores apoptóticos fue significativamente mas alto para las muestras BSS (21.6%±5.0) que para las muestras convencionales NS (14.6%±1.2). El porcentaje de espermatozoides con una alta fragmentación de la cromatina se vio incrementado por la presencia de $H_2O_2$ en las muestras convencionales NS (H000=4.1%±0.9; H050=9.3%±0.7; and H100=10.9%±2.3) no siendo así para las muestras BSS, que mostraron mas resistencia al estrés oxidativo inducido que las muestras convencionales. Por otro lado la adición de GSH protegió la motilidad espermática en presencia de oxidante tras la incubación para ambos tipos de muestras (NS y BSS). Estos resultados mostraron que el proceso de sorting produce un daño subletal en espermatozoides de ciervo rojo pero a la vez selecciona un población de espermatozoides con una cromatina mas resistente al estrés oxidativo que las muestras convencionales. Por su parte, el GSH a una concentración de 1 mM mostró buenos resultados manteniendo valores mas altos de motilidad en aquellas muestras sometidas a un estrés oxidativo mediante la adición de $H_2O_2$, no siendo así para el TRX que mostro un fuerte efecto inhibiendo la motilidad espermática.

En los capítulos tercero y cuarto de la presente tesis doctoral, hemos evaluado 2 herramientas adicionales (selección espermática por centrifugación con Androcoll-S y la adición de 2 sustancias antioxidantes a los medios de manipulación espermática) para la mejora de la calidad espermática y sus efectos en muestras espermáticas de ciervo rojo, para quizá en un futuro poderlas usar como una parte importante de los protocolos sex sorting con el objetivo de mejorar sus rendimientos.

La selección espermática con coloides monocapa mediante la centrifugación es una técnica de gran utilidad para mejora la calidad espermática. El uso de estos medios de selección tales como el Androcoll-S podria convertirse en un herramienta de gran utilidad para la mejora de la calidad espermática y en consecuencia para mejorar otras técnicas de reproducción asistida como el se sorting espermático o la fecundación *in-vitro* (FIV). Por estos motivos, el objetivo del tercer capitulo fue evaluar el efecto del Androcoll-S en muestras espermáticas de ciervo rojo obtenidas mediante electroeyaculación y post-mortem tras someterlas a un proceso de congelación-descongelación y a una incubación de 2 h a 37 ºC. Las muestras seleccionadas con Androcoll-S, mostraron una importante mejora en los parámetros de movilidad analizados respecto a las muestras no analizadas, tanto en el tiempo 0, nada mas hacer la selección, como tras someter las muestras a la incubación. El mismo efecto se observo en otros parámetros como la viabilidad, la actividad mitocondrial o la integridad acrosomal que mejoraron significativamente ($P<0.05$) respecto a las muestras no seleccionadas, y mantuvieron el margen de mejora respecto de dichas muestras tras la incubación. Tras los buenos resultados obtenidos, el Androcoll-S se perfila como una herramienta de gran utilidad que puede ayudarnos a mejorar los protocolos de otras técnicas de reproducción asistida.

Por ultimo, en el cuarto capitulo de la presente tesis doctoral evaluamos el efecto de la adición de 2 sustancias antioxidantes (GSH y TRX) a muestras espermáticas de ciervo obtenidas mediante electroeyaculación y sometidas a un proceso de congelación-descongelación. Para poder evaluar el efecto de los antioxidantes, una vez descongeladas y suplementadas las muestras se sometieron a una incubación de 2 h a 39 ºC. El efecto del GSH en la motilidad espermática fue muy positivo, al contrario que el TRX cuya principal acción fue la inhibición de la misma. Por el contrario el uso de TRX a 1 y 5 mM mostro valores inferiores de espermatozoides con marcadores apoptóticos (12.4 ± 1.1% and 11.7 ± 0.9%) respecto a las muestras suplementadas con GSH a 1 y 5 mM (15.2 ± 1% and 14.6 ± 1.1%) y respecto al las muestras control no suplementadas (16.9 ± 1.2%). Además, el uso de GSH a ambas concentraciones se tradujo en un mantenimiento de valores mas altos de la

actividad mitocondrial respecto las muestras control y las suplementadas con TRX. Por tanto, lo s resultado de este estudio perfilan al GSH como un aditivo de interés para la suplementación de los medios de manejo espermático de ciervo rojo., mientras que el TRX, al menos en el rango mM, no representa una buena opción, dado que su principal acción sobre los espermatozoides de ciervo rojo fue inhibir la motilidad.

# SUMMARY

This doctoral thesis studied the effect of the sex sorting process (using the SX MoFlo, DakoCytomation Inc., Fort Collins, CO, USA) on the thawed and incubated red deer sperm, and the possible differences in several sperm parameters and fertility among sex-sorted, bulk sorted and non-sorted samples. Samples were obtained by electroejaculation from 10 mature stags during breeding season. There were 4 different experimental groups: Bulk sorted spermatozoa (submitted to the sorting process but not sexed) (BSS), samples with high purity of X-spermatozoa (XSS) and with high purity of Y-spermatozoa (YSS), and a control group consisting in non-sorted (NS) sperm. All of them were frozen in liquid nitrogen vapours. After thawing, YSS sperm did not show significantly differences ($P \leq 0.05$) for the total motility in relation with NS and bulk sorted sperm, although XSS was lower ($P \leq 0.05$) respect to the previous treatments. Total motility for all treatments was similar after 2 h of incubation at 37 ºC. The values of apoptotic spermatozoa after thawing were higher ($P \leq 0.05$) for XSS sperm than for the other types of samples. The chromatin stability (%DFI) was similar among treatments after thawing and the values increased after incubation for all treatments except for YSS sperm, being different and lower ($P \leq 0.05$) from the other treatments. Since, there were no differences between YSS and the others experimental groups for most of the sperm parameters assessed, and the parameters of DNA status were even better for YSS sperm, an artificial insemination trial was performed with YSS spermatozoa and was compared with bulk sorted sperm to assess the effect of sex-sorted in thawed sperm samples from red deer. One week before the starting of parturition, the hinds inseminated with the different treatments were housed in separate pen in a shed until the end of parturition. All calves were identified for sex at birth. The percentage of males born from YSS semen was 93%. Fertility was higher ($P<0.05$) for bulk sorted sperm (51%) in relation to YSS sperm (43%) and the percentage of males born was higher (93%) for this previous treatment.

The aims of the second work were assessing the susceptibility to oxidative stress of bulk sorted sperm samples and evaluating the effect of two antioxidants: reduced glutathione and trolox. Sperm samples from 3 stags were collected by electroejaculation. For each male, half of the sample was subjected

to a sorting process but not sexed (Bulk sorted sperm; BSS) and then, both samples; bulk sperm (BSS) and non-sorted (NS) sperm were frozen-thawed. Susceptibility to oxidative stress was assessed in thawed samples by the addition of $H_2O_2$ ($H_2O_2$ 0 mM = H000; $H_2O_2$ 50 mM = H050; $H_2O_2$ 100 mM = H100) in the extender media during an incubation of 2 hours at 37ºC. Just after thawing, the motility of sperm samples showed a significant difference ($p < 0.05$) between both treatments, being NS (59%±3.3) better than BSS (36.9%±5.8). Moreover, the percentage of apoptotic sperm was significantly higher ($p < 0.05$) for BSS sperm (21.6%±5.0) than NS sperm (14.6%±1.2). The DNA damage was increased by the presence of $H_2O_2$ on NS sperm (H000=4.1%±0.9; H050=9.3%±0.7; and H100=10.9%±2.3), but not for BSS sperm. The motility was improved by the addition of GSH in presence of oxidant for both sperm samples (NS and BSS). These results showed that sorting process performs sublethal effects, but selects a sperm population with a more resistant chromatin to oxidative stress than non-sorted sperm. GSH at 1 mM could be a good option to maintain the quality in stressed samples, but not Trolox, which showed a high ability to inhibit sperm motility.

In the third and fourth chapters of this doctoral thesis we tried to evaluate 2 additional tools and its effect on red deer sperm in order to improve the sperm quality and maybe in the future use them as an important part in the sex sorting sperm protocols of red deer.

Single layer centrifugation is a useful technique to select sperm with good quality. The use of selection methods such as the Androcoll could become an important tool to improve the quality of sperm samples and therefore to improve other artificial reproductive techniques such as sperm sex sorting, *in-vitro fertilization* or AI. The aim of the third chapter was to evaluate the effect of a Single Layer Centrifugation with Androcoll-S on the sperm quality on red deer sperm samples of two different origins, electroejaculated samples and epididymal samples obtained post-mortem, after thawing and after an incubation for 2h at 37ºC. Sperm motility, viability, membrane permeability, mitochondrial activity, acrosomal status and DNA fragmentation were determined for all samples. The samples selected by Androcoll-S showed an improvement in sperm kinematics compared to unselected samples after

thawing and after the incubation. The same effect was observed in parameters such as viability, mitochondrial activity or acrosomal status, which were improved after the selection. In contrast, no difference was found in DNA fragmentation between selected and unselected samples within the same sperm type. After these positive results, we conclude that sperm selection by SLC with Androcoll-S after thawing for red deer sperm is a suitable technique that allows sperm quality in both kinds of sperm samples to be improved; thereby improving other assisted reproductive techniques.

In the last chapter, the potential protective effect of reduced glutathione (GSH) and trolox (TRX), an analogue of vitamin E, supplementation during *in vitro* culture (2 h, 39 ºC) of electroejaculated frozen-thawed red deer sperm was investigated. Cryopreserved sperm were thawed and incubated with no additive (Control) and 1 mM or 5 mM of each antioxidant to find out whether these supplementations can maintain the sperm quality, considering the use of thawed samples for *in vitro* techniques such as *in vitro* fertilization (IVF), sperm sex sorting or refreezing. The effect of GSH on sperm motility was positive compared to TRX, which was negative ($P<0.001$). After 2 h of incubation at 39 ºC, use of GSH improved motility while TRX supplementation reduced sperm motility compared with Control samples without antioxidant. Use of TRX at both concentrations (1 and 5 mM; TRX1 and TRX5) resulted in lesser percentages of apoptotic sperm (12.4 ± 1.1% and 11.7 ± 0.9%) than GSH1, GSH5 (15.2 ± 1% and 14.6 ± 1.1%) and Control samples (16.9 ± 1.2%) ($P<0.001$). Use of GSH at both concentrations (1 and 5 mM) resulted in greater mitochondrial activity as compared with findings for the Control, TRX1 and TRX5 groups. The results of this study indicate that GSH is a suitable supplement for electroejaculated red deer sperm. It would be necessary to conduct fertility trials (*in vivo* and *in vitro*), to assess whether GSH supplementation of thawed red deer sperm could improve fertility rates.

# INTRODUCCIÓN GENERAL

El uso de técnicas de reproducción asistida en cérvidos durante los últimos años ha experimentado un importante avance no solo con propósitos conservacionistas (Jabbour *et al.* 1997; Pukazhenthi y Wildt 2003), sino también con propósitos económicos (carne, velvet y trofeos) cuya importancia es alta en algunos países como España, Nueva Zelanda, Australia o China (Asher *et al.* 2000). Esta importancia económica ha generado un interés creciente por parte de los criadores de ciervo a la hora de buscar asesoramiento de especialistas con el fin de desarrollar técnicas que les permitan optimizar sus rendimientos y producciones.

Dentro de este contexto, existe una gran variedad de técnicas de reproducción asistida como la electroeyaculación, la sincronización del estro a través del uso de implantes de progestágenos, la inseminación artificial (IA), la transferencia de embriones o la fecundación in vitro (IVF) que han sido objeto de investigación en varias especies de cérvidos (Asher *et al.* 1999), siendo la más importante tanto para los criadores como para los investigadores el ciervo rojo (*Cervus elaphus*).

Uno de los principales objetivos de la presente tesis doctoral ha sido evaluar el rendimiento y efectos que tiene el proceso de sex-sorting, mediante el uso del sistema (SX MoFlo, DakoCytomation Inc., Fort Collins, CO, USA) modificado para selección espermática, en eyaculados de ciervo rojo obtenidos mediante electroeyaculación. Dicho proceso nos permite separar y clasificar los espermatozoides de eyaculados en fracciones enriquecidas de espermatozoides Y- y espermatozoides X- con una pureza superior al 90%. Estudios previos en cérvidos (Gao *et al.* 2011) han demostrado que es posible obtener fracciones enriquecidas de espermatozoide Y- con capacidad fecundante y obtener descendencia mediante inseminación artificial en hembras superovuladas.

Esta tecnología de sexado espermático es especialmente interesante en especies como el ciervo, en las cuales el interés productivo y en consecuencia el rendimiento económico se obtiene de uno de los sexos. Dado que en el ciervo rojo la principal producción es la cuerna, los machos son los que poseen un elevado valor económico. La posibilidad de elegir el sexo de la descendencia, supondría una enorme optimización de los medios de

producción. Además, desde un punto de vista tanto ético como productivo nos permite evitar la eliminación masiva de hembras en las explotaciones.

Por estos motivos el sex-sorting espermático se presenta como una herramienta de gran utilidad en la producción de cérvidos. Sin embargo, apenas se han realizado estudios en cérvidos que analicen *in-vitro* los diferentes efectos que por su amplitud y complejidad puede tener el proceso de sexado sobre espermatozoides de cérvidos. El único ejemplo que podemos encontrar es el trabajo llevado a cabo por Kjelland *et al.* (2011) donde monitorizan la cinética de fragmentación del DNA y la motilidad espermática en muestras de ciervo de cola blanca (*Odocoileus virginianus*). Durante el proceso de sex-sorting los espermatozoides se exponen a numerosos factores estresantes tales como; altas tasas de dilución, tinción con sondas fluorescentes, procesos mecánicos a lo largo del citómetro de flujo, la aplicación de cargas eléctricas a las gotas donde se encuentran sumergidos los mismos en el momento de la separación, o la centrifugación necesaria para reconcentrar las muestras. Se presupone que todos estos factores reducen la viabilidad de los espermatozoides y por lo tanto reducen la capacidad de éxito de dicha tecnología (Rath *et al.* 2009).

Además se realizó un segundo estudio en muestras espermáticas sometidas al proceso de sorting sin ser separadas en pureza (BSS) contra semen convencional (NS), ambos sometidos a un proceso de congelación-descongelación. Después de descongelar las muestras, estas se sometieron a un test de estrés que consistió en suplementar las muestras con un agente oxidante ($H_2O_2$) dejando un control sin suplementar, y a su vez suplementar las muestras oxidadas y no oxidadas con antioxidantes (Glutatión reducido (GSH) y Trolox) dejando una vez más un control sin oxidante y sin antioxidante. Posteriormente se sometió a las muestras a una incubación de 2 h a 37ºC. El objetivo de este segundo estudio era averiguar si las muestras espermáticas sometidas al proceso de sexado eran más o menos susceptibles al estrés oxidativo y además si esos efectos negativos producidos por un exceso de ROS podían ser contrarrestados con el uso de antioxidantes.

Por un lado, un estudio previo (Gosálvez *et al.* 2011) llevado a cabo en la especie bovina sugirió que tras el proceso de sex-sorting el porcentaje de

espermatozoides con DNA dañado disminuía, lo que puede explicarse debido a uno de los pasos intrínsecos del proceso de sex-sorting por el cual se descartan los espermatozoides no viables y mal orientados. Este hecho estaría en consonancia con Lopes M.Sc. *et al.* (1998) donde concluye que en muestras con mala calidad espermática, el porcentaje de fragmentación del DNA es mayor.

Por otro lado, todos los factores estresantes para el espermatozoide comentados anteriormente y que tienen lugar durante el proceso de sexado nos pueden hacer pensar en un aumento de la susceptibilidad al estrés oxidativo. Además, el proceso de congelación al que son sometidas las muestras en la mayoría de los casos debido a razones logísticas, nos hace pensar en un aumento del estrés oxidativo como se ha demostrado previamente en ganado equino (Ball *et al.* 2001) o en bovino (Chatterjee y Gagnon 2001). Por este motivo nos pareció muy importante, averiguar el grado de susceptibilidad al estrés oxidativo de muestras espermáticas sometidas al proceso de sorting respecto a muestras espermáticas convencionales, y si el uso de antioxidantes pudiera tener efectos beneficiosos a la hora de prevenir daños y mantener la calidad espermática.

Otro de los objetivos de la presente tesis doctoral ha sido profundizar en la mejora de los procesos de manejo de muestras espermáticas de ciervo rojo mediante el uso de técnicas como la centrifugación de dichas muestras en gradientes de selección espermática de una sola capa (Androcoll-S) o la adición de sustancias antioxidantes *in vitro* con el fin de lograr una mejora de la calidad espermática en diferentes etapas de su manipulación.

Dentro de este contexto podemos decir que el uso de gradientes de selección de una sola capa como Androcoll® puede convertirse en una herramienta muy importante a la hora de mejorar la calidad espermática en muestras con cierto deterioro, ayudándonos así a seleccionar únicamente células útiles (motiles y viables) y descartando aquellas deterioradas sin función alguna. Esta técnica por lo tanto, podría permitirnos mejorar por un lado los bancos de recursos genéticos (BRG), permitiéndonos una gran optimización de los mismos debido a la capacidad para seleccionar solo aquellos espermatozoides útiles y descartando aquellos que no lo son. Por otro lado nos

permite mejorar otras técnicas de reproducción asistida como por ejemplo la *fertilización in vitro (FIV)*, la inseminación artificial o el proceso de sex-sorting. Centrándonos en este último, el uso de gradientes de selección (Androcoll-S en este caso) puede ofrecernos varias ventajas. En primer lugar, nos permitiría llevar a cabo el proceso de sex-sorting en muestras previamente congeladas donde la completa eliminación del medio de congelación es un requisito fundamental puesto que dicho gradiente va a permitirnos seleccionar de la muestra solamente los espermatozoides motiles, descartando en su totalidad el medio de congelación (Glicerol, yema…etc.) (Hollinshead *et al.* 2004; Morton *et al.* 2006). Además, el uso de este gradiente va a permitirnos hacer una selección previa, descartando así la gran mayoría de células muertas, y optimizando el proceso de sexado al poder partir de muestras con porcentajes de viabilidad muchos mayores. No hay que olvidar que trabajar con muestras con un bajo porcentaje de células vivas es uno de los mayores problemas del proceso de sex-sorting. Con esta herramienta facilitamos el proceso, haciendo una selección previa y pudiendo aumentar la velocidad y el rendimiento del proceso de sex-sorting per se. De entre los distintos gradientes de selección espermática por centrifugación, hemos seleccionado el Androcoll-S para comprobar su idoneidad en semen de ciervo rojo debido a la gran facilidad de manejo que presenta a priori respecto otros medios de selección espermática bicapa o el swim up. El uso de Androcoll® con formulaciones de coloide especie-especificas ha dado buenos resultados en otras especies tales como: equino (García *et al.* 2009), bovino (Thys *et al.* 2009) o caprino (Jiménez-Rabadán *et al.* 2012). Es por estas razones que desarrollar un gradiente específico para muestras espermáticas de ciervo podría ser una herramienta de gran ayuda como complemento para el desarrollo de otras técnicas de reproducción asistida.

Finalmente y como último objetivo de la presente tesis, hemos querido profundizar en el uso de dos antioxidantes (Trolox (TRX) y Glutatión reducido (GSH)) como aditivos y monitorizar *in-vitro,* en dosis descongeladas de ciervo rojo obtenidas mediante electroeyaculación, el efecto de estos tras someter a dichas muestras espermáticas a un test de estrés mediante una incubación de 2 h a 39ºC.

El Trolox es un análogo hidrosoluble de la Vitamina E con alta capacidad de depuración de ROS (Mickle y Weisel 1993) cuya eficacia pudimos comprobar previamente en muestras epididimarias (Anel-López *et al.* 2012). Habitualmente es usado como antioxidante estándar para comparar la capacidad antioxidante de otros antioxidantes (Lipovac *et al.* 2000; Prior *et al.* 2005). Este antioxidante ha demostrado su utilidad a la hora de mejorar la motilidad y la actividad mitocondrial en espermatozoides de porcino como tras la descongelación como demostró Peña *et al.* (2003). También, ha demostrado su utilidad reduciendo la peroxidación lipídica y preservando la integridad de membrana en espermatozoides epididimarios de ciervo rojo durante una incubación tras la descongelación tanto en muestras con oxidación inducida como en muestras no inducidas (Martinez-Pastor *et al.* 2009; Martínez-Pastor *et al.* 2008). Si bien es cierto, dentro del rango de concentración mM podemos decir que el exceso de antioxidación se tradujo en una reducción de calidad en muestras epididimarias (Anel-López *et al.* 2012).

El GSH es un tripéptido que se encuentra distribuido en las células vivas. Tiene un papel muy importante en la protección de las células contra el estrés oxidativo, actuando directamente o como cofactor de la enzima glutatión peroxidasa (Atmaca 2004) reduciendo el $H_2O_2$ a $H_2O$ y lipoperoxidos a alquil alcoholes. Los buenos resultados obtenidos previamente en muestras epididimarias de ciervo rojo (Anel-López *et al.* 2012) nos parecieron un buen motivo para probar la eficacia de este antioxidante en muestras obtenidas mediante electroeyaculación, y lo han perfilado como un aditivo de gran valor para la mejora de el manejo de espermatozoides de esta especie.

Uno de los principales motivos para profundizar en el uso de antioxidantes ha sido debido a la alta susceptibilidad de los espermatozoides de mamíferos al estrés oxidativo (Baker y Aitken 2004). En este contexto, podríamos definir el estrés oxidativo como la pérdida del balance entre la producción de especies reactivas del oxígeno (ROS) y la capacidad de los antioxidantes de una célula para eliminarlos. Esta alta susceptibilidad por parte de los espermatozoides al estrés oxidativo, se traduce en una pérdida de calidad espermática; perdida de motilidad, perdida de la integridad de la membrana plasmática y perdida de la capacidad fecundante (Aitken 1995;

Aitken *et al.* 2012; Baker y Aitken 2004; Rath *et al.* 2009). Estudios previos, han demostrado que el uso de antioxidantes tuvo efectos beneficiosos sobre muestras espermáticas epididimarias de ciervo rojo tanto en muestras refrigeradas (Fernández‐Santos *et al.* 2009), en muestras suplementadas previamente a la congelación (Anel-López *et al.* 2012) o en muestras suplementadas tras la descongelación (Domínguez-Rebolledo *et al.* 2010). El uso de Antioxidantes como aditivo en los medios de manejo espermático ha de valorarse como una herramienta muy importante que puede ayudarnos a optimizar y mejorar el rendimiento del proceso de sex-sorting con todas las ventajas que esto incluiría tales como; la mejora de los tiempos de separación, la mejora de la calidad espermática tras el sex-sorting, y la mejora de la calidad espermática tras la descongelación de muestras sexadas, con el consiguiente aumento de la fertilidad de dichas muestras.

Debido a la complejidad del proceso de sex-sorting, donde la calidad de las muestras con las que se va a trabajar juega un papel critico a la hora de obtener un buen rendimiento, además de todos los pasos lesivos a los que son sometidos los espermatozoides durante el propio proceso tales como; altas tasas de dilución, tinción con sondas fluorescentes, procesos mecánicos a lo largo del citómetro de flujo, la aplicación de cargas eléctricas a las gotas donde se encuentran sumergidos los mismos en el momento de la separación, o la centrifugación necesaria para reconcentrar las muestras, nos pareció un paso fundamental para poder elaborar un plan de mejora de dichas muestras espermáticas, realizar un análisis descriptivo de la fisiología de los espermatozoides de ciervo rojo tras ser sometidos a al proceso de sex-sorting.

Por estos motivos, quisimos evaluar además, el efecto de 2 herramientas que podrian ayudarnos en un futuro a mejorar los protocolos de sex-sorting espermático. Por un lado, comprobar in vitro el efecto de 2 antioxidantes en muestras espermáticas de ciervo rojo obtenidas mediante electroeyaculación y sometidas a un proceso de congelación-descongelación. Por otro lado, evaluar el efecto del la técnica de selección espermática por centrifugación con Androcoll-S. Esta técnica, podria ayudarnos a realizar el proceso de sexado en muestras que por motivos logísticos hayan tenido que ser previamente congeladas, ya que nos permite desechar por completo el

medio de congelación, quedándonos solo con aquellas células útiles (viables y motiles). Además, nos permite mejorar la calidad espermática de una muestra deteriorada, desechando aquellos espermatozoides muertos, lo que podria ser de gran ayuda a la hora de mejorar el rendimiento del proceso de sex-sorting, ya que la buena calidad de la muestra de partida es un factor critico a la hora de obtener buenos rendimientos con esta técnica.

# OBJETIVOS

1. Evaluar y comparar los efectos del proceso de sex-sorting sobre la calidad espermática de muestras de ciervo rojo, obtenidas mediante electroeyaculación y sometidas al proceso de sorting pero no separadas (BSS), muestras con alta pureza de espermatozoides X (XSS) y muestras con alta pureza de espermatozoides Y (YSS) tras someterlas a un proceso de congelación-descongelación

2. Evaluar y comparar las diferencias de fertilidad en muestras espermáticas de ciervo rojo, obtenidas mediante electroeyaculación y sometidas a un proceso de sorting sin sexar (BSS) y muestras sexadas en pureza Y (YSS) tras un proceso de congelación-descongelación

3. Evaluar la susceptibilidad del estrés oxidativo inducido a muestras espermáticas de ciervo rojo, obtenidas mediante electroeyaculación y sometidas a un proceso de sorting (BSS) respecto a muestras espermáticas convencionales obtenidas también mediante electroeyaculación (NS), tras someterlas a un proceso de congelación-descongelación y determinar si el uso de 2 antioxidantes (Glutatión reducido y Trolox) puede proteger a las muestras espermáticas de dicho estrés oxidativo

4. Evaluar el efecto como herramienta suplementaria para la mejora de la calidad espermática del Androcoll-S, en muestras de ciervo rojo obtenidas mediante electroeyaculación y en muestras epididimarias obtenidas post-mortem tras ser sometidas a un proceso de congelación-descongelación

5. Evaluar en muestras espermáticas de ciervo rojo obtenidas mediante electroeyaculación, tras someterlas a un proceso de congelación-descongelación, el efecto de 2 antioxidantes (Glutatión reducido y Trolox) a 2 concentraciones (1 y 5 mM), tras someter las muestras a una incubación de 2 h a 39ºC

# METODOLOGÍA Y RESULTADOS

En el presente apartado procedemos a describir en detalle la metodología y los resultados obtenidos en cada uno de los cuatro capítulos que componen la presente tesis doctoral.

Los dos primeros capítulos se centran en evaluar el efecto del proceso de sex sorting en muestras espermáticas de ciervo rojo, así como las diferencias en la susceptibilidad de al estrés oxidativo de dichas muestras y si la adición de antioxidantes al los medios de manejo puede ayudar a prevenir los daños derivados del estrés oxidativo.

Los capítulos tercero y cuarto, por su parte se centran en evaluar el efecto de 2 herramientas para la mejora de la calidad espermática de ciervo rojo; por un lado un medio de selección espermática por centrifugación con Androcoll-S y por otro lado el uso de 2 sustancias antioxidantes (glutatión reducido y trolox) como aditivos de los medios de manejo espermático.

# CAPITULO 1

**Effect of sex-sorting and cryopreservation on sperm quality of Iberian red deer thawed spermatozoa.**

L. Anel-López[a], O. García-Álvarez[a], I. Parrilla[b], D. Del Olmo[b], A. Maroto-Morales[a], J.A. Ortiz[c], A.J. Soler[a], D.V. Alkmin[b], T. Tarantini[b], J. Roca[b], E.M. Martínez[b], J.M. Vazquez[b], J.J. Garde[a].

[a]SaBio IREC (CSIC-UCLM- JCCM), Campus Universitario s. n. 02071 Albacete, Spain

[b]Department of Animal Medicine and Surgery, University of Murcia, Murcia, Spain

[c]Medianilla S.L. Finca Las Lomas, Vejer de la Frontera, Cádiz, Spain.

**Abstract**

This work studied the effect of the sex sorting process (using the SX MoFlo, DakoCytomation Inc., Fort Collins, CO, USA) on the thawed and incubated red deer sperm, and the possible differences in several sperm parameters and fertility among sex-sorted, bulk sorted and non-sorted samples. Samples were obtained by electroejaculation from 10 mature stags during breeding season. There were 4 different experimental groups: Bulk sorted spermatozoa (submitted to the sorting process but not sexed) (BSS), samples with high purity of X-spermatozoa (XSS) and with high purity of Y-spermatozoa (YSS), and a control group consisting in non-sorted (NS) sperm. All of them were frozen in liquid nitrogen vapours. After thawing, YSS sperm did not show significantly differences (P≤0.05) for the total motility in relation with NS and bulk sorted sperm, although XSS was lower (P≤0.05) respect to the previous treatments. Total motility for all treatments was similar after 2 h of incubation at 37 ºC. The values of apoptotic spermatozoa after thawing were higher (P≤0.05) for XSS sperm than for the other types of samples. The chromatin stability (%DFI) was similar among treatments after thawing and the values increased after incubation for all treatments except for YSS sperm, being different and lower (P≤0.05) from the other treatments. Since, there were no differences between YSS and the others experimental groups for most of the sperm parameters assessed, and the parameters of DNA status were even better for YSS sperm, an artificial insemination trial was performed with YSS spermatozoa and was compared with bulk sorted sperm to assess the effect of sex-sorted in thawed sperm samples from red deer. One week before the starting of parturition, the hinds inseminated with the different treatments were housed in separate pen in a shed until the end of parturition. All calves were identified for sex at birth. The percentage of males born from YSS semen was 93%. Fertility was higher (P<0.05) for bulk sorted sperm (51%) in relation to YSS sperm (43%) and the percentage of males born was higher (93%) for this previous treatment. In conclusion, it was possible to obtain X or Y enriched sperm from red deer, although the sperm quality after thawing was different depending on the type of sample. For the sex-sorted samples, YSS sperm showed the best values for some sperm parameters, although the fertility was

lower than for bulk-sorted sperm. More studies are still needed in order to improve fertility outcomes.

Key-words: Red deer, sex-sorted sperm, insemination, X and Y-chromosomes

**1. Introduction**

Iberian red deer is a deer subspecies inhabits only Iberian Peninsula. In last years, deer farms have increased in Spain, becoming an alternative livestock production. The principal interest of this production is the trophy, so the males have the highest economic value. The possibility of breeding males only would result in a considerable cost savings and would avoid the massive slaughter of females.

The embryos production by MOET (multiple ovulation and embryo transfer) and the sex determination before transfer is a possibility to produce only embryos of desired sex. Nevertheless, the low inefficiency of this technique by the high discard of the embryos of unwanted sex limits its use. Nowadays, the only accurate and potentially cost effective to obtain the desired sex offspring is sperm sexing by flow cytometry [1]. Pre-selection of spermatozoa based on the relative DNA difference between X- and Y- chromosomes has become one of the most important reproductive technologies to improve the production in farms of mammalians [1-3].

The principal drawback of using this technique is the low fertility register with sex-sorted semen. A reason could be the low sperm number per insemination used, being needed increase it. However, some studies have showed that even increasing the number of spermatozoa in similar way than non-sexed semen the fertility did not improve [4]. Sex-sorted sperm could be damaged by the sex-sorting procedure. Through of this process, spermatozoa are exposed to some stressors such as the incubation with the fluorescent dye, high dilution rate, mechanical processing, and the subsequent passage through the electric field to be sorted [2]. Capacitation-like changes have been observed after sorting of both ram and boar spermatozoa [5-8] reducing their fertilizing lifespan in vivo [9]. Unlike capacitation status, the motility, acrosome and DNA integrity are usually high because prior sorting the cells are stained with a food

dye that discriminate the nonviable cells [10]. Nevertheless, these parameters could decrease after a period of incubation that mimics the temperature occurring in the female tract [11-13].

In addition, sex-sorted spermatozoa are often cryopreserved for logistic reasons. It has been showed that cryopreservation lead to cell damage decreasing the fertility [14]. Thus, after cryopreservation capacitation-like changes, motility impairment and oxidative damage occurs [14]. The combination of low level of antioxidant in samples highly diluted in the sex sorting procedure, with the high production of reactive oxygen species in sorted and frozen-thawed samples could be the causes of low sperm fertility in sexed samples.

Sperm sexing in conjunction with artificial insemination have been used in Sika deer obtaining lower values of fertility for X and Y spermatozoa in relation to non-sorted sperm [15]. In that studied sperm characteristics were not assessed and the reasons for the low fertility were unknown. In other study, Kjelland *et al.* [16] assessed the DNA fragmentation in sex sorted white-tailed deer sperm by comet assay, showed the sexed-sorted samples showed similar values of fragmentation of DNA to those non-sexed. Nevertheless, the study mentioned previously [15] was not implemented with the fertilization ability of sex-sorting samples, not knowing if the sex-sorted semen could be a viable alternative method for the deer industry.

For all these reasons, and due the higher demand of this type of samples in the deer industry, the objective of this study was assess the sperm quality in different steps of sex-sorting procedure and examine the fertility of sex-sorted-frozen-thawed samples.

## 2. Material and Methods

### 2.1. Reagents and media

Fluorescence probes YO-PRO-1, Hoechst33342 and Mitotracker Deep Red were purchased from Invitrogen (Barcelona, Spain), propidium iodide (PI) and PNA-FITC were acquired from Sigma (Madrid, Spain) and acridine orange (chromatographically purified) was purchased from Polysciences (Warrington,

PA, USA). Stock solutions of the fluorescence probes were: PI: 7.5 mM; PNA-FITC: 0.2 mg/mL; YO-PRO-1: 50 µM; Mitotracker Deep Red: 1 mM. All fluorescent stocks were prepared in DMSO —except for PI, Hoechst 33342 and PNA-FITC, which were prepared in water— and kept at -20 ºC and in the dark until needed with the exception of the Hoechst, which is stored at 5 ºC. The stock solution of acridine orange was prepared in water at 1 mg/mL and kept in the dark at 5 ºC. Flow cytometry equipment, software and consumables were purchased from Beckman Coulter (Fullerton, CA, USA) or Becton Dickinson (San Jose, CA, USA).

The collecting medium during sorting was a Tris-Citrate-Glucose (TCG) (pH: 7.3 and pOsm: 380 mOsm/kg) that was composed by a mixture of: glucose (250 mM), sodium citrate (12 mM), EDTA (1.6 mM), tris (0.00033 mM), lactose (5.1 mM), egg yolk at 5% (V/V) penicillin (0.7 mM), and streptomycin (1.14 mM). The washing medium of ejaculates consists in the same extender for transport with egg yolk at 2.5% (V/V). The transport medium was a Tris-Citrate-Fructose (TCF) (pH: 7.3 and pOsm: 330 mOsm/kg) that was composed by: Tris (213 mM), Citric acid monohydrate (71.83 mM), Fructose (55.51 mM), egg yolk at 20% (V/V), penicillin (0.7 mM), and streptomycin (1.14 mM). The work medium for cytometry assessment was the bovine gamete medium (BGM-3) which was composed by 87 mM NaCl, 3.1 mM KCl, 2 mM CaCl2, 0.4 mM MgCl2, 0.3 mM $NaH_2PO_4$, 40mM HEPES, 21.6 mM sodium lactate, 1 mM sodium pyruvate, 0.017 mM kanamycin, 28.22 mM phenol red and 6mg/mL BSA (pH 7.5). Solutions for SCSA (Sperm Chromatin Structure Assay) were prepared following [17]: TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4), acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.4) and acridine orange solution (0.1 M citric acid, 0.2 M Na2HPO4, 1 mM EDTA, 0.15 M NaCl, pH 6.0; acridine orange was added from the stock up to 6 µg/mL). These solutions were kept at 5 ºC in the dark.

### 2.2. Stags, ejaculate collection and sperm sample preparation

Samples were obtained from 10 mature stags during breeding season (mid-September). Animals were housed in a semi-free ranging regime at Las

Lomas Farm (Medianilla S.L., Cadiz, Spain). Animal handling and electroejaculation were performed in accordance with Spanish normative about care and use of experimentation animals (RD 53/2013) that conforms to European Union regulation 2010/63. Electroejaculation procedure was carried out as described Martínez-Pastor *et al.* [18]. Briefly, males were anesthetized with 0.75 mg/Kg of Xylacine (Rom-pun® 2%; Bayer AG, Leverkusen, Germany). The rectum was cleared of faeces and the prepucial area was shaved and washed with physiological saline serum. A three-electrode probe connected to a power source that allowed voltage and amperage control was used (P.T. Electronics, Boring, OR, USA). Probe diameter, probe length and electrode length were 3.2, 35.0 and 6.6 cm respectively. The electroejaculation regime consisted of consecutive series of 5 pulses of similar voltage and separated by 5 sec. The initial voltage was 1V that was increased in each series until a maximum of 5V. Semen was collected by fractions in graduated glass tubes. We discarded the fractions with urine contamination that were positive to Urea Test Strips (Diagnostic Systems GmbH, Holzheim, Germany). Fractions with total motility under 80% were discarded.

Semen was diluted 1:3 in TCG 2.5% egg yolk and then centrifuged at 600xg 5 minutes. The supernatant was removed and the concentration of the pellet was calculated. Sperm concentration was assessed using a hemocytometer (Bürker chamber; Brand Gmbh, Germany), after diluting the sample in a glutaraldehyde solution (5 mL of sample in 500 mL of 2% glutaraldehyde solution—29 g/L glucose monohydrate, 10 g/L sodium citrate tribasic dihydrate and 2 g/L sodium bicarbonate). Once concentration was determined sperm aliquots were individually diluted to a concentration of 800 x $10^6$ sperm/mL in TCF medium supplemented with 20% (v/v) of egg yolk and transported to the sorting facilities (about 8h at 17ºC). At its arrival to the laboratory semen samples were split in two aliquots. One of these aliquots was used for performing the control groups (non-sorted samples; NS); as it is described below, while the other one was used for sperm sorting (sorted samples; BSS, X-enriched sperm; XSS and Y-enriched sperm; YSS). Sperm samples for sorting were re-diluted to 100 x $10^6$ sperm/mL with Tris-Citrate-Glucose (0% egg yolk) medium and stained with 2.6 µL of H-42 (Stock solution:

25 mg/mL) during 50 minutes at 34ºC as described previously by Parrilla *et al.* [19].

### 2.3. Flow cytometry sperm sex sorting

Just prior to flow sorting stained sperm samples, were filtered through a 30 µM nylon mesh filter and 1µL of food dye (0.002% w/v; FD&C #40, Warner Jenkinson Company Inc., St. Louis, MO, USA) was added to each sample for quenching the fluorescence of H-42 in sperm with compromised cell membranes, allowing them to be gated out during the sorting process [20]. Spermatozoa for performing the different experimental groups described above were separated according to the Beltsville Sperm Sorting Technology method [21] using a high-speed cell sorter (SX MoFlo, DakoCytomation Inc., Fort Collins, CO, USA) modified for sperm sorting. The cell sorter was operated at 40 psi and was equipped with a UV-laser set at an output of 175 mW (Spectra Physics 1330, Terra Bella Avenue, Mountain View, California). The samples were sorted in the presence of HEPES-buffer based sheath as Buss *et al.* [22] supplemented with 0.1% of EDTA (w/v) and were collected in 50 mL tubes prefilled with 2.5 mL of collection medium (Tris-Citrate-Glucose medium containing 5% (v/v) of EY. A total of 20 x $10^6$ sorted spermatozoa were collected per tube in an approximate volume of 10 mL with a purity of ≥90%.

### 2.4 Sperm cryopreservation

Sorted spermatozoa were centrifuged at 3000-x g for 4 min at 21ºC. The supernatant was discarded, and the pellets were re-extended to 40 x $10^6$ sperm/mL using Triladyl® (Minitüb, Tiefenbach, Germany) supplemented with 20% (v/v) of EY. Then sperm samples were immersed in a programmable temperature-controlled water bath (Programmable Model 9612, PolyScience, Niles, IL, USA) and slowly cooled from 21ºC to 5 ºC over 90 minutes, and left for an equilibration time of 2h. After this period semen was packaged in 0.25 mL straws (Minitüb, Tiefenbach, Germany) and frozen in nitrogen vapours (4 cm above liquid nitrogen) for 10 min, and then plunged into the liquid nitrogen for storage.

A control group consisting in non-sorted (NS) spermatozoa frozen under the same conditions as the sorted spermatozoa was included in this experiment. For performing the control groups, semen aliquots were highly diluted gradually using HEPES-buffer based sheath fluid to 1 x $10^6$ sperm/mL in presence of collection medium, mimicking the conditions at which sorted sperm are exposed. After dilution sperm samples were stored at room temperature (21–22°C) for approximately 4 h before being processed for freezing together with the sorted samples as described above.

### 2.5. Sperm analyses

2 straws per stag and semen type (NS, BSS, XSS and YSS) were thawed and the following parameters were assessed at thawing and after an incubation for 2 h at 37°C. The objective of carrying out the incubation was evaluated the resistance of the cells in similar conditions of temperature to the reproductive system of the female.

#### 2.5.1. Motility analysis by CASA

A CASA system was used in order to assess the motility characteristics. Samples were loaded into a Makler counting chamber (10 µm depth) at 37ºC. The casa system consisted of a triocular optical phase contrast microscope (Eclipse E400; Nikon, Tokyo, Japan), equipped with a warming stage at 37 ºC and a Basler A312fc digital camera (Basler Vision Technologies, Ahrensburg, Germany). The camera was connected to a computer by an IEEE 1394 interface. Images were captured and analysed using the ISAS software v. 1.2 (Proiser, Valencia, Spain). Sampling was carried out using a X10 negative phase contrast objective (no intermediate magnification). Image sequences were saved and analysed afterwards. The standard parameter settings were: 50 frames/s; 20 to 90 µm2 for head area. For each sperm sample, the following parameters were measured: the percentage of motile spermatozoa (TM;%), velocity according to the actual path (VCL;µm/sec), straightness (STR;%), amplitude of the lateral displacement of the sperm head (ALH;µm). The total motility (TM) was defined as the proportion of spermatozoa with VCL>10 µm/s.

### 2.5.2. Sperm analyses by flow cytometry

Several physiological traits were assessed by using fluorescent probes and flow cytometry, which have been previously described for red deer [18, 23]. Samples were diluted down to 1 X $10^6$ spermatozoa/mL in BGM-3, and stained using the flourophore combinations PI/YO-PRO-1 for studying membrane permeability and viability, PI/PNA-FITC for studying the acrosomal status and YO-PRO-1/Mitotracker deep red for studying mitochondrial activity. The PI/PNA-FITC and YO-PRO-1/PI/Mitotracker deep red combinations were analysed as previously described for red deer [18,23]. Hoechst 33342 was added at 5 mM, in order to discriminate debris in both of them. Spermatozoa stained in these two solutions were incubated 10 minutes in the dark before the analysis.

Flow cytometry analyses were carried out with a CyAn ADP flow cytometer (Beckman Coulter, Brea, CA, USA), with semiconductor lasers emitting at 405 nm (violet; Hoechst 33342), 488 nm (blue; YO-PRO-1 and PI). Filters used for each fluorochrome were 450/50 (blue) for Hoechst 33342, 530/40 (green) for YO-PRO-1 and 613/20 (red) for PI. The system and event analyses were controlled using the Summit software provided with the cytometer. All the parameters were read using logarithmic amplification. For each sample, 5000 spermatozoa were recorded, saving the data in flow cytometry standard (FCS) v. 3 files. The analysis of the flow cytometry data was carried out using WEASEL v. 3 (WEHI, Melbourne, Australia). YO-PRO-1(-)/PI(-) were considered as live spermatozoa with intact plasmalemma (Live_NotApo, %), YO-PRO-1(+)/PI(-) as apoptotic spermatozoa (Apoptotic, %), YO-PRO-1(-)/Mitotracker (+) as live spermatozoa with active mitochondria (Mito_Act, %) and PI(-)/PNA-FITC(-) as live spermatozoa with intact acrosome (Live_Acro, %).

### 2.5.3. Chromatin stability

Chromatin stability was assessed following the SCSA (Sperm Chromatin Structure Assay) [17], as performed previously with epididymal samples from red deer [24]. Acridine orange (AO) fluorescence green when combined with double stranded DNA (DNAds) and red when combined with single stranded

(denatured) (DNAss). Samples were diluted in TNE buffer to a final concentration of $1x10^6$ cells/mL, and stored at -80ºC. For analysis, the samples were thawed on crushed ice and sublime to acid-induced denaturation of DNA in situ and staining with AO. A volume of 200 µL of sample was pipetted in a flow cytometry tube, and it was immediately mixed with 0.4 mL of the acid-detergent solution. After 30 s, 1.2 mL of the AO solution was added to the tube. The tube was kept on ice 3 min before flow cytometry analysis.

We analysed at least 1000 events per sample, exciting the acridine orange with the Ar-ion 488 nm laser and using a 530/30 filter for the green fluorescence of DNAds-bound acridine orange (AO), and a 650 long pass filter for the red fluorescence of DNAss-bound AO. Data was saved in saving the data in flow cytometry standard (FCS) v. 2 files, which were processed using the R statistical environment.

We calculated the DNA Fragmentation Index (DFI) for each spermatozoon as the ratio of red fluorescence respect to total fluorescence (red and green). High values of DFI, indicates chromatin abnormalities. We also calculated %DFI, as the percentage of spermatozoa with DFI >25, and High DNA Stainability (HDS) as the percentage of the spermatozoa with green fluorescence higher than channel 600 (of 1024 channels).

**2.6. Artificial insemination**

Since, males are the main interest in the production systems of Iberian red deer and there were no differences among YSS, NS and BSS sperm, we carried out a trail of artificial insemination with YSS sperm. We used BSS sperm as control of processed samples by sorting flow cytometry. Moreover, artificial inseminations with semen conventionally frozen from nine males were carried out. Semen from these males was collected by electroejaculation, diluted with Triladyl at 100 x $10^6$ spermatozoa/mL and cryopreserved as described by Anel-López *et al.* [25].

The trial was conducted in the farm Medianilla in Cádiz (Spain) during the months of September 2010, 2011, 2012 and 2013. Six hundred seventy-six Iberian deer hinds (4-6 years old; 75-101 kg live weight) were housed in outdoor enclosures that provide exposure to natural fluctuations in light and

temperature. The oestrus of hinds was synchronized as described Garde *et al.* [26]. Briefly, single controlled internal drug releasing (CIDR) devices (type G, 330 mg progesterone per device, InterAg Effective Agricultural System, Hamilton, NZ) were inserted vaginally for a total period of 12 days. The devices were replaced with a new one in each animal on day 9 to ensure that progesterone concentration remained high throughout the CIDR device insertion period. At CIDR withdrawal the hinds received 250 IU of eCG (Folligon, Intervet, Salamanca, Spain) intramuscularly. The hinds were intracervically inseminated with BSS or YSS sperm at concentration $40x10^6$ spermatozoa/mL. One week before the starting of parturition, each hind that had been inseminated was housed in separate pen in a shed until the end of parturition. All calves were identified for sex at birth. Fertility rate was assessed after delivery.

### 2.6. Statistical analysis

Data were analysed using the SAS™ V.9.1. Package (SAS Institute Inc., Cary, NC, USA). Results are shown as means and standard errors of the mean. For the statistical analyses of the sperm data linear mixed-effects models (MIXED procedure, ML method) were carried out, including sample (NS, BSS, XSS and YSS) and incubation time after thawing (0 vs. 2 hours) as fixed factors, and the males as random effect. Comparisons of means were carried out using Tukey test. A significance level of $P \leq 0.05$ was used. For the statistical analyses of sperm fertility a generalized lineal model (GLM) was carried out, including type of sperm sample (BSS vs. YSS) and male within type of semen as fixed factors and fertility as dependent variable. The number of inseminated hinds was considered as weight variable. Bonferroni test was used for the comparison of means. A significance level of $P \leq 0.05$ was used.

## 3. Results

After thawing, TM was not different ($P \leq 0.05$) among NS, BSS and YSS sperm, although for XSS sperm TM was lower (39%, $P \leq 0.05$) than NS (54%) and BSS (50%) but similar (47%, $P > 0.05$) to YSS sperm (Figure 1a). However,

there were no differences (P>0.05) among treatments for TM after incubation time, decreasing the values for this parameter with the time for all treatments (Figure 1a).

In relation to kinetics parameters, there were no significant differences between types of sperm samples after thawing and incubation for VCL and ALH (Figure 1b, d). However, STR was lower (55%, P≤0.05) for NS sperm than BSS (66%), XSS (62%) and YSS (63%) sperm, although this difference disappeared with the incubation time (Figure 1c).

For most sperm parameters assessed by flow cytometry (Live_NotApo, Mito_Act and Live_Acro), the values were similar among treatments for both times of evaluation (Figure 2a, c, d). However, the percentage of apoptotic spermatozoa was higher (17%, P≤0.05) for XSS sperm than NS (12%), BSS (13%) and YSS (14%) sperm, being similar (P>0.05) throughout the time for BSS (8%), XSS (9%) and YSS (9%) sperm (Figure 2b).

The %DFI was similar (P>0.05) among types of sperm samples after thawing (Figure 3a). After incubation, values for this parameter increased in relation to those thawed, except for YSS sperm that showed similar values (P>0.05) to sperm samples just after thawing. Moreover, the %DFI was lower (P≤0.05) for YSS sperm than the other types of sperm (Figure 3a). Likewise, the HDS was lower (P≤0.05) for YSS sperm for both evaluation times in relation to NS, BSS and XSS sperm (Figure 3b).

In spite of the quality for YSS sperm, being the best for most of the sperm parameters assessed, the fertility for this type of sample was lower (P≤0.05) than for BSS sperm (51% vs. 44%, Figure 4). In addition, these values were even lower than for semen untreated, whose fertility is around 70% (data not shown). The ninety-three and fifty five percent of calves born from YSS and BSS sperm respectively were males.

## 4. Discussion

The commercialization of sexed semen is already possible for some species such as cattle or stallion. However, for other species such as deer, this reproductive technology is not so developed.

Some researchers have showed that is possible to produce Sika and Wapiti hybrid calves after artificial insemination with low numbers of sex-sorted cryopreserved Sika sperm [15]. Nevertheless, in that study the fertility of sex-sorted samples was low in relation to non-sexed sperm, not knowing if this could be due to the low number of spermatozoa used or to the damage produced to the sperm during sex-sorting procedure.

The aim of our study was to evaluate the effect of sex sorting and cryopreservation processes on sperm quality and fertilizing ability. For this purpose we evaluated different sperm quality parameters in bulk sorted sperm (BSS), and X and Y sperm populations sorted following the sorting criteria for purities reaching at least 93% for each one (XSS and YSS). In addition, we compared the in vivo fertility rates between YSS and BSS from Iberian red deer. We showed it is possible to obtain X and Y sperm samples with a high purity rates using flow cytometry and then, using Y sperm for artificial insemination with satisfactory results. One of the many problems of carrying out sex-sorting procedure is that the flow cytometers are often away from the farms. In addition, the sexing procedure is extended for days and the spermatozoa are usually frozen. In our study, spermatozoa from red deer were able to survive through the complete process in spite of time spent and the damage inflicted by the sex-sorting and freezing procedures. It has been suggested that the damage in the sorted sperm may initially decrease due to the removal of non-viable cells. Some authors have found a high percentage of motile sperm after sex-sorting because the cells were stained with viability stains and non-viable sperm were eliminated [27].

Our results showed that the XSS sperm motility was especially affected after thawing. However, the differences among types of sperm samples disappeared after the incubation. In the same way, values for most of the kinematic parameters were similar among NS, BSS, XSS and YSS sperm after thawing and incubation, except for STR with lower values for NS sperm after thawing. Results in other studies about the effect of sex-sorting procedure on kinematics characteristics are very confusing. Some authors have reported in different species that sorting process reduced VCL and ALH [28,29]. In contrast, Parrilla *et al.* [30] found that previously mentioned parameters increased after

sorting and others authors even found no differences in motility characteristics evaluated by CASA [31]. These dissimilarities among studies could be due to the different treatments performed during the sex-sorting procedure or even to species. It is known that some species such as rams are highly resistant to sorting process, exhibiting improved motility compared with non-sorted spermatozoa [32]. In the same way, the addition of substances such as seminal plasma in the collection medium improved the motility and viability of sex-sorted bull and boar spermatozoa [33-34] in spite of the presence of seminal plasma during staining and sorting decreased the percentage of live spermatozoa [35].

In our study, VCL and ALH did not change through of incubation, indicating that spermatozoa of this species are resistant to this procedure. It has been showed in some species that capacitation-like changes occur after sex-sorting [5,8]. The kinematics parameters such as VCL and ALH, increase after capacitation [36]. In deer seems that capacitation-like changes did not happen after sex-sorting and during incubation time since these parameters did not showed changes, although it is also possible that the evaluation times were too short (0 and 2 h after thawing) to detect any effect.

In relation to sperm parameters assessed by flow cytometry, the percentage of apoptotic spermatozoa increased after sex-sorting for XSS sperm in relation to NS, BSS and YSS samples. In addition, sex-sorting samples showed higher values for this parameter than NS sperm after incubation although the values were generally much lower than initially. Nevertheless, the viability, the percentage of live spermatozoa with active mitochondria and live spermatozoa with intact acrosome were similar among types of sperm samples after thawing and after the incubation, decreasing between times for all types of samples assessed. Balao da Silva *et al.* [37] found that the percentage of intact spermatozoa from stallion was reduced after sex-sorting, while the percentage of spermatozoa showed a very early stage of apoptosis increasing. In addition, these authors located many proteins related to apoptosis in the sex-sorted stallion spermatozoa.

Regarding to %DFI, in our study the YSS samples showed lower percentage of this parameter after incubation. Moreover, the percentage of HDS was lower for that sperm samples in relation to the other types of semen, and

even the sex-sorted samples exhibited the lower values for this parameter in relation to NS. Our results agree with those by [16] who carried out a study with white-tailed deer sperm. In that study could be concluded that the sperm subpopulation obtained after sex sorting showed a greater DNA stability than conventionally prepared samples and was suggested that sorted samples had decreased concentrations of highly selected viable spermatozoa with a reduced propensity toward sperm mortality.

So far, there are few studies which have assessed the sperm characteristics from possible types of spermatozoa obtained after sex-sorting procedure and no study has been carried out in red deer. Thus, studies have been more focused on the effect of sex-sorting on sperm quality of the all sperm population, independently if they are X- or Y-spermatozoa. The differences found in this study between X- and Y- spermatozoa in several sperm parameters could be due to differences among size, shape or differentially expressed proteins between these types of spermatozoa doing them more susceptible to sex-sorting and freezing procedures. Chen *et al.* [38] showed 14 differentially expressed proteins between X- and Y-spermatozoa which may affect energy metabolism, stress resistance, composition of cytoskeleton, activity of serine proteases, binding and fusion of sperm/oocytes, and development of the zygotic embryo. In that research, was showed a low expression in X-spermatozoa of one protein, the glutathione S-transferase family (GSTM3), which may protect cells against acute toxicity attack [39] and was hypothesized that the ability of X-spermatozoa to resist and remove toxic substances may be decreased, rendering them more vulnerable to environmental changes like sex-sorting and freezing procedures. In addition, N-SMase activation associated factor (NSMAF) displayed higher expression in X sperm [38]. NSMAF can combine with tumour necrosis factor (TNF) and active neutral sphingomyelinase (N-SMase) [40], which can induce cell apoptosis [41,42]. Therefore, the X-spermatozoa may be more sensitive to processes that induce cellular apoptosis such as sex- sorting or cryopreservation procedures as was suggested by [38]. Finally, Cytochrome b-c1 complex subunit 1, mitochondrial (UQCRC1) is a component of the ubiquinol-cytochrome c reductase complex (complex III) and plays an important role in maintaining the

structural stability of complex III. However, complex I/III are major sources of ROS, and the overexpression of UQCRC1 in X-sperm, as has been showed by [38] may damage mitochondrial morphology and physiological function and induce cellular apoptosis [43].

With this background, it could be explained the higher percentage of apoptosis and %DFI in X-samples obtained in our study. This type of cells could be more vulnerable to stressors such as sex-sorting and freezing, generating more ROS and leading to cell apoptosis and DNA damage. In future, would be interesting to assess the amount of ROS generated by this type of samples. In addition, we should keep in mind that though Y-spermatozoa showed the best quality for many of parameters assessed, after artificial insemination the fertility was lower than for BSS sperm. This suggests that other sperm characteristics, which have not been evaluated in our study, and being essential to carry out the fertilization, could be compromised after sex-sorting and freezing procedures.

In conclusion, red deer spermatozoa can be sex-sorted successfully, showing the Y-spermatozoa better quality than X- spermatozoa, even sometimes higher than NS samples. Nevertheless, the fertility of Y-spermatozoa was lower than bulk sorted samples. More studies are needed in order to determine why the Y-spermatozoa, showing quality results very similar to the bulk sorted sperm, reported worse results of fertility than the bulk sorted sperm.

**References:**

[1] Johnson L. Sexing mammalian sperm for production of offspring: The state-of-the-art. Anim Reprod Sci 2000; 60:93-107.

[2] Garner DL. Flow cytometric sexing of mammalian sperm. Theriogenology 2006; 65(5):943-57.

[3] Evans G, Hollinshead F, Maxwell W. Preservation and artificial insemination of sexed semen in sheep. Reprod Fert Dev 2004; 16(4):455-64.

[4] DeJarnette JM, Leach MA, Nebel RL, Marshall CE, McCleary CR, Moreno JF. Effects of sex-sorting and sperm dosage on conception rates of Holstein heifers: Is comparable fertility of sex-sorted and conventional semen pausible? J Dairy Sci 2011; 94:3477-83.

[5] Catt SL, O'Brien JK, Maxwell WMC, Evans G. Assessment of ram and boar spermatozoa during cell-sorting by flow cytometry. Reprod Dom Anim 1997; 32:251–58.

[6] Maxwell, WMC, Welch GR, Johnson LA. Viability and membrane integrity of spermatozoa after dilution and flow cytometric sorting in the presence or absence of seminal plasma. Reprod Fertil Dev 1997; 8:1165–78.

[7] Maxwell WMC, Johnson LA. Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling or cryopreservation. Mol Reprod Dev 1997; 46:408–18.

[8] Maxwell WMC, Johnson LA. Physiology of spermatozoa at high dilution rates: the influence of seminal plasma. Theriogenology 1999; 52:1353–62.

[9] Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev 1995; 7:871–91.

[10] Maxwell WMC, Evans G, Hollinshead FK, Bathgate R, de Graaf SP, Eriksson BM, Gillan L, Morton KM, O'Brien JK. Integration of sperm sexing technology into the ART toolbox. Anim Reprod Sci 2004; (82-83):79-95.

[11] Gólsalvez J, Ramirez MA, López-Fernández C, Crespo F, Evans KM, Kjelland ME, Moreno JF. Sex-sorted bovine spermatozoa and DNA damage: I. Static features. Theriogenology 2011; 75:197-205.

[12] Gólsalvez J, Ramirez MA, López-Fernández C, Crespo F, Evans KM, Kjelland ME, Moreno JF. Sex-sorted bovine spermatozoa and DNA damage: II. Dynamic features. Theriogenology 2011; 75:206-11.

[13] Rath D, Ruiz S, Sieg B. Birth of female piglets following intrauterine insemination of a sow using flow cytometrically sexed boar semen. Vet Rec 2003; 152:400–1.

[14] Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000; (60–61):481–92.

[15] Gao QH,Wei HJ, han CM, Du HZ,Zhang ZG, Zhao WG, Zhan Y, Li S. Successful low dose insemination of flow cytometrically sorted Sika (Cervus Nippon) sperm in Wapiti (*Cervus elaphus*). Anim Reprod Sci 2010; 118:89-93.

[16] Kjelland ME, González-Marín C, Gosálvez J, López-Fernández C, Lenz RW, Evans KM, Moreno JF. DNA fragmentation kinetics and post-thaw motility of flow cytometric-sorted white-tailed deer sperm. J Anim Sci 2011; 89:3996-4006.

[17] Evenson D, Jost L. Sperm chromatin structure assay is useful for fertility assessment. Met Cell Sci 2000; 22(2):169-89.

[18] Martínez-Pastor F, Martínez F, García-Macías V, Esteso MC, Anel E, Fernández-Santos MR, Soler AJ, de Paz P, Garde JJ, Anel L. A pilot study on post-thawing quality of Iberian red deer spermatozoa (epididymal and electroejaculated) depending on glycerol concentration and extender osmolality. Theriogenology 2006; 66(5):1165-72.

[19] Parrilla I, Del Olmo D, Bisbal A, Ortiz JA, Maroto A, Garcia-Alvarez O, Roca J, Martínez EA, Garde JJ, Vázquez JM: Optimization of protocols for Iberian red deer sperm sex sorting by flow cytometry.2012;47 (Suppl. 5):102-3.

[20] Johnson LA, Welch GR, Rens W. The Beltsville sperm sexing technology: high-speed sorting gives improved sperm output for IVF and AI. J Anim Sci 1999; 77(Suppl. 2):213–20.

[21] Johnson L, Welch G. Sex preselection: High-speed flow cytometric sorting of X and Y sperm for maximum efficiency. Theriogenology 1999; 52(8):1323-41.

[22] Buss H. Verbesserung Der Tiefgefrierfähigkeit Geschlechtsspezifisch Sortierter Hengstspermien 2005. Doctoral Thesis.

[23] Martínez-Pastor F, Fernández-Santos M, Del Olmo E, Domínguez-Rebolledo A, Esteso M, Montoro V, Garde JJ. Mitochondrial activity and forward scatter vary in necrotic, apoptotic and membrane-intact spermatozoan subpopulations. Reprod Fert Dev 2008; 20(5):547-56.

[24] Martínez-Pastor F, Aisen E, Fernández-Santos MR, Esteso MC, Maroto-Morales A, García-Álvarez O, Garde JJ. Reactive oxygen species generators affect quality parameters and apoptosis markers differently in red deer spermatozoa. Reproduction 2009; 137(2):225-35.

[25] Anel-López L, García-Álvarez O, Maroto-Morales A, Iniesta-Cuerda M, Ramón M, Soler AJ, Fernández-Santos MR, Garde JJ. Reduced glutathion addition improves both the kinematics and physiological quality of post-thawed red deer sperm. Anim Reprod Sci 2015. Doi:10.1016/j.anireprosci.2015.09.012.

[26] Garde JJ, Martínez-Pastor F, Gomendio M, Malo AF, Soler AJ, Fernández-Santos MR, Esteso MC, García AJ, Anel L, Roldán ERS. The application of reproductive technologies to natural populations of red deer. Reprod Dom Anim 2006; 41 (Suppl.2) 93-102.

[27] Evans G, Hollinshead FK, Maxwell WMC. Preservation and artificial insemination of sexed semen in sheep. Reprod, Fert Dev 2004; 16:455-64.

[28] Lindsey AC, Muckle LK, Squires EL. Effect of caffeine stimulation on stallion sperm motion characteristics following 18-h storage, flow sorting, and cryopreservation. Theriogenology 2003; 59:510.

[29] Suh TK, Schenk JL. Pressure during flow sorting of bull sperm affects post-thaw motility characteristics. Theriogenology 2003; 59:516.

[30] Parrilla I, Vazquez JM, Gil MA, Caballero I, Almiñana C, Roca J, Martínez EA. Influence of storage time on functional capacity of flow cytometrically sex-sorted boar spermatozoa. Theriogenology 2005; 64:86-98.

[31] Brogliatti BR, Barreiro G, Larraburu G, Laborde A. CASA evaluation of sexed and non-sexed frozen bull semen. Reprod Fertil 2004; 16:127-28.

[32] de Graaf SP, Evans G, Maxwell WMC, Downing JA, O'Brien JK. Successful low dose insemination of flow cytometrically sorted ram spermatozoa in sheep. Reprod Dom Anim 2006; 42:648-53.

[33] Caballero I, Vazquez JM, Mayor GM, Almiñana C, Calvete JJ, Sanz L, Roca J. PSP-I/PSP-II spermadhesin exert a decapacitation effect on highly extended boar spermatozoa. Int J Androl 2009; 32(5):505-13.

[34] Garner DL. Sex-sorting mammalian sperm: concept to application in animals. J Androl 2001; 22(4):519-26.

[35] Burroughs CA, Graham JK, Lenz RW, Seidel GE Jr. Seminal plasma effect on sex-sorting bovine sperm. Theriogenology 2013; 79(3):551-7.

[36] Mortimer ST, Maxwell WM. Kinematic definition of ram sperm hyperactivation. Reprod Fertil Dev 1999; 11(1):25-30.

[37] Balao da Silva C, Ortega Ferrusola C, Morillo Rodriguez A, Gallardo Bolaños J, Plaza Dávila M, Morrell JM, Rodriguez-Martínez H, Tapia JA, Aparicio IM, Peña FJ. Sex sorting increases the permeability of the membrane of stallion spermatozoa. Anim Reprod Sci 2013; 138(3):241-51.

[38] Chen X,Zhu H, Wu C, Han W, HAo H, Zhao X, Du W, Qin T, Liu Y, Wang D. Indentification of differentially expressed proteins between bull X and Y spermatozoa. J Proteom 2012; 77:59-67.

[39] Seidegard J, Ekstrom G. The role of human glutathione transferases and epoxide hydrolases in the metabolism of xenobiotics. Environ Health Perspect 1997; 105(Suppl.4):791-9.

[40] Marchesini N, HannunYA. Acid and neutral sphingomyelinases: roles and mechanisms of regulation. Biochem Cell Biol 2004; 82(1):27-44.

[41] Ruvolo PP, Gao F, Blalock WL, Deng X, May WS. Ceramide regulates protein synthesis by a novel mechanism involving the cellular PKR activator RAX. J Biol Chem 2001; 276(15):11754-8.

[42] Ruvolo PP. Intracellular signal transduction pathways activated by ceramide and its metabolites. Pharmacol Res 2003; 47(5):383-92.

[43] Kunet T, Wang Z, Michal JJ, Daniels TF, Magnuson NS, Jiang Z. Functional UQCRC1 polymorphisms affect promoter activity and body lipid accumulation. Obesity (Silver Spring) 2007; 15(12):2896-901.

**Acknowledgements**

This work was supported by Spanish Ministry of Economy and Competitiveness (AGL2010-21487 and IPT-2012-1066-060000). García-Álvarez O and Anel-López L were supported by a fellowship of CYTEMA-UCLM and Junta de Castilla y La Mancha (PRE123/2010), respectively.

**Figure 1:**

The diagram shows the differences between treatments; non-sorted (NS), sorted (BSS) and samples with high purity of X-sperm (XSS) and of Y-sperm (YSS) after thawing and after incubation (2 h at 37ºC).

Data showed: a) TM (%): Total motility; b) VCL (µm/sec): Curvilinear velocity; c) STR (%): Straightness; d) ALH (µm): Amplitude of the lateral displacement of the sperm head

Different textures show significant differences ($P<0.05$) in the same type sample between 0 and 2 h of incubation at 37ºC. Different letters show differences ($P<0.05$) between samples at the same time of incubation (0 or 2 h).

**Figure 2:**

The diagram shows the differences between treatments; non-sorted (NS), sorted (BSS) and samples with high purity of X-sperm (XSS) and of Y-sperm (YSS) after thawing and after incubation (2 h at 37ºC).

Data showed: a) Live_NotApo (%): Live sperm; b) Apoptotic (%): Apoptotic sperm; c) Mito_act (%): Spermatozoa with active mitochondria; d) Live_Acro (%): Live sperm with intact acrosome.

Different textures show significant differences ($P<0.05$) in the same sample between 0 and 2 h of incubation at 37ºC. Different letters show differences ($P<0.05$) between samples at the same time of incubation (0 or 2 h).

**Figure 3:**

The diagram shows the differences between treatments; non-sorted (NS), sorted (BSS) and samples with high purity of X-sperm (XSS) and of Y-sperm (YSS) after thawing and after an incubation of 2 h at 37ºC.

Data showed: a) %DFI (%): DNA fragmentation index; b) HDS (%): vHigh DNA Stainability

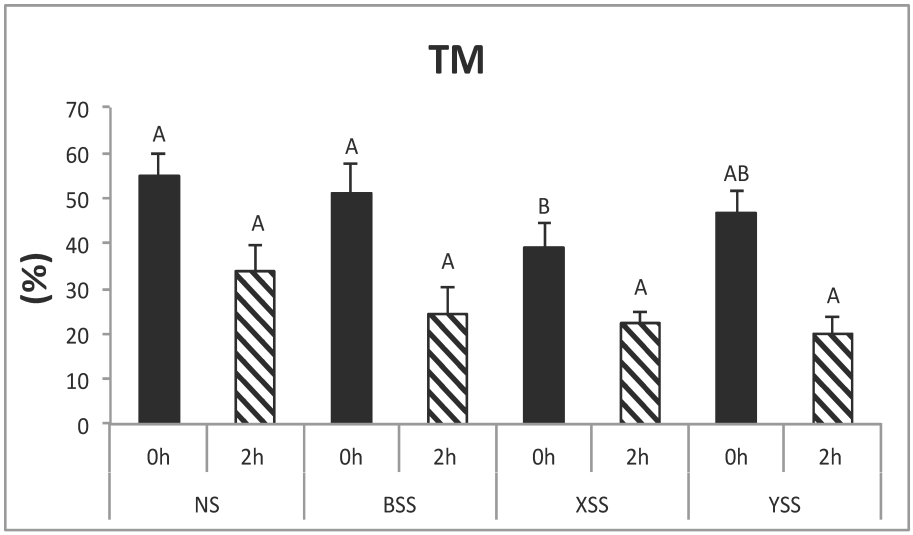
Different textures show significant differences ($P<0.05$) in the same sample between 0 and 2 h of incubation at 37ºC. Different letters show differences ($P<0.05$) between samples at the same time of incubation (0 or 2 h).

**Figure 4:**

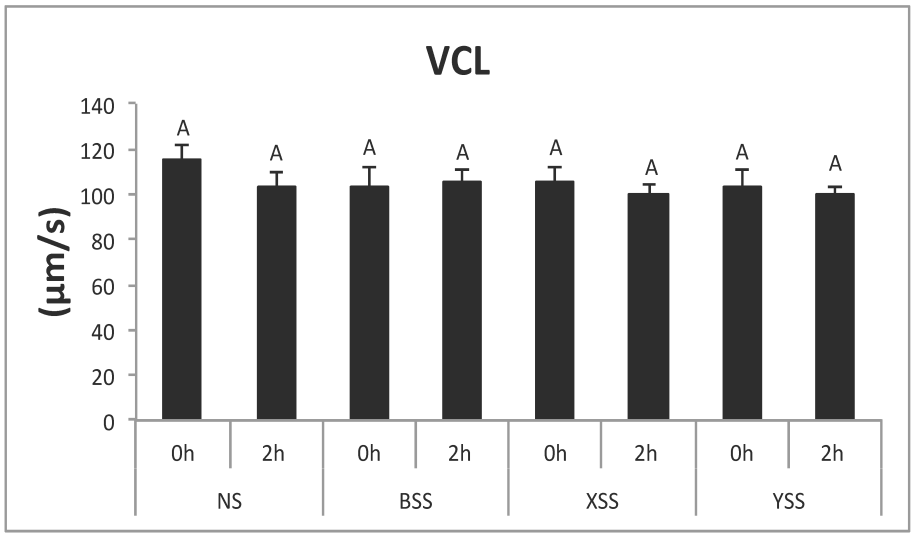
The diagram shows the percentage of fertility after delivery for different treatments: BSS: sorted samples; YSS: samples with high purity of Y-sperm. Different letters show differences ($P<0.05$) among types of sperm samples.

**Figure 1**

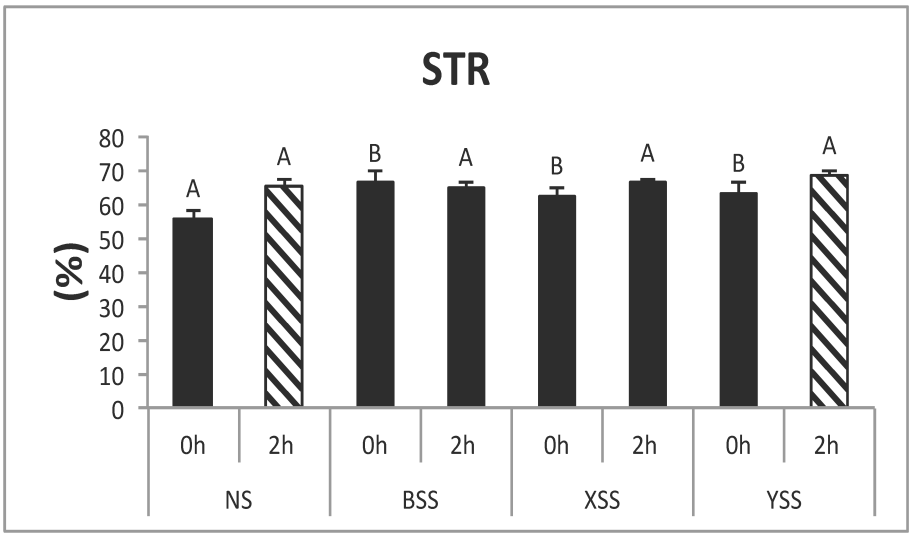
a)


TM
(%)
70
60
50
40
30
20
10
0
A
A
A
A
B
A
AB
A
0h
2h
0h
2h
0h
2h
0h
2h
NS
BSS
XSS
YSS

b)


VCL
(µm/s)
140
120
100
80
60
40
20
0
A
A
A
A
A
A
A
A
0h
2h
0h
2h
0h
2h
0h
2h
NS
BSS
XSS
YSS

c)


STR
(%)
80
70
60
50
40
30
20
10
0
A
A
B
A
B
A
B
A
0h
2h
0h
2h
0h
2h
0h
2h
NS
BSS
XSS
YSS

d)

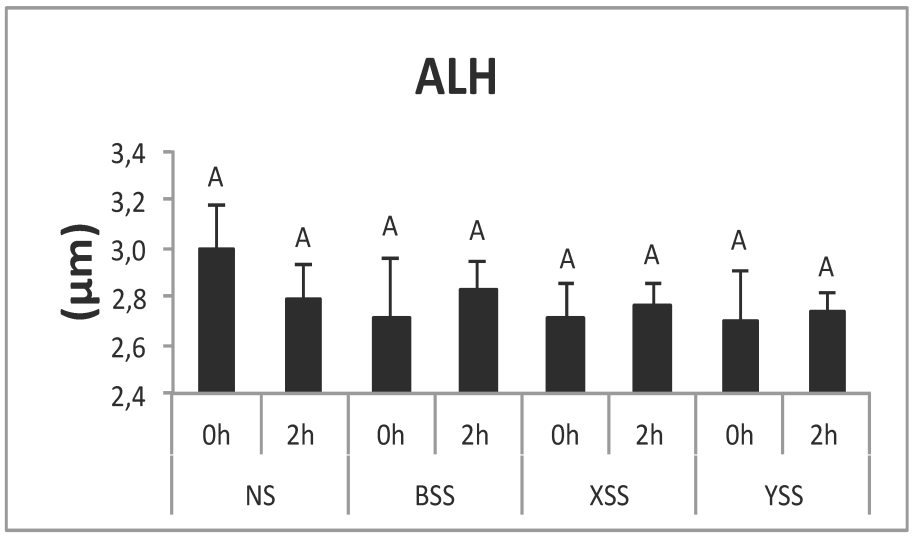
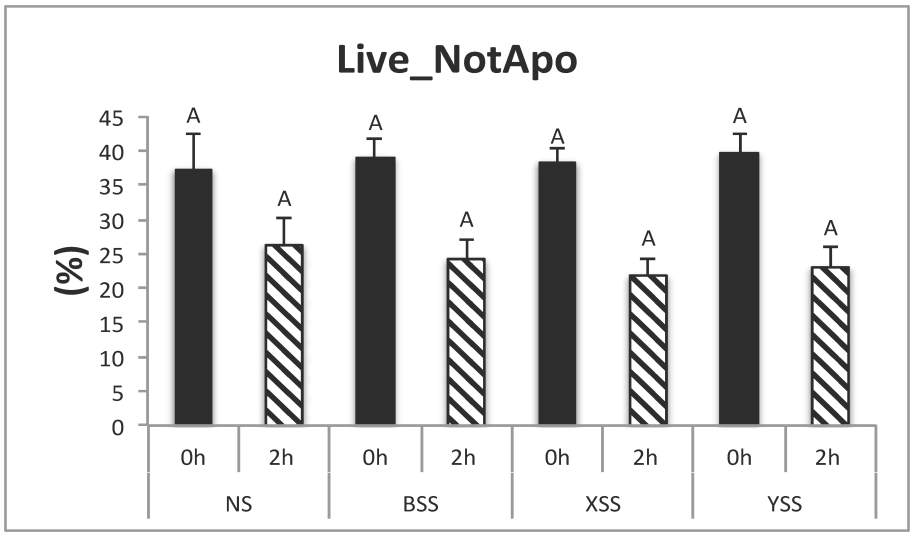
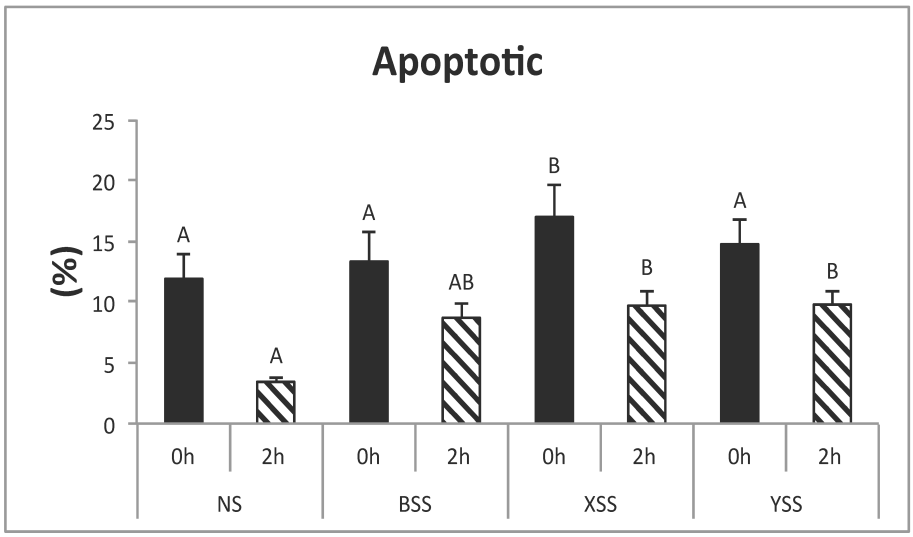

ALH
(µm)
3,4
3,2
3,0
2,8
2,6
2,4
A
A
A
A
A
A
A
A
0h
2h
0h
2h
0h
2h
0h
2h
NS
BSS
XSS
YSS

**Figure 2:**

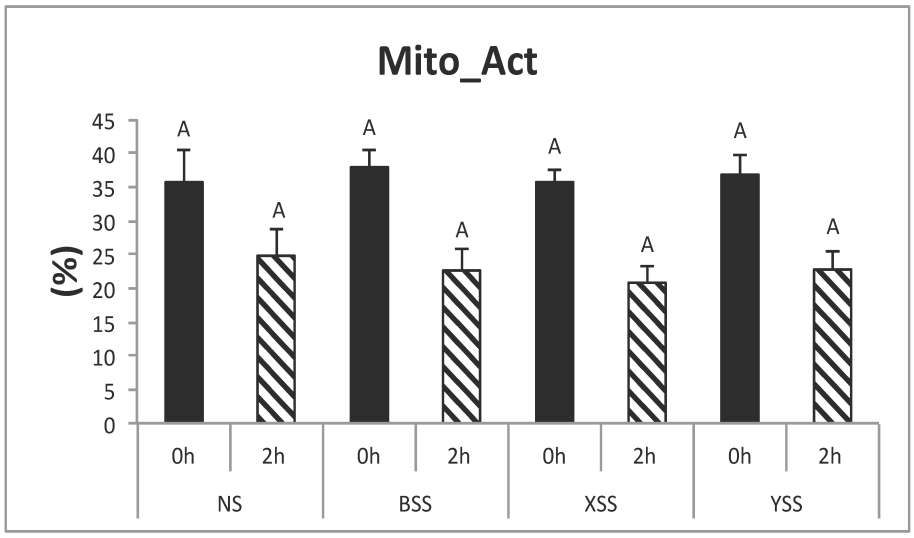
a)


Live_NotApo
(%)
45
40
35
30
25
20
15
10
5
0
A
A
A
A
A
A
A
A
0h
2h
0h
2h
0h
2h
0h
2h
NS
BSS
XSS
YSS

b)


Apoptotic
(%)
25
20
15
10
5
0
A
A
A
AB
B
B
A
B
0h
2h
0h
2h
0h
2h
0h
2h
NS
BSS
XSS
YSS

c)


Mito_Act
(%)
45
40
35
30
25
20
15
10
5
0
A
A
A
A
A
A
A
A
0h
2h
0h
2h
0h
2h
0h
2h
NS
BSS
XSS
YSS

d)

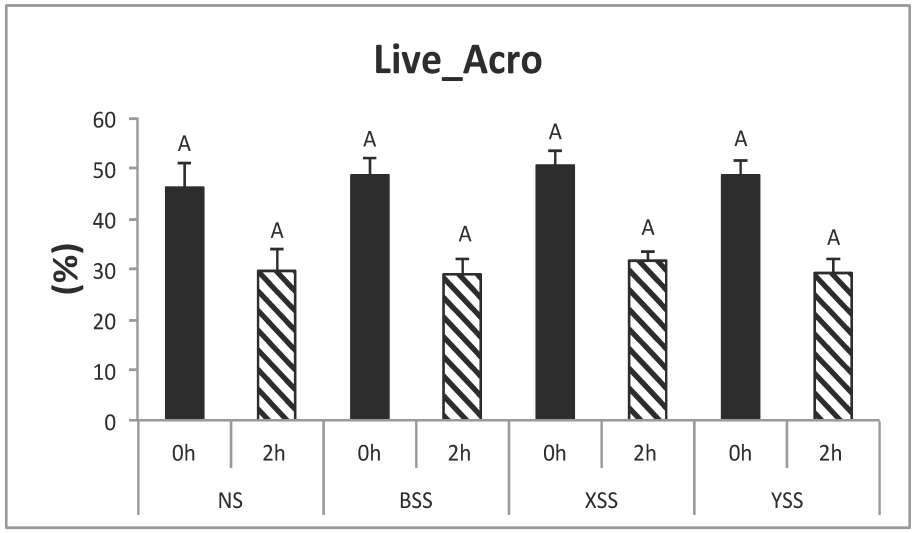
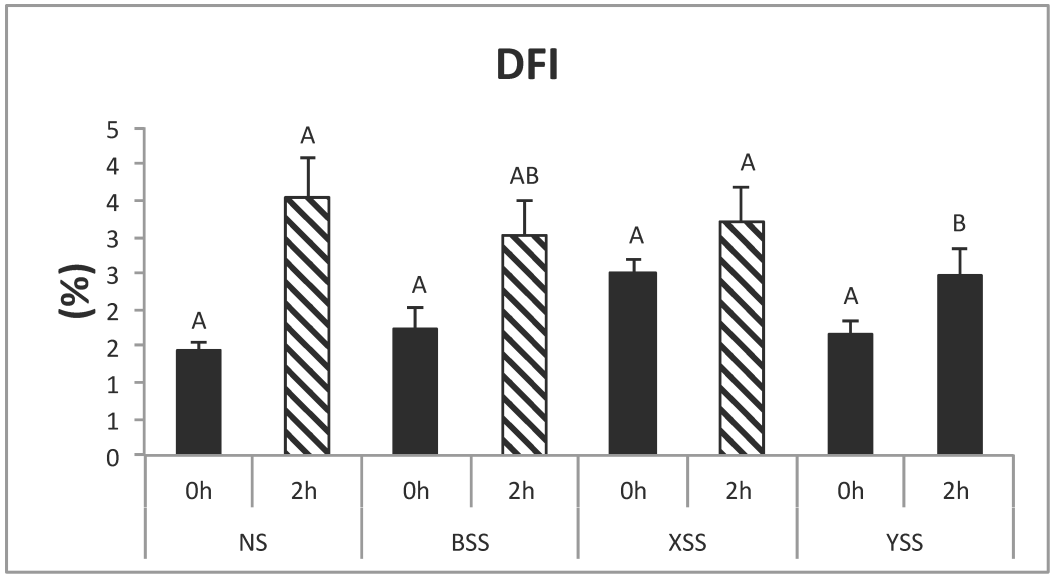

Live_Acro
(%)
60
50
40
30
20
10
0
A
A
A
A
A
A
A
A
0h
2h
0h
2h
0h
2h
0h
2h
NS
BSS
XSS
YSS

**Figure 3:**

a)


DFI
(%)
5
4
4
3
3
2
2
1
1
0
A
A
A
AB
A
A
A
B
0h
2h
0h
2h
0h
2h
0h
2h
NS
BSS
XSS
YSS

b)

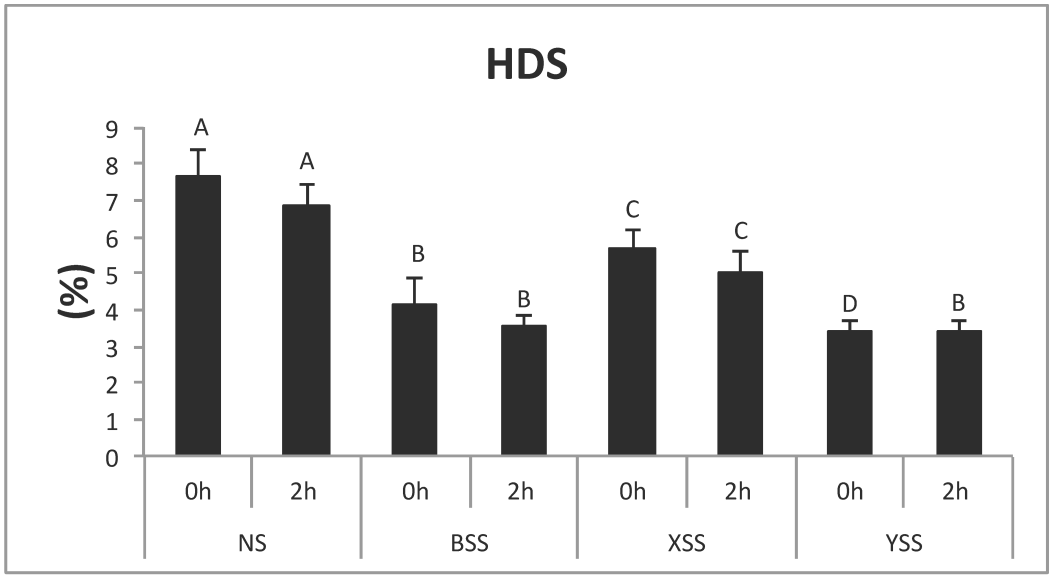
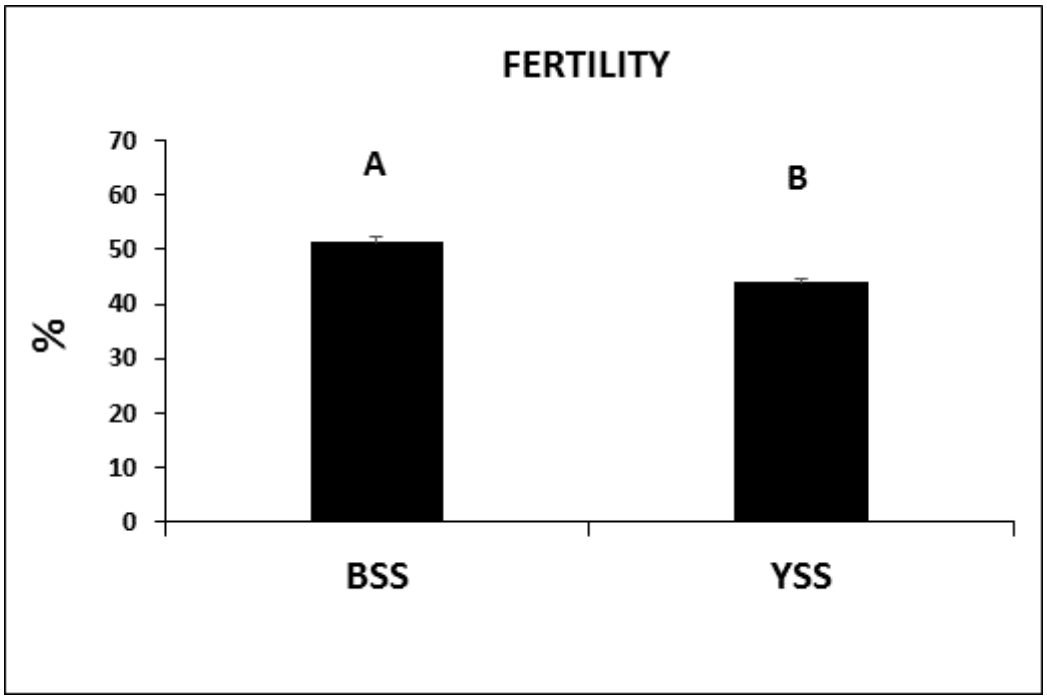

HDS
(%)
9
8
7
6
5
4
3
2
1
0
A
A
B
B
C
C
D
B
0h
2h
0h
2h
0h
2h
0h
2h
NS
BSS
XSS
YSS

**Figure 4:**



# CAPITULO 2

**The impact of oxidative stress on thawed bulk sorted red deer sperm**

L Anel-López[1], O García-Álvarez[1], I Parrilla[2], D Del Olmo[2], MR Fernández-Santos[1], AJ Soler[1], A Maroto-Morales[1], JA Ortiz[3], DV Alkmin[2], T. Tarantini[2], J. Roca[2], EA Martínez[2], JM Vazquez[2], JJ Garde[1].

[1] SaBio IREC (CSIC-UCLM- JCCM), Campus Universitario s. n. 02071 Albacete, Spain

[2] Department of Animal Medicine and Surgery, University of Murcia, Murcia, Spain

[3] Medianilla S.L. Finca Las Lomas, Vejer de la Frontera, Cádiz, Spain.

**Abstract**

The aims of this work were assessing the susceptibility to oxidative stress of sorted sperm samples and evaluating the effect of two antioxidants: reduced glutathione and trolox. Sperm samples from 3 stags were collected by electroejaculation. For each male, half of the sample was subjected to a sorting process but not sexed (Bulk sorted sperm; BSS) and then, both samples; bulk sperm (BSS) and non-sorted (NS) sperm were frozen-thawed. Susceptibility to oxidative stress was assessed in thawed samples by the addition of $H_2O_2$ ($H_2O_2$ 0 mM = H000; $H_2O_2$ 50 mM = H050; $H_2O_2$ 100 mM = H100) in the extender media during an incubation of 2 hours at 37ºC. Just after thawing, the motility of sperm samples showed a significant difference ($p < 0.05$) between both treatments, being NS (59%±3.3) better than BSS (36.9%±5.8). Moreover, the percentage of apoptotic sperm was significantly higher ($p < 0.05$) for BSS sperm (21.6%±5.0) than NS sperm (14.6%±1.2). The DNA damage was increased by the presence of $H_2O_2$ on NS sperm (H000=4.1%±0.9; H050=9.3%±0.7; and H100=10.9%±2.3), but not for BSS sperm. The motility was improved by the addition of GSH in presence of oxidant for both sperm samples (NS and BSS). These results showed that sorting process performs sublethal effects, but selects a sperm population with a more resistant chromatin to oxidative stress than non-sorted sperm. GSH at 1 mM could be a good option to maintain the quality in stressed samples, but not Trolox, which showed a high ability to inhibit sperm motility.

Keywords: Red deer, sex-sorting, cryopreservation, oxidative stress, antioxidant, reduced glutathione, trolox.

## 1. Introduction

Pre-selection of sperm based on the relative DNA difference between X- and Y- chromosomes has become one of the most important reproductive technologies to improve the production in farms of mammalians (Evans *et al.* 2004; Garner 2006). Sex sorting process by flow cytometry, is an established method that has been introduced into commercial cattle production (Garner and Seidel Jr. 2008). The use of this technology can help farmers to get an optimal proportion of males and females in their animal production system with the advantages that this entails. This becomes especially interesting in the production of red deer for hunting since, only males have trophy and therefore economic value. However, nowadays the studies carried out to know the effect of sex sorting process in this species are limited. In the same way, the study of the positive effect that the use of antioxidant could perform in sorted samples is very limited too.

Preview studies (Gosálvez *et al.* 2011) showed that after sex-sorting process, DNA damage decrease in the sperm sample due to the sorting methodology include a step that removes nonviable and non-flow orientated sperm. In addition, mammalian sperm with flattened, oval heads tend to be more readily oriented in a sperm sorter using hydrodynamics than those gametes possessing more rounded or angular shaped heads (Garner 2006). In the same way, Dean *et al.* (1978) discussed that morphologically abnormal sperm could not align properly in the flow stream, and Sun *et al.* (1998) show that high level of DNA fragmentation is increased in poor-quality sperm samples.

On the other hand, during sex sorting, sperm are exposed to some stressors such as the incubation with the fluorescent dye Hoechst 33342 at 36ºC, high dilution, mechanical processing, and the subsequent passage through the electric field to be sorted. All of these stressors may cause oxidative stress in the seminal samples. In addition, sex-sorted sperm are often cryopreserved for logistic reasons. Freeze-thaw damage has been reported to increase the sperm susceptibility to ROS in other species such as stallion (Ball *et al.* 2001) or bull (Chatterjee and Gagnon 2001). The most common reactive

oxygen species (ROS) generated by sperm are superoxide anion (O-2), hydrogen peroxide ($H_2O_2$) and hydroxyl radical (OH-), being $H_2O_2$ the most toxic ROS for sperm because of its ability to penetrate biological membranes (Aitken 1995). Oxidative stress in the sperm results in a loss of motility, membrane integrity or fertilizing capability (Aitken 1995; Aitken and Baker 2004; Domínguez-Rebolledo *et al.* 2011). In this context, antioxidants could help us to prevent theses damages. Antioxidants have an important role in maintaining the motility and the DNA integrity of sperm against oxidative stress and damage (Hughes *et al.* 1998). Extenders can be supplemented with antioxidants, before freezing (Peña *et al.* 2004; Roca *et al.* 2005; Fernández-Santos *et al.* 2007; Anel-López *et al.* 2012) or just after thawing (Fernández-Santos *et al.* 2009; Domínguez-Rebolledo *et al.* 2010), which scavenge the excess of ROS.

One of the antioxidants widely used has been the reducted glutathione (GSH). The GSH is a tripeptid distributed in living cells. It has an important role in cell protection from the noxious effect of oxidative stress, directly and as a cofactor of glutathione peroxidases (Atmaca 2004). This enzyme uses GSH to reduce hydrogen peroxide to H2O and lipoperoxides to alkyl alcohols. The addition of GSH to cryopreservation extender has had variable results in several species (Câmara *et al.* 2011; Anel-López *et al.* 2012). The supplementation with GSH to epididymal red deer sperm before freezing (Anel-López *et al.* 2012) and to electroejaculated red deer sperm after thawing (Anel-López *et al.* 2015) has been showed as a high value additive increasing the sperm quality in this species.

On the other hand, Trolox is an analogue of vitamin E with high capacity to capture free radicals (Mickle and Weisel 1993), and usually it is used such as standard to check the antioxidant capacity of others molecules (Lipovac 2000; Ronald *et al.* 2005). The supplementation of extender with TRX was showed to improve sperm motility and mitochondrial membrane integrity during post-thaw incubation in ejaculated boar sperm after thawing (Peña *et al.* 2003). Furthermore, we have demonstrated in previews studies that Trolox reduced intracellular reactive oxygen species, lipid peroxidation, and preserved membrane integrity of red deer epididymal sperm during post-thaw incubation, either with or without induced oxidative stress (Martínez-Pastor *et al.* 2008;

Martínez-Pastor *et al.* 2009) in epididymal samples. We have also reported that Trolox protected motility and viability and abolished DNA damage in samples submitted to oxidative stress after thawing and washing (Domínguez-Rebolledo *et al.* 2009). In contrast, a recent study (Anel-López *et al.* 2015) has showed that using Trolox in the millimolar range in electroejaculated red deer sperm samples as an additive after thawing has a negative effect in the motility. With this background, the aims of the present study were:

(1) Assessment of the effect of sex sorting process on red deer sperm and how this process affects sperm status after thawing.

(2) Assessment of the effect of oxidative stress on bulk sorted (BSS) and non-sorted (NS) red deer sperm induced by the action of $H_2O_2$.

(3) Determine if the use of antioxidants (GSH and TRX) at different concentrations can provide protection over the sperm against ROS damage.

## 2. Materials & Methods

### 2.1. Reagents and media

Fluorescence probe YO-PRO-1 and Hoeschst 33342 were purchased from Invitrogen (Barcelona, Spain), propidium iodide (PI) was acquired from Sigma (Madrid, Spain) and acridine orange (chromatographically purified) was purchased from Polysciences (Warrington, PA, USA). Stock solutions of the fluorescence probes were: PI: 1.5 mM; YO-PRO-1: 50 µM. YO-PRO-1 stock was prepared in DMSO. PI and Hoeschst33342 were prepared in water. PI and YO-PRO-1 were stored at -20 ºC and Hoeschst33342 was stored at 5 ºC, all of them in the dark. The stock solution of acridine orange was prepared in water at 1 mg/mL and kept in the dark at 5 ºC. Flow cytometry equipment, software and consumables were purchased from Beckman Coulter (Fullerton, CA, USA) or Becton Dickinson (San Jose, CA, USA). The stock solutions of the antioxidants were prepared at 100 mM in DMSO (Trolox) or in water (reduced glutathione, GSH) and stored at -20 ºC.

The collecting medium during sorting was a Tris-Citrate-Glucose (TCG) (pH: 7.3 and pOsm: 380 mOsm/kg) that was composed by a mixture of: glucose

(250 mM), sodium citrate (12 mM), EDTA (1.6 mM), tris (0.00033 mM), lactose (5.1 mM), egg yolk at 5% (V/V) penicillin (0.7 mM), and streptomycin (1.14 mM). The washing medium of ejaculates consists in the same extender for transport with egg yolk at 2.5% (V/V). The transport medium was a Tris-Citrate-Fructose (TCF) (pH: 7.3 and pOsm: 330 mOsm/kg) that was composed by: Tris (213 mM), Citric acid monohydrate (71.83 mM), Fructose (55.51 mM), egg yolk at 20% (V/V), penicillin (0.7 mM), and streptomycin (1.14 mM). The work medium for cytometry assessment was the bovine gamete medium (BGM-3) which was composed by 87 mM NaCl, 3.1 mM KCl, 2 mM CaCl2, 0.4 mM MgCl2, 0.3 mM $NaH_2PO_4$, 40mM HEPES, 21.6 mM sodium lactate, 1 mM sodium pyruvate, 0.017 mM kanamycin, 28.22 mM phenol red and 6mg/mL BSA (pH 7.5). Solutions for SCSA® (Sperm Chromatin Structure Assay) were prepared following Evenson and Jost (2000): TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4), acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.4) and acridine orange solution (0.1 M citric acid, 0.2 M Na2HPO4, 1 mM EDTA, 0.15 M NaCl, pH 6.0; acridine orange was added from the stock up to 6 µg/mL). These solutions were kept at 5 ºC in the dark.

**2.2. Stags, ejaculate collection and sperm sample preparation**

Samples were obtained from 3 mature stags during breeding season (mid-September). Animals were housed in a semi-free ranging regime at Las Lomas Farm (Medianilla S.L., Cadiz, Spain). Animal handling and electroejaculation were performed in accordance with Spanish Animal Protection Regulation RD53/2013 which conforms to European Union Regulation 2010/63/UE. Electroejaculation procedure was carried out as described Martínez *et al.* (2008). Males were anesthetized with Xylacine (0.75 mg/Kg) (Rompun® 2%; Bayer AG, Leverkusen, Germany). The rectum was cleared of faeces and the prepucial area was shaved and washed with physiological saline serum. A three-electrode probe connected to a power source that allowed voltage and amperage control was used (P.T. Electronics, Boring, OR, USA). Probe diameter, probe length and electrode length were 3.2, 35.0 and 6.6 cm respectively. The electroejaculation regime consisted of

consecutive series of 5 pulses of similar voltage and separated by 5 sec. the initial voltage was 1V which was increased in each series until a maximum of 5V. Semen was collected by fractions in graduated glass tubes. Sperm concentration was assessed using a hemocytometer (Bürker chamber; Brand Gmbh, Germany), after diluting the sample in a glutaraldehyde solution (5 mL of sample in 500 mL of 2% glutaraldehyde solution—29 g/L glucose monohydrate, 10 g/L sodium citrate tribasic dihydrate and 2 g/L sodium bicarbonate). We discarded the fractions with urine contamination, which were positive to Urea Test Strips (Diagnostic Systems GmbH, Holzheim, Germany). Fractions with total motility under 80% were discarded.

Semen was diluted 1:3 in TCG 2.5% egg yolk and then centrifuged at 600xg 5 minutes. The supernatant was removed and then the concentration of the pellet was calculated. Once concentration was determined sperm aliquots were individually diluted to a concentration of 800 x $10^6$ sperm/mL in TCF medium supplemented with 20% (v/v) of egg yolk and transported to the sorting facilities (about 8h at 17ºC). At its arrival to the laboratory sperm samples were split in two aliquots. One of these aliquots was used for performing the control groups (non-sorted samples; NS), as it is described below, while the other one was used for sperm sorting (bulk sorted samples; BSS). Sperm samples for sorting were re-diluted to 100 x $10^6$ sperm/mL with Tris-Citrate-Glucose (0% egg yolk) medium and stained with 2.6 µL of H-42 (Stock solution: 25 mg/mL) during 50 minutes at 34ºC as it has been previously described by Parrilla *et al.* (2012).

### 2.3. Flow cytometric sperm sex sorting

Just prior to flow sorting, stained sperm samples were filtered through a 30 µm nylon mesh filter and 1µL of food colour dye (0.002% w/v; FD&C #40, Warner Jenkinson Company Inc., St. Louis, MO, USA) was added to each sample for quenching the fluorescence of H-42 in sperm with compromised cell membranes, allowing them to be gated out during the sorting process. X and Y-chromosome-bearing sperm were separated (bulk sorting) according to the Beltsville Sperm Sorting Technology method (Johnson and Welch 1999) using a

high-speed cell sorter (SX MoFlo, DakoCytomation Inc., Fort Collins, CO, USA) modified for sperm sorting. The cell sorter was operated at 40 psi and was equipped with a UV-laser set at an output of 175 mW (Spectra Physics 1330, Terra Bella Avenue, Mountain View, California). The samples were sorted in the presence of HEPES-buffer based sheath fluid, as Buss (2005) described, supplemented with 0.1% of EDTA (w/v) and were collected in 50 mL tubes prefilled with 2.5 mL of Tris-Citrate-Glucose medium containing 5% (v/v) of EY. A total of 20 x $10^6$ of bulk sperm were collected per tube in an approximate volume of 25 mL.

**2.4 Sperm cryopreservation**

Sorted sperm were centrifuged at 3000 x g for 4 min at 21ºC. The supernatant was discarded, and the pellets were re-extended to 20 x $10^6$ sperm/mL using Triladyl® (Minitüb, Tiefenbach, Germany) supplemented with 20% (v/v) of EY. Then sperm samples were immersed in a programmable temperature-controlled water bath (Programmable Model 9612, PolyScience, Niles, IL, USA) and slowly cooled from 21ºC to 5ºC over 90 minutes, and left for an equilibration time of 2h. After this period sperm were packaged in 0.25 mL straws (Minitüb, Tiefenbach, Germany) and frozen in nitrogen vapours (4 cm above liquid nitrogen) for 10 min, and then plunged into the liquid nitrogen for storage. After 1 year of storage, two straws from each group were thawed in a circulating water bath at 37ºC for 30 s.

A control group consisting in non-sorted (NS) sperm frozen under the same conditions as the sorted sperm was included in this experiment. For performing the control groups, sperm aliquots were highly diluted gradually using HEPES-buffer based sheath fluid to 1 x $10^6$ sperm/mL in presence of collection media, mimicking the conditions at which sorted sperm are exposed. After dilution sperm samples were stored at flow cytometer room temperature (21–22°C) for approximately 4 h before being processed for freezing together with the sorted samples. Storage and thawing was performed as described for sorted samples.

### 2.5. Experimental design

After thawing, three replicates of 3 stags from the same ejaculate were used in this work. Seven straws per stag, replicate and sperm type (NS or BSS) were thawed by dropping them into a water bath with saline solution at 37ºC for 30 s. After that, straws were pooled by sperm type NS and BSS and assessed as follow:

Experiment 1: Assessment of the, effect of sex sorting process on red deer sperm just after thawing (0h) and after 2 hours of incubation at 37ºC (2h).

Experiment 2: Assessment of the susceptibility of NS and BSS sperm to oxidative stress. Just after thawing, each group (NS and BSS) was divided in three aliquots and then supplemented with $H_2O_2$ to a final concentration of 50 µM and 100 µM (H050 and H100); a sample without $H_2O_2$ was left as a control (H000). Then, the samples were submitted to an incubation of 2 h at 37 ºC and evaluated.

Experiment 3: Determination of the benefits from the addition of GSH and TRX to a final concentration of 1 and 2 mM added after thawing in BSS and NS sperm against oxidative stress. After thawing, each $H_2O_2$ group (H000, H050 and H100) was divided into 5 aliquots and then they were supplemented with GSH and Trolox respectively to a final concentration of 1 and 2 mM each one. An aliquot without any antioxidant was used as a control. Then, the samples were submitted to an incubation of 2 h at 37 ºC and evaluated.

### 2.6. Motility analysis by CASA

Motility characteristics of all sperm samples after thawing and after 2 hours of incubation at 37ºC were objectively assessed by using CASA systems. Samples were loaded into a Makler counting chamber (10 µm depth) at 37ºC. The casa system consisted of a triocular optical phase contrast microscope (Eclipse E400; Nikon, Tokyo, Japan), equipped with a warming stage at 37ºC and a Basler A312fc digital camera (Basler Vision Technologies, Ahrensburg, Germany). The camera was connected to a computer by an IEEE 1394 interface. Images were captured and analysed using the ISAS software v. 1.2 (Proiser, Valencia, Spain). Sampling was carried out using a x 10 negative

phase contrast objective (no intermediate magnification). Image sequences were saved and analysed afterwards. The standard parameter settings were: 50 frames/s; 20 to 90 μm2 for head area; VCL > 10 μm/s to classify a spermatozoon as motile. For each sperm, the software rendered the percentage of motile sperm, three velocity parameters (VCL: velocity according to the actual path; VSL: velocity according to the straight path; VAP: velocity according to the smoothed path), three track linearity parameters (LIN: linearity; STR: straightness: WOB: wobble), the ALH (the amplitude of the lateral displacement of the sperm head) and the BCF (head beat-cross frequency). We also defined total motility (TM) as the proportion of sperm with VCL>10 μm/s, and progressive motility as the proportion of sperm with VCL>25 μm/s and STR>80%.

### 2.7. Flow cytometry analyses: evaluation of sperm viability and apoptotic markers

Several physiological traits were assessed by using fluorescent probes and flow cytometry, which have been previously described for red deer (Anel-López *et al.* 2012). Samples were diluted down to $10^6$ mL-1 in BGM-3, and stained using the flourophore combinations PI/YO-PRO-1 for studying membrane permeability and viability. PI at 6 μM, YO-PRO-1 at 0.1 μM. In all cases, Hoechst 33342 was added at 5 mM, in order to discriminate debris. Sperm stained in these two solutions were incubated for 10 minutes in the dark before being analysed by flow cytometry. The sperm populations showed in this paper were: PI negative (Viability), PI negative\YO-Pro-1 positive (Apoptotic).

Flow cytometry analyses were carried out with a CyAn ADP flow cytometer (Beckman Coulter, Brea, CA, USA), with semiconductor lasers emitting at 405 nm (violet; Hoechst 33342), 488 nm (blue; YO-PRO-1 and PI). Filters used for each fluorochrome were 450/50 (blue) for Hoechst 33342, 530/40 (green) for YO-PRO-1 and 613/20 (red) for PI. The system and event analyses were controlled using the Summit software provided with the cytometer. All the parameters were read using logarithmic amplification. For each sample, 5000 sperm were recorded, saving the data in flow cytometry

standard (FCS) v. 3 files. The analysis of the flow cytometry data was carried out using WEASEL v. 3 (WEHI, Melbourne, Australia). The YO-PRO-1/PI/ Hoeschst33342 combination was analysed as previously described for red deer (Anel-López *et al.* 2012).

**2.8. Sperm chromatin structure assay**

Chromatin stability was assessed following the SCSA® (Sperm Chromatin Structure Assay), based on the susceptibility of sperm DNA to acid-induced denaturation in situ and on the subsequent staining with the metachromatic fluorescent dye acridine orange (Evenson *et al.* 2002). Acridine orange (AO) fluorescence shifts from green (dsDNA; double strand) to red (ssDNA; single strand). Samples were diluted in TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA and pH 7.4) to a final sperm concentration of 2 x $10^6$ cells/mL. Samples were frozen (-80 ºC) until needed. For analysis, the samples were thawed in crushed ice. Acid-induced denaturation of DNA in situ was achieved by adding 0.4 mL of an acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.4) to 200 µL of sample. After 30 s, the cells were stained by adding 1.2 mL of an acridine orange solution (0.1 M citric acid, 0.2 M Na2HPO4, 1 mM EDTA, 0.15 M NaCl, 6 µg/mL acridine orange, pH 6.0). The stained samples were analysed by flow cytometry exactly at 3 min after adding the acridine orange solution.

A tube with 0.4 mL of detergent-acid solution and 1.2 mL of acridine orange solution was run through the system before running any samples and between samples. For the analysis of SCSA, we used a FACScalibur flow cytometer (Becton Dickinson) and the acquisition software CellQuest v. 3. At the beginning of each session, a standard sperm sample was run through the cytometer, and settings were adjusted in order that mean fluorescence values (0-1023 linear scale) for FL-1 and FL-3 were 475 and 125, respectively. Results of the DNA denaturation test were processed to obtain the ratio of red fluorescence versus total intensity of the fluorescence (red/ [red+green] ×100), called DFI (DNA fragmentation index; formerly called αt) for each sperm, representing the shift from green to red fluorescence. High values of DFI

indicate chromatin abnormalities. Flow cytometry data was processed to obtain %DFI (% of sperm with DFI>25).

### 2.9. Statistical analysis

Data were analysed using the SAS™ V.9.1. package (SAS Institute Inc., Cary, NC, USA). Results are shown as means and standard errors of the mean. Analyses of the data were carried out using linear mixed-effects models (MIXED procedure, ML method), including sample (non-sorted vs. sorted), incubation time after thawing (0 vs. 2 hours), $H_2O_2$ concentration (0, 50 and 100 µM) and kind and concentration of antioxidant (GSH and TRX at 1 and 2 mM) as fixed factors, and the replica (pool of sperm samples) as random effect. Significant fixed effects were further analysed using multiple comparisons of means with Tukey contrasts. A significance level of $p < 0.05$ was used.

## 3. Results

**Experiment 1: effect of sex sorting process on red deer sperm just after thawing and after 2 hours of incubation at 37ºC.**

After thawing, the motility of sperm samples showed a significant difference ($p < 0.05$) with higher values for NS sperm (59%±3.3) than the BSS sperm (36.9%±5.8) (Table 1). In addition, there was a higher percentage of apoptotic sperm ($p < 0.05$) for BSS sperm (21.6%±5) than NS sperm (14.6%±1.2) after thawing (Table 1). However, viability and %DFI did not show differences (Table 1). After the incubation, the motility did not show a detrimental effect neither for NS nor for BSS sperm. Although, the viability decreased significantly ($p < 0.05$) and the %DFI increased ($p < 0.05$) over time for both sperm samples. However, viability and %DFI did not show differences between both types of samples after incubation (Table 1).

**Experiment 2: Susceptibility of non-sorted and sorted sperm to oxidative stress.**

In presence of $H_2O_2$, motility was clearly affected, showing a significant decreased for both sperm samples (Figure 1a). In contrast, $H_2O_2$ at 100 µM showed a significant positive effect ($p < 0.05$) for viability on NS sperm against absence of $H_2O_2$ (41.7%±2.4 vs. 31.6%±1.5). In presences of $H_2O_2$ viability was higher ($p < 0.05$) for NS sperm than BSS sperm (Fig. 1b) being the percentage of apoptotic cells higher in BSS sperm than NS sperm in presence of oxidant (Fig. 1c). However, the %DFI was not affected by the presence of oxidant for BSS sperm, increasing this parameter ($p < 0.05$) with the concentration of $H_2O_2$ for NS sperm (H050=9.3±0.7 and H100=10.9%±2.3 Vs. H000=4.1±0.9) (Fig 4d).

**Experiment 3: The effect of the addition of antioxidants GSH and TRX at different concentrations against oxidative stress**

The addition of GSH at concentrations of 1 and 2 mM (GSH1 and GSH2) had a beneficial effect on sperm motility in both type of sperm samples in presence of oxidant (Table 2). At H050, over NS sperm GSH1 (44.5%±4.8) and GSH2 (47.7%±6.6) kept the motility significantly higher ($p < 0.05$) than Control (21.1%±3.9), At H100 the same effect was showed (Table 2). However, the TRX at concentrations of 1 and 2 mM (TRX1 and TRX2) had not a beneficial effect on sperm motility or decreased this parameter in relation to control in samples with 50 or 100 µM of $H_2O_2$ on NS sperm (Table 2). However, the antioxidants at different concentrations did not have any effect on viability in presence of oxidant for both types of sperm samples (NS and BSS) (Table 2). The addition of GSH at 2 mM increased the percentage of apoptotic sperm at both concentrations of oxidant in BSS sperm, while TRX at 1mM decreased this value in relation to Control for this type of sperm (BSS) for both concentrations of oxidant (Table 2). Finally, the %DFI kept lower values ($p < 0.05$) by the presence of antioxidants in samples with oxidant for NS sperm (H050_GSH1 4.3%±0.6; H050_TRX1 6.2%±0.6 vs. H050_Control 9.3%±0.7). The same effect was observed in NS sperm at a concentration of 100 µM of $H_2O_2$. However,

none effect was showed in %DFI for the BSS sperm by the addition of antioxidants, with similar values to the Control (Table 2).

**4. Discussion**

The aim of this work was to assess the effect of sex-sorting process in thawed sperm samples of red deer. For that, sperm samples were sorted and then cryopreserved. Just after thawing, viability and %DFI did not show differences between sperm non-sorted (NS) and bulk sorted (BSS) sperm samples. However, the percentage of apoptotic sperm was higher for BSS sperm than NS sperm, whereas the percentage of motile sperm was significant lower for BSS than NS sperm. The increase of apoptotic sperm after sex sorting process is agreed with Balao da Silva *et al.* (2013) who showed that sex sorting process increased the permeability of the membrane of stallion sperm. It could be the reason why the sorted sperm showed a detrimental effect on motility after thawing than NS sperm and an increment in apoptotic cells. After incubation, viability was higher for NS sperm and the percentage of apoptotic cells was lower too.

In relation to oxidative stress, both sperm samples (NS and BSS) were susceptible as evidenced by the decrease of total motility in the presence of oxidant. In a previous study of our own group (Martínez-Pastor *et al.* 2009), it was found that 10 μM $H_2O_2$ did not affect the quality of thawed sperm, but 100 μM and 1 mM depressed motility within 1 h of incubation in epididymal red deer sperm. It is not know the exact mechanism by which $H_2O_2$ inhibits the motility, but it is know that it inhibits enzymes such as glucose-6-phosphate dehydrogenase (Maneesh and Jayalekshmi 2006).

Viability did not decrease in presence of $H_2O_2$. In fact, NS sperm at H100 showed a significant increase of viability. It is know that ROS are essentials factors for many metabolic pathways, so it could be that the ROS produced by the addition of $H_2O_2$ had a positive effect. In addition, similar results were reported by Leahy *et al.* (2010) who reported a beneficial effect by the addition of 45 μM $H_2O_2$ in ram thawed sperm over the proportion of viable sperm after 3

hours of incubation at 37ºC. In fact, their authors reported that it was the first report of a beneficial effect of $H_2O_2$ on the viability of sperm.

DNA damage was strongly increased by the presence of $H_2O_2$ in NS sperm samples but not in BSS. Thus, sorted sperm were more resistant to oxidative stress than NS sperm, which showed an increment of %DFI in presence of oxidant after 2 hours of incubation at 37ºC. This effect may be due to a step of sorting process which is conducted to remove the dead sperm subpopulation. Boe-Hansen *et al.* (2005) showed significant differences in the DNA damage between conventional bull sperm samples (higher) and sorted sperm samples (lower) when DNA integrity was measured using sperm chromatin structure assay (SCSA®) and neutral comet assay. The authors suggested that this effect could be linked to the sorting process by excluding non-viable sperm. Later, this fact was confirmed by Gosálvez *et al.* (2011) who demonstrated that the damaged sperm were accumulated in the sorted dead subpopulation. It is true that in our study just after thawing and after incubation NS and BSS sperm did not show significant differences in DNA damage but in presence of $H_2O_2$ the effect was so strong. Probably, the sorting process, which includes a step, that removes nonviable and non-flow orientated sperm, selected indirectly a subpopulation with a higher resistant chromatin to oxidative stress than those sperm samples non-sorted. In addition, a recent study (Aitken *et al.* 2015) has showed that capacitation and apoptosis are linked processes joined by their common dependence on the continued generation of ROS. Because of the higher number of sperm with apoptotic markers in BSS samples, we can hypothesize that the sorting process could induce some capacitation like process as it has been previously described in ram or in boar (Catt *et al.* 1997; Maxwell and Johnson 1999; Maxwell *et al.* 1998) .

In the last experiment of this study we assessed the effect of the addition of 2 antioxidants in order to prevent the damaged induced by the addition of $H_2O_2$. Viability was not affected by GSH, but apoptotic cells were increased by GSH 2mM at H050 and H100. In contrast GSH improved total motility for both types of sperm (NS and BSS) in presence of $H_2O_2$, and kept low values of %DFI for NS sperm. Probably the beneficial effect over BSS sperm did not show after incubation due to the low values of DNA damage. These good results in total

motility were in according to a preview study of our group (Anel-López *et al.* 2012) where GSH showed a beneficial effect on motility in thawed epididymal red deer sperm after 6 hours of incubation at 37ºC. In the same way, a recent study carried out in red deer sperm obtained by electroejaculation (Anel-López *et al.* 2015), the GSH supplementation after thawing has showed a protective effect, keeping higher sperm quality than those samples with no antioxidant, after an incubation of 2 h at 39ºC. However, results with GSH in other ruminants have not been so encouraging. Foote *et al.* (2002) reported in bull sperm that GSH at a concentration of 0.5 mM had some improvement in motility only after 12 h of incubation with superoxide dismutase and Tuncer *et al.* (2010) found low values of DNA fragmentation. Similarly, studies on ram sperm have yielded few positive results when using GHS (Bucak *et al.* 2008; Câmara *et al.* 2011).

On the other hand, Trolox appeared as an attractive option in order to keep the quality of deer sperm. Previews studies reported that Trolox at 1 mM greatly decrease the susceptibility of epididymal deer sperm to oxidative stress after thawing and washing (Domínguez-Rebolledo *et al.* 2009). In addition, Trolox showed a high free radical scavenging activity in red deer sperm at only 10 µM (Martínez-Pastor *et al.* 2009). In contrast, the present study showed that Trolox at the low millimolar range (1 and 2 mM) was not a good option to maintain the quality of NS and BSS sperm after incubation in absence or in presence of oxidative stress. Its main effect was to inhibit the motility of samples. These results were according with some preview studies of our group in epididymal sperm of red deer (Fernandez-Santos *et al.* 2007) and in sperm samples obtained by electroejaculation (Anel-López *et al.* 2015), which showed a detrimental effect in motility. In the opposite, TRX at 1 mM showed a protective effect in sperm membranes (lower apoptotic cells) over BSS sperm in presence of both concentrations of $H_2O_2$. Peña *et al.* (2004) found positive results cryopreserving boar sperm with Trolox at 100 and 200 µM, finding a protective effect in sperm membranes which depended on the semen fraction frozen.

GSH at 1 mM showed the best results at the study. In presence of an oxidative agent, it kept high values of motility and low values of DNA damage for both sperm types. In addition, this concentration did not perform an increase

of apoptotic cells in BSS sperm as GSH at 2 mM. In contrast, TRX at 1 and 2 mM did not show good result. It protected NS sperm against DNA damage in presence of oxidant, but total motility was inhibited by TRX in absence of oxidative stress and did not show effect in presence of them for both types of sperm samples.

In conclusion, the main findings of this work were that sorting process performs a sublethal effect, which increased the percentage of apoptotic cells, but at the same time sorting process selects a sperm population with a chromatin more resistant to be injured by oxidative stress than non-sorted sperm after incubation. GSH at 1 mM may be a good option to maintain the quality but not Trolox at these concentrations (1 and 2 mM) which showed a high ability to inhibit sperm motility. Regarding the latter, it is interesting the use of an antioxidant to reduce the negative effects of oxidative stress.

**References**

Aitken RJ, Baker MA, 2004: Oxidative stress and male reproductive biology. Reproduction, Fertility and Development 16 581-588.

Aitken RJ, 1995: Free radicals, lipid peroxidation and sperm function. Reproduction, Fertility and Development 7 659-668.

Aitken RJ, Baker MA, Nixon B, 2015: Are sperm capacitation and apoptosis the opposite ends of a continuum driven by oxidative stress? Asian J.Androl. 17 633-639.

Anel-López L, Garcia-Alvarez O, Maroto-Morales A, Iniesta-Cuerda M, Ramón M, Soler A, Fernández-Santos M, Garde J, 2015: Reduced glutathione addition improves both the kinematics and physiological quality of post-thawed red deer sperm. Anim.Reprod.Sci.

Anel-López L, Álvarez-Rodríguez M, García-Álvarez O, Álvarez M, Maroto-Morales A, Anel L, de Paz P, Garde JJ, Martínez-Pastor F, 2012: Reduced glutathione and Trolox (vitamin E) as extender supplements in cryopreservation of red deer epididymal spermatozoa. Anim.Reprod.Sci. 135 37-46.

Atmaca G, 2004: Antioxidant effects of sulfur-containing amino acids. Yonsei Med.J. 45 776-788.

Balao da Silva C, Ortega Ferrusola C, Morillo Rodriguez A, Gallardo Bolaños J, Plaza Dávila M, Morrell J, Rodriguez Martínez H, Tapia J, Aparicio I, Peña F, 2013: Sex sorting increases the permeability of the membrane of stallion spermatozoa. Anim.Reprod.Sci.

Ball BA, Vo AT, Baumber J, 2001: Generation of reactive oxygen species by equine spermatozoa. Am.J.Vet.Res. 62 508-515.

Boe-Hansen GB, Morris ID, Ersbøll AK, Greve T, Christensen P, 2005: DNA integrity in sexed bull sperm assessed by neutral Comet assay and sperm chromatin structure assay. Theriogenology 63 1789-1802.

Bucak MN, Ateşşahin A, Yüce A, 2008: Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze–thawing process. Small Ruminant Research 75 128-134.

Buss H, 2005: Verbesserung Der Tiefgefrierfähigkeit Geschlechtsspezifisch Sortierter Hengstspermien.

Câmara D, Silva S, Almeida F, Nunes J, Guerra M, 2011: Effects of antioxidants and duration of pre-freezing equilibration on frozen-thawed ram semen. Theriogenology 76 342-350.

Catt, S., O'Brien, J., Maxwell, W., Evans, G., 1997. Assessment of Ram and Boar Spermatozoa during Cell-sorting by Flow Cytometry. Reprod. Domest. Anim. 32, 251–258. doi:10.1111/j.1439-0531.1997.

Chatterjee S, Gagnon C, 2001: Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol.Reprod.Dev. 59 451-458.

Dean PN, Pinkel D, Mendelsohn ML, 1978: Hydrodynamic orientation of sperm heads for flow cytometry. Biophys.J. 23 7-13.

Domínguez-Rebolledo ÁE, Fernández-Santos MR, Bisbal A, Ros-Santaella JL, Ramón M, Carmona M, Martínez-Pastor F, Garde JJ, 2010: Improving the effect of incubation and oxidative stress on thawed spermatozoa from red deer by using different antioxidant treatments. Reproduction, Fertility and Development 22 856-870.

Domínguez-Rebolledo A, Fernández-Santos M, García-Álvarez O, Maroto-Morales A, Garde J, Martínez-Pastor F, 2009: Washing increases the susceptibility to exogenous oxidative stress in red deer spermatozoa. Theriogenology 72 1073-1084.

Domínguez‐Rebolledo A, Martínez‐Pastor F, Bisbal A, Ros‐Santaella J, García‐Álvarez O, Maroto‐Morales A, Soler A, Garde J, Fernández‐Santos M, 2011: Response of Thawed Epididymal Red Deer Spermatozoa to Increasing Concentrations of Hydrogen Peroxide, and Importance of Individual Male Variability. Reproduction in Domestic Animals 46 393-403.

Evans G, Hollinshead F, Maxwell W, 2004: Preservation and artificial insemination of sexed semen in sheep. Reproduction, Fertility and Development 16 455-464.

Evenson D, Jost L, 2000: Sperm chromatin structure assay is useful for fertility assessment. Methods in Cell Science 22 169-189.

Evenson DP, LARSON KL, Jost LK, 2002: Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J.Androl. 23 25-43.

Fernandez-Santos MR, Martínez-Pastor F, Garcia-Macias V, Esteso MC, Soler AJ, Paz P, Anel L, Garde JJ, 2007: Sperm characteristics and DNA integrity of Iberian red deer (Cervus elaphus hispanicus) epididymal spermatozoa frozen in the presence of enzymatic and nonenzymatic antioxidants. J.Androl. 28 294.

Fernández‐Santos M, Domínguez‐Rebolledo A, Esteso M, Garde J, Martínez‐Pastor F, 2009: Catalase supplementation on thawed bull spermatozoa abolishes the detrimental effect of oxidative stress on motility and DNA integrity. Int.J.Androl. 32 353-359.

Foote RH, Brockett CC, Kaproth MT, 2002: Motility and fertility of bull sperm in whole milk extender containing antioxidants. Anim.Reprod.Sci. 71 13-23.

Garner D, Seidel Jr G, 2008: History of commercializing sexed semen for cattle. Theriogenology 69 886-895.

Garner DL, 2006: Flow cytometric sexing of mammalian sperm. Theriogenology 65 943-957.

Gosálvez J, Ramirez M, López-Fernández C, Crespo F, Evans K, Kjelland M, Moreno J, 2011: Sex-sorted bovine spermatozoa and DNA damage: I. Static features. Theriogenology 75 197-205.

Hughes CM, Lewis S, McKelvey-Martin VJ, Thompson W, 1998: The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. Human Reproduction 13 1240.

Johnson L, Welch G, 1999: Sex preselection: high-speed flow cytometric sorting of X and Y sperm for maximum efficiency. Theriogenology 52 1323-1341.

Leahy T, Celi P, Bathgate R, Evans G, Maxwell W, Marti J, 2010: Flow-sorted ram spermatozoa are highly susceptible to hydrogen peroxide damage but are protected by seminal plasma and catalase. Reproduction, Fertility and Development 22 1131-1140.

Lipovac MGV, 2000: Antioxidative effect of melatonin on human spermatozoa. Systems Biology in Reproductive Medicine 44 23-27.

Maneesh M, Jayalekshmi H, 2006: Role of reactive oxygen species and antioxidants on pathophysiology of male reproduction. Indian Journal of Clinical Biochemistry 21 80-89.

Martínez A, Martínez-Pastor F, Álvarez M, Fernández-Santos M, Esteso M, De Paz P, Garde J, Anel L, 2008: Sperm parameters on Iberian red deer: Electroejaculation and post-mortem collection. Theriogenology 70 216-226.

Martínez-Pastor F, Aisen E, Fernandez-Santos MR, Esteso MC, Maroto-Morales A, Garcia-Alvarez O, Garde JJ, 2009: Reactive oxygen species generators affect quality parameters and apoptosis markers differently in red deer spermatozoa. Reproduction 137 225.

Martínez-Pastor F, Fernández-Santos M, Del Olmo E, Domínguez-Rebolledo A, Esteso M, Montoro V, Garde J, 2008: Mitochondrial activity and forward scatter vary in necrotic, apoptotic and membrane-intact spermatozoan subpopulations. Reproduction, Fertility and Development 20 547-556.

Maxwell, W.M.C., Johnson, L.A., 1999. Physiology of spermatozoa at high dilution rates: the influence of seminal plasma. Theriogenology 52, 1353–1362. doi:10.1016/S0093-691X(99)00222-8

Maxwell, W.M.C., Long, C.R., Johnson, L.A., Dobrinsky, J.R., Welch, G.R., 1998. The relationship between membrane status and fertility of boar spermatozoa after flow cytometric sorting in the presence or absence of seminal plasma. Reprod. Fertil. Dev. 10, 433. doi:10.1071/RD98102

Mickle DA, Weisel RD, 1993: Future directions of vitamin E and its analogues in minimizing myocardial ischemia-reperfusion injury. Can.J.Cardiol. 9 89-93.

Parrilla I, Del Olmo D, Bisbal A, Ortiz J, Maroto A, Garcia-Alvarez O, Roca J, Martínez E, Garde J, Vazquez J, 2012: Optimization of protocols for Iberian Red Deer sperm sex sorting by flow cytometry. 47 102-103.

Peña F, Johannisson A, Wallgren M, Rodriguez Martínez H, 2003: Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Anim.Reprod.Sci. 78 85-98.

Peña F, Johannisson A, Wallgren M, Martínez HR, 2004: Antioxidant supplementation of boar spermatozoa from different fractions of the ejaculate improves cryopreservation: changes in sperm membrane lipid architecture. Zygote 12 117-124.

Roca J, Rodriguez MJ, Gil MA, Carvajal G, Garcia EM, Cuello C, Vazquez JM, Martínez EA, 2005: Survival and in vitro fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. J.Androl. 26 15.

Ronald L, Wu X, Schaich K, 2005: Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J.Agric.Food Chem. 53 4290-4302.

Sun Ph D J, Jurisicova Ph D A, Meriano B Sc J, Casper MD RF, 1998: Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. Fertil.Steril. 69 528-532.

Tuncer PB, Bucak MN, Büyükleblebici S, Sarıözkan S, Yeni D, Eken A, Akalın PP, Kinet H, Avdatek F, Fidan AF, 2010: The effect of cysteine and glutathione on sperm and oxidative stress parameters of post-thawed bull semen. Cryobiology 61 303-307.

**Acknowledgements**

This work was supported by Spanish Ministry of Economy and Competitiveness (AGL2010-21487 and IPT-2012-1066-060000); CDTI (2008/0478-2008/0825), Spain; Seneca Foundation (GERM, 04543/07), Spain; Sexing Technologies (Texas, USA)

**García-Álvarez O and Anel-López L were supported by a fellowship of CYTEMA-UCLM and Junta de Castilla y La Mancha (PRE123/2010) respectively.**

**Table 1. Effect of sex sorting process on thawed red deer sperm.**

Data are shown as the model-derived mean ± s.e.m. Data showed: Total motility (%TM), Viability, percentage of apoptotic sperm (Apoptotic) and DNA fragmentation index (%DFI). Different letters show significant differences ($p < 0.05$) among treatments (within columns).

| SPERM | TIME AT | TM (%) | Viability (%) | Apoptotic (%) | %DFI |
|---|---|---|---|---|---|
| **NS** | **0 h** | $59\pm3,3^{A}$ | $56,5\pm1,5^{A}$ | $14,6\pm1,2^{A}$ | $2,1\pm0,1^{A}$ |
| | **2 h** | $57,5\pm4,1^{A}$ | $31,6\pm1,5^{B}$ | $3\pm0,3^{B}$ | $4,1\pm0,9^{B}$ |
| **BSS** | **0 h** | $36,9\pm5,8^{B}$ | $56,4\pm4,8^{A}$ | $21,6\pm5^{C}$ | $1,7\pm0,5^{A}$ |
| | **2 h** | $36,2\pm3,8^{B}$ | $22,5\pm1^{C}$ | $6,4\pm0,4^{B}$ | $3,2\pm0,2^{B}$ |

**Table 2. Effect of glutathione reducted (GSH) and Trolox (TRX) in non-sorted (NS) and sorted (BSS) sperm against induced oxidative stress after 2 hours of incubation at 37 ºC.**

Data are shown as the model-derived mean ± s.e.m. Data showed: Total motility (%TM), Viability, percentage of apoptotic sperm (% Apopt) and DNA fragmentation index (%DFI). For each treatment (SPERM + $H_2O_2$ + ANTIOX) an asterisk (*) means significant difference ($p < 0.05$) between the sample and its corresponding Control.

| SPERM | $H_2O_2$ | ANTIOX | (%)TM | (%)Viability | (%)Apopt | %DFI |
|---|---|---|---|---|---|---|
| **NS** | **H050** | **Control** | 21,1±3,9 | 35,6±1,6 | 2,8±0,3 | 9,3±0,7 |
| | | **GSH1** | 44,5±4,8 * | 34,1±2,5 | 2,7±0,4 | 3,4±0,6 * |
| | | **GSH2** | 47,7±6,6 * | 33,6±4,2 | 2,9±0,4 | 2,3±0,1 * |
| | | **TRX1** | 12,1±1,5 * | 35,1±1,1 | 2,4±0,2 | 6,2±0,6 * |
| | | **TRX2** | 13,2±1,8 * | 33,8±1,3 | 2,7±0,1 | 7,2±0,1 * |
| | **H100** | **Control** | 11,3±2,2 | 41,7±2,4 | 3±0,2 | 10,9±2,3 |
| | | **GSH1** | 28,4±2,2 * | 39,8±1,2 | 3±0,1 | 5,9±1,9 * |
| | | **GSH2** | 38,7±3,4 * | 36,9±1,8 | 2,6±0,2 | 5,1±1 * |
| | | **TRX1** | 5,7±1,2 | 35,2±2,5 | 3,2±0,4 | 5,8±1 * |
| | | **TRX2** | 4,5±1,7 | 35,9±3,4 | 3,3±0,3 | 5,5±0,7 * |
| **BSS** | **H050** | **Control** | 8,9±1,8 | 25,8±2,5 | 5,8±0,9 | 3,3±0,2 |
| | | **GSH1** | 33,3±8,1 * | 23,8±0,9 | 6,7±0,5 | 2,4±0,6 |
| | | **GSH2** | 31,4±2,9 * | 25,2±0,8 | 7,3±1,3 * | 1,9±0,4 |
| | | **TRX1** | 7,3±2,2 | 25±1,5 | 4,5±0,5 * | 2±0,3 |
| | | **TRX2** | 9±1,7 | 25,9±1,6 | 4,4±0,4 * | 1,8±0,2 |
| | **H100** | **Control** | 5±0,8 | 27,6±5,8 | 5,4±1,3 | 2,5±0,5 |
| | | **GSH1** | 23±2,3 * | 28,2±1 | 6,5±1,1 | 2±0,2 |
| | | **GSH2** | 28,3±0,5 * | 25,7±0,4 | 6,9±0,2 * | 1,3±0,2 |
| | | **TRX1** | 2,9±0,7 | 26,3±2,2 | 3,8±0,3 * | 1,8±0,2 |
| | | **TRX2** | 3,6±0,3 | 29,3±3 | 4,4±0,8 | 1,8±0,6 |

## Figure Legends

### Figure 1

Diagram to show the differences between different concentration of $H_2O_2$ (H050 (50 µM) and H100 (100 µM) vs. H000 (0 µM)) over non-sorted (NS) and sorted (BSS) sperm after 2 hours of incubation at 37ºC. Data showed: Total motility (%TM) Fig. 1a; Viability (%Viab) Fig.1b; % of apoptotic cells (%Apoptotic) Fig. 1c; DNA fragmentation index (%DFI) Fig. 1d. Different textures show significant differences ($p < 0.05$) between concentrations of $H_2O_2$ into sperm samples. Different capital letters show differences ($p < 0.05$) between sperm samples at the same concentration of oxidant.

**Figure 1**

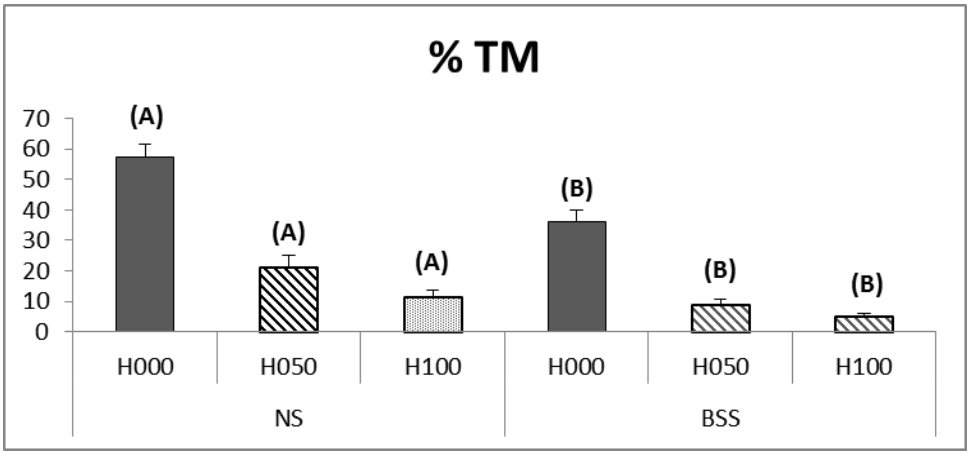



**Figure 1ª**

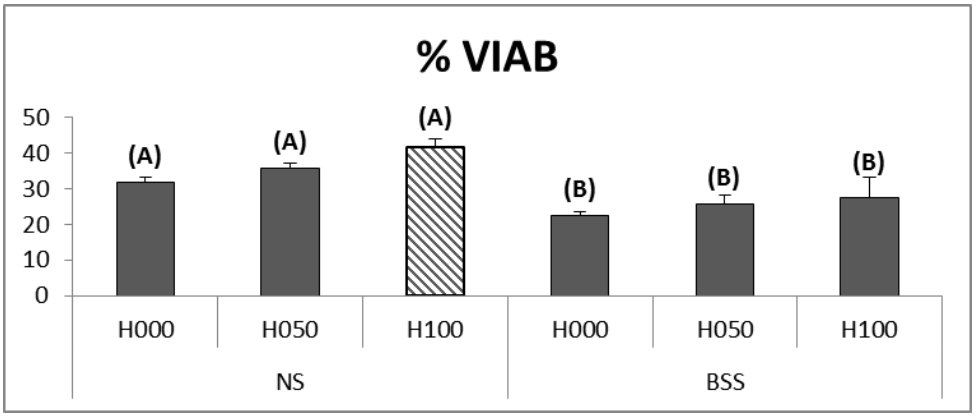



**Figure 1b**

**Figure 1**

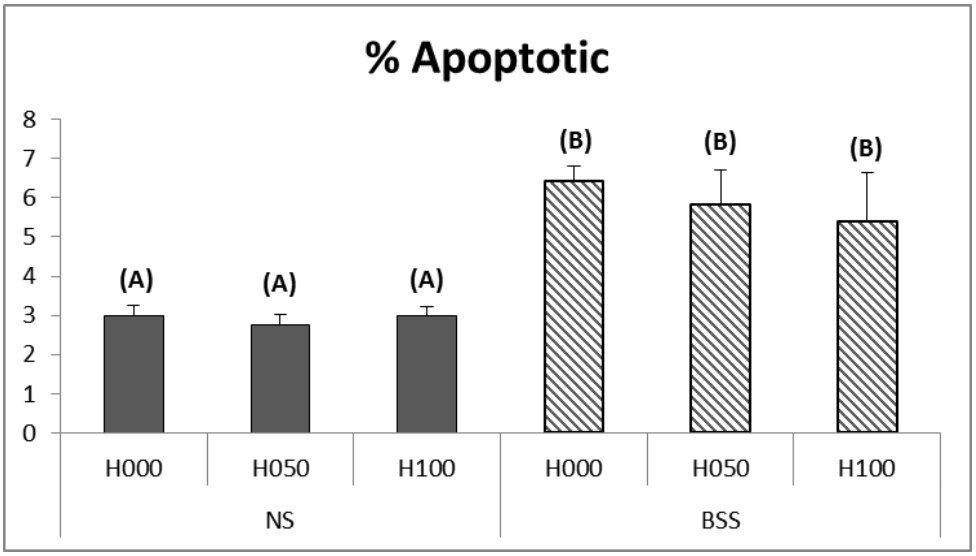



**Figure 1c**

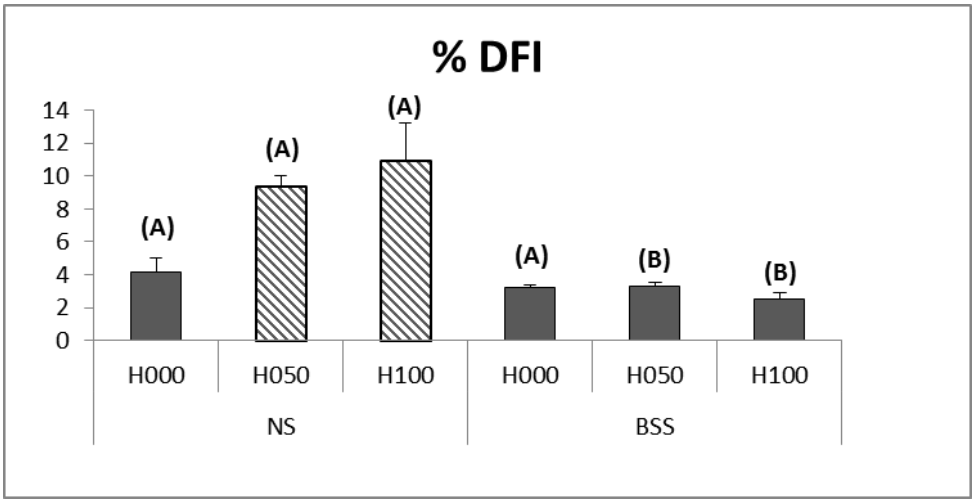



**Figure 1d**

# CAPITULO 3

**Use of Androcoll-S after thawing improves the quality of electroejaculated and epididymal sperm samples from red deer**

L. Anel-López [a], C. Martínez-Rodríguez [b], A.J. Soler [a], M.R. Fernández-Santos [a], J.J. Garde [a], J.M. Morrell [c,*]

[a] SaBio IREC (CSIC-UCLM), Campus Universitario s. n. 02071 Albacete, Spain
[b] ITRA-ULE, INDEGSAL, University of León, 24071 León, Spain
[c] Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden

**Abstract**

Single layer centrifugation is a useful technique to select sperm with good quality. The use of selection methods such as the Androcoll could become an important tool to improve the quality of sperm samples and therefore to improve other artificial reproductive techniques such as sperm sex sorting, *in-vitro fertilization* or AI. The aim of this study was to evaluate the effect of a Single Layer Centrifugation with Androcoll-S on the sperm quality on red deer sperm samples of two different origins, electroejaculated samples and epididymal samples obtained post-mortem, after thawing and after an incubation for 2h at 37ºC. Sperm motility, viability, membrane permeability, mitochondrial activity, acrosomal status and DNA fragmentation were determined for all samples. The samples selected by Androcoll-S showed an improvement in sperm kinematics compared to unselected samples after thawing and after the incubation. The same effect was observed in parameters such as viability, mitochondrial activity or acrosomal status which were improved after the selection. In contrast, no difference was found in DNA fragmentation between selected and unselected samples within the same sperm type. We conclude that sperm selection by SLC with Androcoll-S after thawing for red deer sperm of both types is a suitable technique that allows sperm quality in both kinds of sperm samples to be improved; thereby improving other assisted reproductive techniques. Further studies (IVF and in vivo fertilization) are required to determine whether this improvement can increase fertility, as has been shown for other species.

Key Words: Spermatozoa Selection, Androcoll, Sperm Centrifugation, red deer, Cryopreserved sperm.

## 1. Introduction

During the last few decades the use of artificial reproductive techniques for wild ungulates has become important not only for conservation purposes (Jabbour *et al.*, 1997, Pukazhenthi and Wildt, 2003) but also for economic purposes (deer meat, velvet and trophies), since some species are commercially important in several countries (Asher *et al.*, 1999). The increasing

use of artificial insemination (AI) with frozen-thawed sperm in red deer requires improved sperm quality in frozen samples. Cryopreservation protocols have to be adjusted to take into account the differences between samples obtained by electroejaculation and those obtained post-mortem from the epididymal cauda. Previous studies (Garcia-Macias *et al.*, 2006) assessed differences between ejaculated and epididymal samples. Moreover, some studies (Fernandez-Santos *et al.*, 2007,Domínguez-Rebolledo *et al.*, 2010, Anel-López *et al.*, 2012) were carried out to improve sperm quality by the addition of antioxidant in the extender. Since both electroejaculation and epididymal sperm samples are sources of sperm for different artificial reproductive techniques in this species, it is important to assess the effect that Single Layer Centrifugation with Androcoll has on such samples.

The use of selection methods such as Androcoll could become an important tool to improve the quality of sperm samples and therefore to improve other artificial reproductive techniques such as sperm sex sorting, *in-vitro fertilization* (IVF) or AI. During the sex sorting process, the slow speed of sorting is a significant problem due to samples with high numbers of dead sperm. The correct application of Androcoll centrifugation before the sex sorting process could improve sorting speed due to an increase in the proportion of viable sperm in some samples. In addition, the use of selection methods enables removal of all the freezing extender from the sample, allowing the use of thawed samples without contamination with cryoextender (Hollinshead *et al.*, 2004, Morton *et al.*, 2006). Thus, it is necessary to check the capability of Androcoll-S to select red deer sperm. Processing with Androcoll is easier than other methods such as discontinuous density gradient centrifugation or swim up. The use of Androcoll® with species-specific colloid formulations has been successfully reported in stallion (Macías García *et al.*, 2009), bull (Thys *et al.*, 2009) or buck (Jiménez-Rabadán *et al.*, 2012)

The aim of this study was to evaluate the effect of Androcoll on sperm quality of thawed red deer samples obtained by two collection methods; electroejaculated samples (EE) and epididymal samples obtained post-mortem (EP).

## 2. Material and methods

### 2.1. Reagents and media

Fluorescence probes YO-PRO-1 and Mitotracker Deep Red were purchased from Invitrogen (Barcelona, Spain), propidium iodide (PI) and PNA-FITC were acquired from Sigma (Madrid, Spain) and acridine orange (chromatographically purified) was purchased from Polysciences (Warrington, PA, USA). Stock solutions of the fluorescence probes were: PI: 7.5 mM; PNA-FITC: 0.2 mg/mL; YO-PRO-1: 50 µM; Mitotracker Deep Red: 1 mM. All fluorescent stocks were prepared in DMSO —except for PI and PNA-FITC, which were prepared in water— and kept in the dark at -20 ºC until needed. The stock solution of acridine orange was prepared in water at 1 mg/mL and kept in the dark at 5 ºC. Flow cytometry equipment, software and consumables were purchased from Beckman Coulter (Fullerton, CA, USA) or Becton Dickinson (San Jose, CA, USA). The stock solutions of the antioxidants were prepared at 100 mM in DMSO (Trolox) or in water (reduced glutathione, GSH) and stored at -20 ºC.

The work medium for cytometry assessment was bovine gamete medium (BGM-3) composed of 87 mM NaCl, 3.1 mM KCl, 2 mM CaCl2, 0.4 mM MgCl2, 0.3 mM NaH2PO4, 40 mM HEPES, 21.6 mM sodium lactate, 1mM sodium pyruvate, 50µg/mL kanamycin, 10µg/mL phenol red and 6mg/mL BSA (pH 7.5). Solutions for SCSA (Sperm Chromatin Structure Assay) were prepared following (Evenson and Jost, 2000): TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4), acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.4) and acridine orange solution (0.1 M citric acid, 0.2 M Na2HPO4, 1 mM EDTA, 0.15 M NaCl, pH 6.0; acridine orange was added from the stock up to 6 µg/mL). These solutions were kept at 5 ºC in the dark.

### 2.2. Ejaculate collection and cryopreservation

Samples were obtained from 9 mature stags during the breeding season (mid-September). Animals were housed in a semi-free ranging environment at Las Lomas Farm (Medianilla S.L., Cadiz, Spain). Animal handling and

electroejaculation were performed in accordance with Spanish Harvest Regulation RD 1201/2005 that conforms to European Union regulation 2010/63. The electroejaculation procedure was carried out as described (Martínez *et al.*, 2008a). Males were anesthetized with Xylacine (0.75 mg/Kg) (Rom-pun® 2 %; Bayer AG, Leverkusen, Germany). The rectum was cleared of faeces and the preputial area was shaved and washed with physiological saline solution. A three-electrode probe connected to a power source that allowed voltage and amperage control was used (P.T. Electronics, Boring, OR, USA). Probe diameter, probe length and electrode length were 3.2, 35.0 and 6.6 cm respectively. The electroejaculation regimen consisted of consecutive series of 5 pulses of similar voltage and separated by 5 sec. The initial voltage was 1V and was increased in each series up to a maximum of 5 V. Semen was collected in fractions in graduated glass tubes. Sperm concentration was assessed using a hemocytometer (Bürker chamber; Brand Gmbh, Germany), after diluting the sample in a glutaraldehyde solution (5 mL of sample in 500 mL of 2% glutaraldehyde solution—29 g/L glucose monohydrate, 10 g/L sodium citrate tribasic dihydrate and 2 g/L sodium bicarbonate). We discarded the fractions with urine contamination that were positive to Urea Test Strips (Diagnostic Systems GmbH, Holzheim, Germany). Fractions with total motility under 80% were discarded.

Seminal samples were extended to a final concentration of 100 x $10^6$ sperm/mL using Triladyl® (Minitüb, Tiefenbach, Germany) supplemented with 20% (v/v) of egg yolk (EY). Then sperm samples were immersed in a programmable temperature-controlled water bath (Programmable Model 9612, PolyScience, Niles, IL, USA) and slowly cooled from 21ºC to 5ºC over 90 minutes, and left for an equilibration time of 2h. After this period semen was packaged in 0.25 mL straws (Minitüb, Tiefenbach, Germany) and frozen in nitrogen vapour (4 cm above liquid nitrogen) for 10 min, before plunging into liquid nitrogen for storage.

### 2.3. Animals, epididymal collection and cryopreservation

For this study, we used spermatozoa recovered from the epididymides of 9 mature stags that were legally hunted in their natural habitat on the same game reserve. The harvest plan was made following Spanish Harvest Regulation, (RD 1201/2005), which conforms to European Union Regulation (86/609).

All of these stags were shot in 2012 from November to December. Immediately upon removal, testes in the scrotum were placed in plastic bags and cooled to five degrees. From two testes of each pair, spermatozoa were recovered from the epididymis by dissection. Before starting sperm collection, the superficial blood vessels of the epididymis were pierced, wiping out the blood. The collection was done from the distal portion of the epididymis by repeated longitudinal and transverse cuts with a surgical scalpel. The sperm mass was diluted 1:1 with Triladyl® 20% EY. After equilibrating for 2 h at 5ºC, samples were diluted down to a concentration of 100 x $10^6$ sperm/mL and packed in 0.25-mL French straws. Freezing was carried out using a programmable biofreezer (Kryo-10-16 II Planer TM) at -20°C/min down to -100°C and then transferred to liquid nitrogen containers.

### 2.4. Experimental design

Two straws from each male and treatment were thawed and pooled to avoid differences in the starting concentration. An aliquot of 150 µL was used as a control unselected, and another aliquot of 150 µL was layered on top of the column (1 mL of Androcol-S in a 1.5 mL Eppendorf® tube). The centrifugation was carried out at 300 x g/20 min. The resulting sperm pellet (separated sperm population) was harvested and then resuspended in a fixed amount of 50 µL of Triladyl®. Selected samples and unselected samples were assessed, then incubated for 2 h at 37ºC and assessed again. The number of spermatozoa was counted before and after SLC to determine the yield of the separated sperm population.

### 2.5. Computer-Assisted Sperm Analysis

The sample was diluted to 30 x $10^6$ spermatozoa/mL in BGM to check the motility. A warmed Makler counting chamber was loaded with 5 µL of sample. The analysis was carried out by CASA (Computer Assisted Sperm Analysis). The CASA system consisted of an optical phase-contrast microscope (Nikon Labophot-2) (and owed with negative phase-contrast objectives and a warming stage at 37ºC), a Basler A312fc camera (Basler, Germany), and a PC with the sperm Class Analyser software (ISAS v. 1.2; Proiser, Valencia, Spain). The magnification was 10X. At least five fields per sample were acquired, recording at least 200 motile sperm. Image sequences were saved and analysed afterwards. CASA acquisition parameters were 50 images acquired at an acquisition rate of 50 images per second. The following parameters were used for the study: total motility (%; TM), progressive motility (%; PM), curvilinear path velocity (µm/s; VCL), linearity (%; LIN), amplitude of the lateral movement of the head (µm; ALH), wobble (%; WOB) and beat cross frequency (Hz; BCF).

Samples were corrected and analysed using the editing facilities provided by ISAS. Spermatozoa were considered motile when VCL > 25 µm/sec. Events other than spermatozoa were removed, and settings were adjusted in each case to assure a correct track analysis.

### 2.6. Evaluation of sperm viability, acrosomal status, mitochondrial activity and apoptotic markers

Several physiological traits were assessed using fluorescent probes and flow cytometry, as described in a previous study (Anel-López *et al.*, 2012). Samples were diluted to $10^6$ $mL^{-1}$ in BGM and stained using three or four flourophore combinations. Sperm viability and acrosomal status were assessed with 100 µg/mL PNA-FITC, 6 µM PI and 5 µM Hoechst. Mitochondrial activity and apoptotic status were assessed by combining 0.1 µM YO-PRO-1, 0.1 µM Mitotracker Deep Red, 6 µM PI and 5 µM Hoechst. Spermatozoa stained in these two solutions were incubated 10 minutes in the dark before analysis with a CyAn ADP flow cytometer (Beckman Coulter, Fullerton, CA, USA).

### 2.7. Sperm chromatin structure assay

We used the SCSA technique (Evenson *et al.*, 1985) to assess chromatin stability using the metachromatic stain Acridine Orange (AO; Polysciences Inc., Warrington, PA). This dye fluoresces green when combined with double-stranded DNA, and red when combined with single-stranded DNA. Samples were diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris–HCl, 1 mM EDTA; pH 7.4) into cryotubes at a final sperm concentration of 1–2 x $10^6$ cells/mL. Samples were frozen immediately in LN2 and stored in an ultracold freezer (-80 °C) until needed. For analysis, samples were thawed on crushed ice and mixed with 40 ml of an acid-detergent solution (0.08 N HCl, 150 mM NaCl, 0.1% Triton X 100; pH 1.4). Exactly 30 s later, we added 1.20 ml of acridine orange staining solution (37 mM citric acid, 126 mM $Na_2HPO_4$, 1.1 mM disodium EDTA, 150 M NaCl; pH 6.0) containing 6 mg/ml electrophoretically purified acridine orange. The stained samples were analysed just 3 min after acridine orange staining as previously described (Garcia-Macias *et al.*, 2006). At the beginning of each session, a standard semen sample was run though the cytometer and settings were adjusted in order that mean fluorescence values (0-1023 linear scale) for FL-1 and FL-3 were 475 and 125 respectively. Results of the DNA denaturation test were processed to obtain the ratio of red fluorescence versus total intensity of the fluorescence (red/[red+green] x 100), called the DNA fragmentation index (DFI) for each spermatozoon, representing the shift from green to red fluorescence. Flow cytometry data was processed to obtain %DFI (percentage of sperm with DFI > 25).

### 2.8. Statistical analysis

Data were analysed using the SAS™ V.9.1. Package (SAS Institute Inc., Cary, NC, USA). Results are shown as means and standard errors of the mean. Analyses of the data were carried out using linear mixed-effects models (MIXED procedure, ML method), including kind of sample (electroejaculated vs. epididymal), incubation time after thawing (0 vs. 2 hours) and selection with Androcol-S as fixed factors, and males as random effect. Significant fixed

effects were further analysed using multiple comparisons of means with Tukey contrasts. A significance level of $P<0.05$ was used.

## 3. Results

### 3.1. Sperm recovery after SLC

The straws had a sperm concentration of 100 x $10^6$ $mL^{-1}$. After SLC, the recovery task was 22.8±3.7% for EE sperm, and 26.3±2.7% for EP sperm (Tab.1). No significant difference ($P>0.05$) was found between types of sample.

### 3.2. Effect of SLC on sperm kinematics

Red deer sperm samples selected with Androcol-S showed an improvement in sperm kinematics (Table 1). There was no difference between treatments in total motility after thawing. In contrast, the SLC-selected samples showed higher total motility than the corresponding control within the same treatment group, both immediately after thawing. After 2 h of incubation at 37ºC, the SLC-selected EE samples maintained higher values than unselected, although, the EP samples did not show any difference ($P>0.05$) between unselected and selected samples. The progressive motility showed the same behaviour as the total motility (Table1).

### 3.3. Effect of SLC on viability, apoptosis, mitochondrial activity and acrosomal status.

Frozen-thawed sperm prepared by colloid centrifugation showed a significant increase ($P<0.05$) in the proportioned of viable non apoptotic spermatozoa; 67.3±3.1% vs. 34.6±4.8% on EE samples and 49.2±6.6% vs 24.3±5.5% on EP samples. In addition, EE samples were significantly better than EP immediately after thawing (Table 2). After 2 h of incubation at 37ºC, both EE and EP SLC-selected samples showed higher viability than unselected samples (Table 2). Both EE and EP had similar proportions of apoptotic cells

after SLC, which were significantly lower than the unselected samples (Table 2).

The mitochondrial activity showed a significant increase just after thawing and after the SLC selection; 34.8±4.3% vs 67±4.1% on EE and 23.3±5.6% vs. 47.7±6.9% on EP, being significantly better for the EE than the EP (Table 2). After the incubation for 2 h at 37ºC, the mitochondrial activity for both types of sperm samples (EE and EP) maintained higher values selected samples than unselected samples (Table 2). The live sperm with intact acrosome showed a significant improvement for the selected samples just after thawing and after 2 hours of incubation at 37ºC vs. the unselected samples (Table 2). No differences were found between EE and EP samples.

### 3.4. Effect of SLC on sperm DNA fragmentation.

Although there were differences in %DFI between the two types of sperm samples for the same treatment, no difference was found between treatments within the same type of sample.

## 4. Discussion

The aim of this study was to assess the effect of a SLC with Androcoll-S on red deer sperm samples obtained in vivo from electroejaculation or post-mortem from the caudal epididymis, immediately after thawing and after an incubation of 2h at 37ºC. The sperm selection to improve the sperm quality of red deer could become an important tool in sperm preparation and sperm handling for different purposes. In this context, SLC with Androcoll-S becomes especially interesting because its use is much easier than other methods such as swim up or density gradients. In this specie, both *in vivo* and *post-mortem* samples have to be considered as a useful source of cells (Martínez *et al.*, 2008b) to practice the different reproductive techniques such as cryopreservation, IVF, AI or sperm sex sorting. In this context, it is interesting to assess the effect of a SLC with Androcoll-S on these two kinds of sperm samples (EE and EP) and its suitability.

In our study, the SLC using Androcoll-S® significantly improved most of the sperm parameters studied in red deer frozen-thawed sperm and this improvement was maintained after an incubation of 2h at 37ºC with respect to the unselected samples. The improvement in motility after selection was very high for both types of samples. These results are in accordance with other studies that showed an improvement on motility sperm quality after selection with Androcoll in dog (Dorado *et al.*, 2013), stallion (Macías García *et al.*, 2009), or buck (Jiménez-Rabadán *et al.*, 2012). Moreover, Martinez-Alborcia *et al.* (Martinez-Alborcia *et al.*, 2013) showed an improvement in sperm motility on boar after thawing, when the sperm were processed with Androcoll before freezing.

Previous studies showed freezing and thawing cause damage to the spermatozoa, especially in their membranes (plasma and organelle membrane), not only in red deer (Esteso *et al.*, 2003), but also in other species such as boar (Peña *et al.*, 2003b, Peña *et al.*, 2003a). The results of our study showed that the selection with Androcoll-S after thawing improved the percentage of live sperm with intact membrane and reduced the percentage of apoptotic sperm in both kinds of sample (EE and EP) compared with unselected samples. In addition, the proportion of live sperm with intact membrane in selected samples was maintained at a higher value than unselected samples after 2h of incubation at 37ºC. These results are in agreement with other studies such as Macías García *et al.* in stallion (Macías García *et al.*, 2009) where there was an increase in the population of live sperm with intact membranes and a decrease in the Yo-Pro-1+ population after the selection of thawed samples by a SLC with Androcoll. Similar results were found for Blanca Celtiberica Buck by Rabadán *et al.* (Jiménez-Rabadán *et al.*, 2012), these authors found a significant improvement in the viability when they used the SLC with Androcoll after thawing.

Mitochondria are considered as being one of the structures in the spermatozoa most sensitive to cold shock (Ortega-Ferrusola *et al.*, 2008). The mitochondrial activity was strongly improved by the selection with Androcoll-S after thawing and also maintained higher values than unselected samples after the incubation. This fact is an important finding because mitochondria are an

essential organelle of the spermatozoa. Some studies have demonstrated that the mitochondrial ribosomes are closely involved in protein translation in sperm (Gur and Breitbart, 2006). Moreover, the inhibition of protein translation could significantly reduce sperm functions such as sperm motility or sperm capacitation and thus result in a reduction of fertility. Furthermore, high mitochondrial activity has been showed as a marker for fertilizing potential in humans (Gallon *et al.*, 2006). These improvements in motility and mitochondrial activity are related between them, taking into account their strength relationship. It has showed that this kind of selection with Androcoll-S for red deer sperm samples (EE and EP) is highly related with a high motility quality, which is known to be connected with a high mitochondrial activity as (Paoli *et al.*, 2011) showed.

The same improvement was observed in the percentage of live sperm with intact acrosome after selection with Androcoll-S. In addition, selected samples maintained significantly higher values than unselected for each kind of sperm (EE and EP). The beneficial effect on this parameter showed similar values between unselected samples just after thawing and selected samples after the incubation for 2h at 37ºC.

The DNA fragmentation revealed equally low values for EE sperm and for EP sperm. No difference was found between selected and unselected samples either just after thawing or after the incubation within the same treatment (EE or EP). Jimenez-Rabadán (Jiménez-Rabadán *et al.*, 2012) showed similar results. These authors did not find any difference in %DFI when they compared unselected samples vs. selected samples in buck sperm just after thawing. Moreover, their values of %DFI were quite low. In this respect, some authors (Love, 2005, Evenson and Wixon, 2006, Didion *et al.*, 2009) have reported that %DFI thresholds that impact fertility in different species must be much higher than those obtained in our work.

After this experience, we can conclude that sperm selection by SLC with Androcoll-S after thawing for red deer sperm obtained by EE or post mortem (EP) is a suitable technique that can allow us to improve the sperm quality after thawing in both kinds of sperm samples and therefore to improve other assisted reproductive techniques. Further studies (IVF and in vivo fertilization) are

required in this specie to determine whether this improvement is also reflected in improved fertility.

**Acknowledgments**

This work was supported by Spanish ministry of science and innovation (MICINN) with the project (AGL2010-21487) and by Junta de Castilla y La Mancha (PRE123/2010) and by the Veterinary Faculty at SLU (JM Morrell).

## References

Anel-López, L., Álvarez-Rodríguez, M., García-Álvarez, O., Álvarez, M., Maroto-Morales, A., Anel, L., de Paz, P., Garde, J.J., Martínez-Pastor, F., 2012. Reduced glutathione and Trolox (vitamin E) as extender supplements in cryopreservation of red deer epididymal spermatozoa. Anim. Reprod. Sci. 135, 1-4, 37-46.

Asher, G.W., Monfort, S.L., Wemmer, C., 1999. Comparative reproductive function in cervids: implications for management of farm and zoo populations. J. Reprod. Fertil. Suppl. 54, 143-156.

Didion, B.A., Kasperson, K.M., Wixon, R.L., Evenson, D.P., 2009. Boar fertility and sperm chromatin structure status: a retrospective report. J. Androl. 30, 655-660.

Domínguez-Rebolledo, Á.E., Fernández-Santos, M.R., Bisbal, A., Ros-Santaella, J.L., Ramón, M., Carmona, M., Martínez-Pastor, F., Garde, J.J., 2010. Improving the effect of incubation and oxidative stress on thawed spermatozoa from red deer by using different antioxidant treatments. Reproduction, Fertility and Development 22, 856-870.

Dorado, J., Gálvez, M., Morrell, J., Alcaráz, L., Hidalgo, M., 2013. Use of single-layer centrifugation with Androcoll-C to enhance sperm quality in frozen-thawed dog semen. Theriogenology 80, 955-962.

Esteso, M.C., Fernández-Santos, M.R., Soler, A.J., Garde, J.J., 2003. Head dimensions of cryopreserved red deer spermatozoa are affected by thawing procedure. CryoLetters 24, 260-267.

Evenson, D., Jost, L., 2000. Sperm chromatin structure assay is useful for fertility assessment. Methods in Cell Science 22, 169-189.

Evenson, D.P., Higgins, P.J., Grueneberg, D., Ballachey, B.E., 1985. Flow cytometric analysis of mouse spermatogenic function following exposure to ethylnitrosourea. Cytometry 6, 238-253.

Evenson, D.P., Wixon, R., 2006. Clinical aspects of sperm DNA fragmentation detection and male infertility. Theriogenology 65, 979-991.

Fernandez-Santos, M.R., Martinez-Pastor, F., Garcia-Macias, V., Esteso, M.C., Soler, A.J., Paz, P., Anel, L., Garde, J.J., 2007. Sperm characteristics and DNA integrity of Iberian red deer (Cervus elaphus hispanicus) epididymal spermatozoa frozen in the presence of enzymatic and nonenzymatic antioxidants. J. Androl. 28, 294.

Gallon, F., Marchetti, C., Jouy, N., Marchetti, P., 2006. The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability. Fertil. Steril. 86, 1526-1530.

Garcia-Macias, V., Martinez-Pastor, F., Alvarez, M., Garde, J.J., Anel, E., Anel, L., de Paz, P., 2006. Assessment of chromatin status (SCSA®) in epididymal and ejaculated sperm in Iberian red deer, ram and domestic dog. Theriogenology 66, 1921-1930.

Gur, Y., Breitbart, H., 2006. Mammalian sperm translate nuclear-encoded proteins by mitochondrial-type ribosomes. Genes Dev. 20, 411-416.

Hollinshead, F.K., Evans, G., Evans, K.M., Catt, S.L., Maxwell, W.M., O'Brien, J.K., 2004. Birth of lambs of a pre-determined sex after in vitro production of embryos using frozen-thawed sex-sorted and re-frozen-thawed ram spermatozoa. Reproduction 127, 557-568.

Jabbour, H., Hayssen, V., Bruford, M., 1997. Conservation of deer: contributions from molecular biology, evolutionary ecology, and reproductive physiology. J. Zool. 243, 461-484.

Jiménez-Rabadán, P., Morrell, J., Johannisson, A., Ramón, M., García-Álvarez, O., Maroto-Morales, A., Álvaro-García, P., Pérez-Guzmán, M., Fernández-Santos, M., Garde, J., 2012. Single layer centrifugation (SLC) improves sperm quality of cryopreserved Blanca-Celtibérica buck semen. Anim. Reprod. Sci. 136, 47-54.

Love, C.C., 2005. The sperm chromatin structure assay: a review of clinical applications. Anim. Reprod. Sci. 89, 39-45.

Macías García, B., Morrell, J., Ortega-Ferrusola, C., González-Fernández, L., Tapia, J., Rodriguez-Martinez, H., Peña, F., 2009. Centrifugation on a single layer of colloid selects improved quality spermatozoa from frozen-thawed stallion semen. Anim. Reprod. Sci. 114, 193-202.

Martínez, A.F., Martínez-Pastor, F., Álvarez, M., Fernández-Santos, M.R., Esteso, M.C., de Paz, P., Garde, J., Anel, L., 2008a. Sperm parameters on Iberian red deer: electroejaculation and post-mortem collection. Theriogenology 70, 216-226.

Martinez-Alborcia, M.J., Morrell, J.M., Gil, M.A., Barranco, I., Maside, C., Alkmin, D.V., Parrilla, I., Martinez, E.A., Roca, J., 2013. Suitability and effectiveness of single layer centrifugation using Androcoll-P in the cryopreservation protocol for boar spermatozoa. Anim. Reprod. Sci. 140, 173-179.

Morton, K.M., Rowe, A.M., Chis Maxwell, W., Evans, G., 2006. In vitro and in vivo survival of bisected sheep embryos derived from frozen-thawed unsorted, and frozen-thawed sex-sorted and refrozen-thawed ram spermatozoa. Theriogenology 65, 1333-1345.

Ortega-Ferrusola, C., Sotillo-Galán, Y., Varela-Fernández, E., Gallardo-Bolaños, J., Muriel, A., González-Fernández, L., Tapia, J., Pena, F., 2008. Detection of “Apoptosis-Like” Changes During the Cryopreservation Process in Equine Sperm. J. Androl. 29, 213-221.

Paoli, D., Gallo, M., Rizzo, F., Baldi, E., Francavilla, S., Lenzi, A., Lombardo, F., Gandini, L., 2011. Mitochondrial membrane potential profile and its correlation with increasing sperm motility. Fertil. Steril. 95, 2315-2319.

Peña, F.J., Johannisson, A., Wallgren, M., Rodríguez-Martínez, H., 2003a. Assessment of fresh and frozen–thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity. Theriogenology 60, 677-689.

Peña, F., Johannisson, A., Wallgren, M., Rodriguez Martinez, H., 2003b. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Anim. Reprod. Sci. 78, 85-98.

Pukazhenthi, B.S., Wildt, D.E., 2003. Which reproductive technologies are most relevant to studying, managing and conserving wildlife? Reproduction, Fertility and Development 16, 33-46.

Thys, M., Vandaele, L., Morrell, J.M., Mestach, J., Van Soom, A., Hoogewijs, M., Rodriguez-Martinez, H., 2009. In vitro Fertilizing Capacity of Frozen-thawed Bull Spermatozoa Selected by Single-layer (Glycidoxypropyltrimethoxysilane) Silane-coated Silica Colloidal Centrifugation. Reproduction in Domestic Animals 44, 390-394.

**Figure Legend**

**Figure 1.**

DNA fragmentation index (%DFI) in selected (SLC) and unselected (Unsel) samples for electroejaculated samples (EE) and epididymal samples (EP) just after thawing and after an incubation of 2h at 37ºC.

**Table 1. Effect of single-layer centrifugation (SLC) on sperm motility of electroejaculated (EE) and epididymal samples (EP) from red deer spermatozoa.**

Treatments: unselected samples 0 and 2 h of incubation at 37ºC (Unsel_0 and Unsel_2), selected samples 0 and 2 h of incubation (SLC_0 and SLC_2). Data are shown as the model-derived mean ± s.e.m.

| Sperm | Treatment_Time | Yield | | %TM | | %PM | |
|---|---|---|---|---|---|---|---|
| EE | Unsel_0 | | | 26.9±3.8 | a,A | 8.8±1.8 | a,A |
| | Unsel_2 | | | 5.8±2.2 | b,A | 0.9±0.4 | b,A |
| | SLC_0 | 22.8±3.7 | A | 38.8±5.6 | c,A | 12.1±3.6 | c,A |
| | SLC_2 | | | 21.8±4.4 | a,A | 5.8±1.2 | a,A |
| EP | Unsel_0 | | | 22.9±4.5 | a,A | 0.9±0.2 | a,B |
| | Unsel_2 | | | 15.9±4.1 | a,A | 1.1±0.3 | a,A |
| | SLC_0 | 26.3±2.7 | A | 34.2±5.7 | b,A | 4.4±0.9 | b,B |
| | SLC_2 | | | 17.6±5.4 | a,A | 1±0.4 | a,A |

Lower case letters show differences between treatments for the same kind of sperm. Capital letters show differences for the same treatment between kinds of sample.

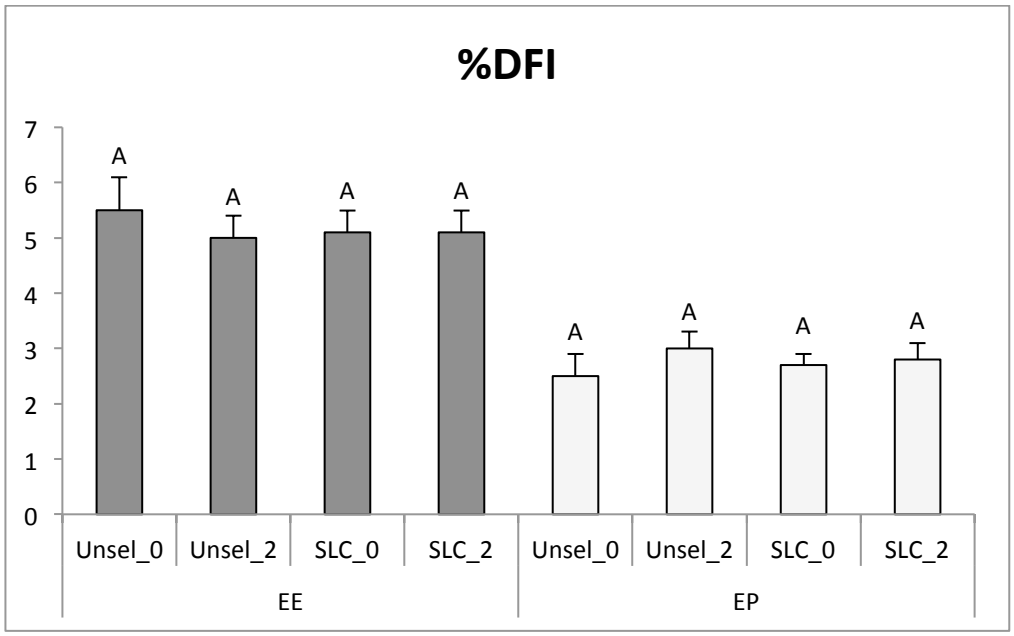
**Table 2. Effect of single-layer centrifugation (SLC) on sperm viability, apoptotic and acrosomal status and mitochondrial activity of electroejaculated (EE) and epididymal samples (EP) from red deer.**

Data are shown as the model-derived mean ± s.e.m.

| Sperm | Treatment Time | %Live_NotApop | | %Apoptotic | | %Mito_Act | | %Live_Acro | |
|---|---|---|---|---|---|---|---|---|---|
| EE | Unsel_0 | 34.6±4.8 | a,A | 20.2±2.2 | a,A | 34.8±4.3 | a,A | 46±2.5 | a,A |
| | Unsel_2 | 15±2.2 | b,A | 12.9±1.5 | b,A | 10.8±2.6 | b,A | 26.5±2.1 | b,A |
| | SLC_0 | 67.3±3.1 | c,A | 11.3±1.1 | b,A | 67±4.1 | c,A | 65.7±6.1 | c,A |
| | SLC_2 | 28.4±3.2 | a,A | 13.1±1 | b,A | 27.9±3.2 | a,A | 37.7±4.2 | d,A |
| EP | Unsel_0 | 24.3±5.5 | a,A | 19,5±3 | a,A | 23.3±5.6 | a,A | 43±7.8 | a,A |
| | Unsel_2 | 16.5±4.9 | b,A | 8±2 | b,A | 12.9±3.8 | b,A | 24.7±5.3 | b,A |
| | SLC_0 | 49.2±6.6 | c,B | 8,9±2.2 | b,A | 47.7±6.9 | c,B | 57.9±7.8 | c,A |
| | SLC_2 | 35±5.2 | d,A | 8,3±1.8 | b,A | 32±4.7 | a,A | 45.9±6.1 | a,A |

Treatments: unselected samples 0 and 2 h of incubation at 37ºC (Unsel_0 and Unsel_2), selected samples 0 and 2 h of incubation (SLC_0 and SLC_2). Lower case letters show differences between treatments for the same kind of sperm. Capital letters show differences for the same treatment between kinds of sample.

**Figure 1**




Different textures show significant differences (P<0.05) between kinds of samples (EE or EP) for the same treatment. Different capital letters show differences (P<0.05) between treatments into the same semen type

# CAPITULO 4

**Reduced glutathione addition improves both the kinematics and physiological quality of post-thawed red deer sperm**

L. Anel-López[a], O. Garcia-Álvarez[a], A. Maroto-Morales[a], M. Iniesta-Cuerda[a,] M. Ramón[b], A.J. Soler[a], M.R. Fernández-Santos[a], J.J. Garde[a]

[a]SaBio IREC (CSIC-UCLM- JCCM), Campus Universitario s. n. 02071 Albacete, Spain

[b]CERSYRA, Valdepeñas, Spain

**Abstract**

The potential protective effect of reduced glutathione (GSH) and trolox (TRX), an analogue of vitamin E, supplementation during *in vitro* culture (2 h, 39 ºC) of electroejaculated frozen-thawed red deer sperm was investigated. Cryopreserved sperm were thawed and incubated with no additive (Control) and 1 mM or 5 mM of each antioxidant to find out whether these supplementations can maintain the sperm quality, considering the use of thawed samples for *in vitro* techniques such as *in vitro* fertilization (IVF), sperm sex sorting or refreezing. The effect of GSH on sperm motility was positive compared to TRX which was negative ($P<0.001$). After 2 h of incubation at 39 ºC, use of GSH improved motility while TRX supplementation reduced sperm motility compared with Control samples without antioxidant. Use of TRX at both concentrations (1 and 5 mM; TRX1 and TRX5) resulted in lesser percentages of apoptotic sperm (12.4 ± 1.1% and 11.7 ± 0.9%) than GSH1, GSH5 ( 15.2 ± 1% and 14.6 ± 1.1%) and Control samples (16.9 ± 1.2%) ($P<0.001$). Use of GSH at both concentrations (1 and 5 mM) resulted in greater mitochondrial activity as compared with findings for the Control, TRX1 and TRX5 groups. Results of this study indicate that GSH is a suitable supplement for electroejaculated red deer sperm. It would be necessary to conduct fertility trials (*in vivo* and *in vitro*), to assess whether GSH supplementation of thawed red deer sperm could improve fertility rates.

Keywords: Red deer, Sperm, Cryopreservation, Antioxidant, Reduced glutathione, Trolox

## 1. Introduction

In red deer, post-mortem collection has been considered as a very important germplasm resource because of the hunting activity in Spain during the past few decades (Garde *et al.*, 2006). Studies using this sperm collection approach have provided for significant improvements by implementing different cooling and freezing rates, extender composition or antioxidant supplementation (Fernandez-Santos *et al.*, 2006, Martínez-Pastor *et al.*, 2006a, Martinez-Pastor *et al.*, 2006b, Anel-López *et al.*, 2012).

The production of red deer on farms is becoming an important source of economic resource in many countries. In this production situation, electroejaculation is the obvious and the most suitable choice for sperm collection (Asher *et al.*, 2000). It allows performance of intensive breeding selection and, therefore, more rapid improvement of genetic quality than post-mortem collection. For these reasons the improvement in specific cryopreservation protocols for use of ejaculated and electroejaculated sperm samples should be considered a priority.

Mammalian sperm are known to be especially susceptible to oxidative stress (Baker and Aitken, 2004). Oxidative stress can be defined as the loss of the balance between reactive oxygen species (ROS) production and the ability of antioxidants to scavenge ROS. The susceptibility of sperm to oxidative damage arises as an important problem because this might lead to loss of motility, membrane integrity, fertilising capability and other physiological changes in sperm cells (Aitken, 1995, Aitken and Baker, 2004, Rath *et al.*, 2009, Aitken *et al.*, 2012). Previews studies have demonstrated the use of some antioxidants could enhance the viability of epididymal red deer sperm either in refrigerated storage (Fernández-Santos *et al.*, 2009a), cryopreservation (Fernandez-Santos *et al.*, 2007) or post-thawing incubation (Domínguez-Rebolledo *et al.*, 2010). However, further investigations should be conducted because of the great variability of antioxidant effects. These effects may vary, not only depending on concentration (Domínguez-Rebolledo *et al.*, 2010), but also from differences in sperm responses among species, time of application of antioxidants during sperm processing, and medium or temperature used for sperm storage. For example, in a previous study (Mara *et al.*, 2005)

refrigerating ram semen in presence of the antioxidant TEMPOL improved sperm quality and fertility. In contrast, use of TEMPOL reduced the motility of deer sperm during incubation at 39 ºC, but it was able to protect DNA against oxidative stress (Mata-Campuzano *et al.*, 2012).

TRX is an analogue of vitamin E with high capacity to capture free radicals (Mickle and Weisel, 1993), and usually it is used as the standard to assess antioxidant capacity of other molecules (Lipovac, 2000, Ronald *et al.*, 2005). The supplementation of extender with TRX improved sperm motility and mitochondrial membrane integrity during post-thaw incubation of ejaculated boar sperm after thawing (Pena *et al.*, 2003). Furthermore, use of TRX reduced intracellular reactive oxygen species, lipid peroxidation, and preserved membrane integrity of red deer epididymal sperm during post-thaw incubation, either with or without induced oxidative stress (Martínez-Pastor *et al.*, 2008, Martinez-Pastor *et al.*, 2009) in epididymal samples. TRX also protected motility and viability and abolished DNA damage in samples exposed to oxidative stress after thawing and washing (Domínguez-Rebolledo *et al.*, 2009).

Reduced glutathione (GSH) is a tripeptid distributed within living cells. It has an important role in cell protection from the noxious effect of oxidative stress, directly and as a cofactor of GSH peroxidase (Atmaca, 2004). This enzyme acts on GSH to reduce hydrogen peroxide to $H_2O$ and lipoperoxides to alkyl alcohols. The addition of GSH to cryopreservation extender has had variable results in several species (Anel-López *et al.*, 2012, Câmara *et al.*, 2011). Thus, the supplementation of GSH and TRX to extenders for epididymal red deer sperm was beneficial, although its effect on ejaculated sperm is still unknown.

The main aim of the present study was to assess the effect in electroejaculated frozen-thawed sperm samples of two different antioxidants (GSH and TRX) at two different concentrations (1 and 5 mM) after an incubation of 2 h at 39 ºC. Attempts were also made to ascertain whether these supplementations can maintain sperm quality, considering the use of thawed samples for *in vitro* techniques such as IVF, sperm sex sorting or refreezing.

## 2. Material and methods

### 2.1. Reagents and media

Flow cytometric equipment, software and consumables were purchased form Beckman Coulter (Fullerton, CA, USA). Fluorescence probes YO-PRO-1 and Mitotracker Deep Red were purchased from Invitrogen (Barcelona, Spain), propidium iodide (PI) and Peanut Agglutinin-Fluorescein isothiocyanate (PNA-FITC) were acquired from Sigma (Madrid, Spain) and acridine orange (chromatographically purified) was purchased from Polysciences (Warrington, PA, USA). Stock solutions of the fluorescence probes were: PI: 1.5 mM; PNA-FITC: 0.2 mg/mL; YO-PRO-1: 50 µM; Mitotracker Deep Red: 1 mM. All fluorescent stocks were prepared in DMSO, except for PI and PNA-FITC, which were prepared in distilled water and kept at -20 ºC in the dark until needed. The stock solution of acridine orange was prepared in distilled water at 1 mg/mL and kept in the dark at 5 ºC. The stock solutions of the antioxidants were prepared at 100 mM in DMSO (TRX) or in water (reduced GSH) and stored at -20 ºC.

The work medium for cytometry assessment was bovine gamete medium (BGM-3) composed of 87 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.3 mM $NaH_2PO_4$, 40 mM HEPES, 21.6 mM sodium lactate, 1 mM sodium pyruvate, 50 µg/mL kanamycin, 10 µg/mL phenol red and 6mg/mL BSA (pH 7.5). Solutions for SCSA® (Sperm Chromatin Structure Assay) were prepared following Evenson *et al.* 2000 (Evenson and Jost, 2000): TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4), acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.4) and acridine orange solution (0.1 M citric acid, 0.2 M Na2HPO4, 1 mM EDTA, 0.15 M NaCl, pH 6.0; acridine orange was added from the stock up to 6 µg/mL). These solutions were kept at 5 ºC in darkened conditions.

### 2.2. Ejaculate collection and cryopreservation

Samples were obtained from nine mature stags during breeding season (mid-September). Animals were housed in a semi-free ranging environment at Las Lomas Farm (Medianilla S.L., Cadiz, Spain). Animal handling and

electroejaculation were performed in accordance with Spanish Animal Protection Regulation RD53/2013 which conforms to European Union Regulation 2010/63/UE. The electroejaculation procedure was conducted as described by (Martínez *et al.*, 2008). Males were anesthetized with Xylacine (0.75 mg/Kg) (Rom-pun® 2%; Bayer AG, Leverkusen, Germany). The rectum was cleared of faeces and the prepucial area was shaved and washed with physiological saline solution. A three-electrode probe connected to a power source that allowed voltage and amperage control was used (P.T. Electronics, Boring, OR, USA). Probe diameter, probe length and electrode length were 3.2, 35.0 and 6.6 cm, respectively. The electroejaculation regimen consisted of consecutive series of 5 pulses of similar voltage and separated by 5 s. The initial voltage was 1 V that was increased in each series up to a maximum of 5 V. Semen was collected in fractions in graduated glass tubes at 37 ºC. Sperm concentration was assessed using a hemocytometer (Bürker chamber; Brand Gmbh, Germany), after diluting the sample in a glutaraldehyde solution (5 mL of sample in 500 mL of 2% glutaraldehyde solution- 29 g/L glucose monohydrate, 10 g/L sodium citrate tribasic dihydrate and 2 g/L sodium bicarbonate). Fractions with urine contamination were discarded that were positive to Urea Test Strips (Diagnostic Systems GmbH, Holzheim, Germany). Sperm fractions with a total motility of less than 80% were also discarded.

Seminal samples were extended to a final concentration of 100 x $10^6$ sperm/mL using Triladyl® (Minitüb, Tiefenbach, Germany) supplemented with 20% (v/v) of egg yolk (EY). Then sperm samples were immersed in a programmable temperature-controlled water bath (Programmable Model 9612, PolyScience, Niles, IL, USA) and slowly cooled from 21 ºC to 5 ºC over 90 min, and left for an equilibration time of 2 h. After this period semen was packaged in 0.25 mL straws (Minitüb, Tiefenbach, Germany) and frozen in nitrogen vapour (4 cm above liquid nitrogen) for 10 min, and then plunged into the liquid nitrogen for storage.

### 2.3. Experimental design

The experimental process was replicated three times. For each replicate, four straws per male were thawed and sperm samples were divided into five tubes. One of the tubes was left as a control and analysed immediately, and the other tubes were supplemented with GSH or TRX to final concentrations of 1 and 5 mM. Antioxidant stocks were performed to a concentration of 500 mM (0.3 µL in a volume of 149.7 µL of sample for 1 mM samples and 1.5 µL in a volume of 148.5 µL of sample for 5 mM samples). After the supplementation, aliquots were incubated for 2 h at 39 ºC in a programmable water bath and then assessed again.

### 2.4. Computer-Assisted Sperm Analysis

Samples were diluted to 30 X $10^6$ spermatozoa/mL in BGM-3 to assess sperm motility. A prewarmed Makler counting chamber was subsequently loaded with 5 µL per sample. The analysis was conducted by CASA (Computer Assisted Sperm Analysis). The CASA system consisted of an optical phase-contrast microscope (Nikon Labophot-2; and equipped with negative phase-contrast objectives and a warming stage at 37 ºC), a Basler A312fc camera (Basler, Germany), and a computer with the sperm Class Analyser software (ISAS v. 1.2; Proiser, Valencia, Spain). The magnification was 10X. At least five fields per sample were acquired, recording at least 200 motile sperm. Image sequences were saved and analysed afterward. CASA acquisition variables were 25 images acquired at an acquisition rate of 25 images/s. The following sperm variables were used for the study: total motility (%; TM), progressive motility (%; PM), curvilinear path velocity (µm/s; VCL), linearity (%; LIN), amplitude of the lateral movement of the head (µm; ALH), wobble (%; WOB) and beat cross frequency (Hz; BCF).

Samples were corrected and analysed using the editing facilities provided by ISAS. Sperm cells were considered motile when VCL > 25 µm/s. Different events from sperm were removed and settings were adjusted in each sample to assure a correct track analysis.

### 2.5. Evaluation of sperm viability, acrosomal status, mitochondrial activity and apoptotic markers

Several physiological variables were assessed by using fluorescent probes and flow cytometry, following the protocol described in previous studies (Anel-López *et al.*, 2012). Samples were diluted down to $10^6$/mL in BGM-3, and stained using two or three flourophore combinations. Sperm viability and acrosomal status were assessed with 100 µg/mL PNA-FITC and 6 µM PI. Mitochondrial activity and apoptotic status were assessed by combining 0.1 µM YO-PRO-1, 0.1 µM Mitotracker Deep Red and 6 µM PI. After 15 min of incubation with the fluorescent probes, sperm suspensions were analysed with a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA, USA) controlled with the MXP software (v. 1). Cells were passed through the instrument at 150 to 300 cells/s, collecting data from 10,000 cells. Excitation was performed with a 488-nm Ion-Ar laser, except for Mitotracker deep red, which was excited with a 633-nm He-Ne laser. Fluorescein isothiocytate (FITC) and acridine orange green fluorescence were detected with a 530/28 band-pass filter (FL-1) while propidium iodide and acridine orange red fluorescence were detected with a 620/240 band-filter (FL-3), both in logarithmic scales. Flow cytometry data was analysed with WEASEL software Ver. 3 (WEHI, Melbourne, Australia)

### 2.6. Sperm chromatin structure assay

Chromatin stability was assessed following the SCSA® based on the susceptibility of sperm DNA to acid-induced denaturation in situ and on the subsequent staining with the metachromatic fluorescent dye acridine orange (Evenson *et al.*, 2002). Acridine orange (AO) fluorescence shifts from green (dsDNA; double strand) to red (ssDNA; single strand). Samples were diluted in TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA and pH 7.4) to a final sperm concentration of $2x10^6$ cells/mL. Samples were frozen (-80 ºC) until needed. For analysis, the samples were thawed in crushed ice. Acid-induced denaturation of DNA *in situ* was achieved by adding 0.4 mL of an acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.4) to 200 µL of sample. After 30 s, the cells were stained by adding 1.2 mL of an AO solution

(0.1 M citric acid, 0.2 M $Na_2HPO_4$, 1 mM EDTA, 0.15 M NaCl, 6 µg/mL acridine orange, pH 6.0). The stained samples were analysed by flow cytometry exactly at 3 min after adding the AO solution.

A tube with 0.4 mL of detergent-acid solution and 1.2 mL of acridine orange solution was flushed through the system before inserting any samples and this same procedure was used between sample assessments. At the beginning of each session, a standard semen sample was run through the cytometer, and settings were adjusted in order that mean fluorescence values (0-1023 linear scale) for FL-1 and FL-3 were 475 and 125, respectively. Results of the DNA denaturation test were processed to obtain the ratio of red fluorescence compared with total intensity of the fluorescence (red/ [red+green] ×100), called DFI (DNA fragmentation index; formerly called αt) for each sperm cell, representing the shift from green to red fluorescence. Greater values of DFI indicate chromatin abnormalities. Flow cytometry data was processed to obtain %DFI (% of sperm with DFI>25) and HDS (High DNA Stainability: % of sperm with green fluorescence greater than channel 600, of 1024 channels).

### 2.7. Statistical analysis

Data analysis was performed using R version 3.1.2. (R Core Team 2014). Significance level was established at $P < 0.05$. Prior to data analysis, the assumption of normality was checked and data corrections (arcsin or log) were applied when not satisfied. To estimate how the proposed antioxidants affect sperm quality, a mixed-effects model was used including different treatments as fixed effects and the male as random effect. For multiple comparisons analysis, Bonferroni correction was applied.

## 3. Results

### 3.1. Sperm kinematics assessment

After 2 h at 39 ºC of incubation, all samples had a decrease ($P<0.001$) in most cell motility variables that were assessed, except for LIN, STR, WOB and BCF (Figure 1). The supplementation with antioxidant agents provided different

results depending on the antioxidant used (GSH and TRX). For all samples, the use of GSH resulted in a greater value for sperm motility than relative values when there was TRX supplementation ($P$<0.001). After 2 h of incubation at 39 ºC, samples that were supplemented with GSH (TM; GSH1: 26.6 ± 3.6% and GSH5: 28.6 ± 3.9%) had greater motility than Control samples (12.5 ± 4.3%; $P$<0.001) incubated without antioxidants. In contrast, the supplementation with TRX (TM; TRX1: 1.0 ± 0.3% and TRX5: 1.1 ± 0.3%) resulted in lesser values ($P$<0.001) for sperm motility than Control samples without antioxidant (Figure 1). Except for the TM and PM, no other sperm cell velocity variables are presented for TRX samples because of the absence of any detectable motility in these samples.

### 3.2. Sperm viability, apoptotic status, mitochondrial activity, acrosomal status and DNA fragmentation

The variables related to physiological status of the stored sperm had a pattern similar to that for motility variables (Table 1). After 2 h at 39 ºC of incubation, values for every variable assessed (viability, apoptotic status, mitochondrial activity and acrosomal status) decreased ($P$<0.001). After incubation, there were no differences in the values when different concentrations (1 and 5 mM) of the same antioxidant type (GSH or TRX) were used. After incubation, the percentage of viable sperm did not differ among treatment groups. The use of the TRX treatment at both concentrations (1 and 5 mM), however, resulted in lesser values in percentage of apoptotic sperm (12.4 ± 1.1% and 11.7 ± 0.9%) than the GSH1, GSH5 (15.2 ± 1% and 14.6 ± 1.1%) treatments and Control samples (16.9 ± 1.2%; $P$<0.001). In contrast, use of GSH at both concentrations (1 and 5 mM) resulted in greater values (15.2 ± 2.4% and 14.6 ± 2.4%) of mitochondrial activity than the values for the Control (10.6 ± 2.2%), and TRX1 and theTRX5 samples (12.4 ± 1.7% and 11.7 ± 1.9%; $P$<0.001). There were no differences among Control samples and antioxidant treatments in the percentage of viable sperm with an intact acrosome after incubation. The percentage of sperm with greater amounts of DNA fragmentation was not different among samples after thawing and after

incubation. There were also no differences in DNA fragmentation among treatments (antioxidants and Control samples) after incubation.

**4. Discussion**

The use of antioxidant to improve sperm quality or to protect sperm when using biotechnologies such as cryopreservation or sex sorting has garnered great interest over the last several years. However, results have been inconsistent largely based on species differences and/or the antioxidant/dose studied. For example, butylated hydroxytoluene (BHT) was demonstrated to be a potent antioxidant for use with dog (Neagu *et al.*, 2010) and boar sperm (Roca *et al.*, 2004), but had no beneficial effect on equine sperm (Morillo-Rodríguez *et al.*, 2012). The antioxidant concentrations used in the present study were chosen based on previous research (Anel-López *et al.*, 2012, Fernandez-Santos *et al.*, 2007) and experiences when using various antioxidants under varying conditions.

The freezing-thawing process may induce the production of free radicals, which might add to and worsen the effects of cold shock and cryopreservation (Chatterjee and Gagnon, 2001). Sperm are highly sensitive to lipid peroxidation, but this effect can be prevented or reversed by antioxidant actions in cells or seminal plasma (Storey, 1997). The use of antioxidants as a supplement of red deer semen extenders might be an important tool to sustain and improve the sperm quality during processes such as IVF or sperm sex sorting. This technology is especially interesting because its use can help farmers have an optimal proportion of males and females in production systems. This becomes especially interesting in the production of red deer for hunting because the trophy antlers are only grown by males and are, therefore, of primary economic value.

Many previews studies with epididymal samples in red deer indicate TRX is a useful antioxidant choice for supplementing sperm extenders. Supplementation of extenders for thawed deer epididymal sperm with TRX at 100 µM had a small but positive impact on sperm quality (Martínez-Pastor *et al.*, 2008) probably because of its free radical scavenging activity, even at the 10

µM concentration (Martinez-Pastor *et al.*, 2009). When TRX was used at 1 mM there was a decreased susceptibility of epididymal red deer sperm to oxidative stress after thawing and washing (Domínguez-Rebolledo *et al.*, 2009). In contrast, in a previous study with red deer epididymal sperm (Anel-López *et al.*, 2012), and a preliminary study for the present research indicated there was a detrimental effect on sperm quality when using TRX, especially on motility. Lipid peroxidation was also clearly reduced by use of TTX relative to results for the Control samples and with use of GSH after incubation. The excessive ROS scavenging with use of TRX may have a negative effect on sperm. There were two marked differences with the previous and preliminary studies; time of TRX supplementation (after freezing or posthawing) and kind of sperm (epididymal and electroejaculated), but the same negative effect of TRX was evident for sperm motility in both studies after incubation (Anel-López *et al.*, 2012). Use of TRX at both concentrations had little effect on the physiological variables assessed such as the viability, acrosomal status or mitochondrial activity. This finding suggests that these TRX concentrations negatively affect some metabolic pathways involved with sperm motility while not impacting other sperm viability markers because of an imbalance between ROS production and scavenging capacity of the extender. This is especially important, taking into account the importance of ROS in some metabolic pathways involved in motility, capacitation or acrosome reaction (de Lamirande *et al.*, 1997).

In contrast, use of GSH improved the quality of sperm after incubation as compared with the Control samples and to an even greater extent compared with TRX-treated samples. Treatment with GSH at both concentrations resulted in greater values of sperm motility than Control samples and those where TRX was used to supplement the extender. The improvement in sperm motility with use of GSH compared with TRX at both concentrations was not only on TM but also PM. A previous study on red deer (Anel-López *et al.*, 2012) provided results indicating a beneficial effect on sperm motility after thawing and after incubation for 6 h at 39 ºC when sperm had been frozen with GSH. The improvements in sperm motility could have been the factor in this previous study when these samples were used for artificial insemination, given the importance of sperm velocity on fertility assessments (Malo *et al.*, 2005). In

other studies, undesirable results have occurred with use of GSH (Foote *et al.*, 2002) whereas with cryopreserved bull sperm with the use of 0.5 mM GSH extender supplementation there were some improvements in sperm motility at 12 h of incubation (Tuncer *et al.*, 2010) in cryopreserved bull sperm samples with 0.5 and 2 mM GSH supplementation. In another study (Silva *et al.*, 2011) found that there were negative effects of GSH at 7 mM on ram sperm motility, mitochondrial activity and acrosomal ultrastructure. However, these comparisons should be taken with some caution due to the concentrations of GSH used and the stage of the semen processing when antioxidants were added.

Mitochondria have to be considered as one of the most sensitive cell structures to damage during the sperm cryopreservation process (Ortega-Ferrusola *et al.*, 2008). In addition, the oxidative damage produced during and after the cryopreservation to mitochondrial DNA and membrane architecture is a factor of major importance to explain the impaired fertility and motility with use of frozen-thawed semen (Cummins *et al.*, 1994), which could be prevented by using antioxidants (Peña *et al.*, 2003). In the current study, the mitochondrial functions were protected by use of GSH as evidenced by the greater sperm mitochondrial function values than the comparable values for the Control and TRX samples after incubation. The protection of mitochondrial activity during sperm incubation is especially important given the role of this organelle not only in maintaining the energetic status of the sperm cell, but also in pathways regulating to motility, capacitation, or apoptosis (Peña *et al.*, 2009).

The DNA fragmentation was minimal in the present study. There were no differences after or before incubation nor after incubation when using the different antioxidant treatments. The chromatin of red deer sperm seems to be very resistant to damage even after long periods of storage without antioxidants (Fernández-Santos *et al.*, 2009a, Fernández-Santos *et al.*, 2009b). In addition, it has been reported that %DFI thresholds that impact fertility in different species (Love, 2005, Evenson and Wixon, 2006, Didion *et al.*, 2009) must be much greater than those with the present study.

In conclusion, the use of GSH to supplement semen extenders at 1 and 5 mM concentrations might be useful and could be a candidate for improving

media, either for IVF and similar techniques or other techniques, such as sperm sex sorting of red deer semen. However, the use of GSH for these purposes deserves further research to understand the pathways involving its effects, especially whether these quality improvements can improve fertility rates either in *in-vitro* or *in-vivo* trials. It can be concluded from the present research that the use of TRX in the millimolar range is not suitable as a supplement for electroejaculated red deer because of its negative effect on sperm motility and the absence of a TRX effect for improving physiological variables. The use of TRX deserves further investigations to fully understand the effects involved in the sperm motility suppression in the absence of effects on sperm viability.

**Acknowledgments**

This research was supported by Junta de Castilla y La Mancha (PRE123/2010).

**References**

Aitken, R.J., Jones, K.T., Robertson, S.A., 2012. Reactive oxygen species and sperm function—in sickness and in health. J. Androl. 33, 1096-1106.

Aitken, R.J., 1995. Free radicals, lipid peroxidation and sperm function. Reprod. Fertil. Dev. 7, 659-668.

Aitken, R.J., Baker, M.A., 2004. Oxidative stress and male reproductive biology. Reprod., Fertil. Dev. 16, 581-588.

Anel-López, L., Álvarez-Rodríguez, M., García-Álvarez, O., Álvarez, M., Maroto-Morales, A., Anel, L., de Paz, P., Garde, J.J., Martínez-Pastor, F., 2012. Reduced glutathione and Trolox (vitamin E) as extender supplements in cryopreservation of red deer epididymal spermatozoa. Anim. Reprod. Sci. 135, 37-46.

Asher, G., Berg, D., Evans, G., 2000. Storage of semen and artificial insemination in deer. Anim. Reprod. Sci. 62, 195-211.

Atmaca, G., 2004. Antioxidant effects of sulfur-containing amino acids. Yonsei Med. J. 45, 776-788.

Baker, M.A., Aitken, R.J., 2004. The importance of redox regulated pathways in sperm cell biology. Mol. Cell. Endocrinol. 216, 47-54.

Câmara, D., Silva, S., Almeida, F., Nunes, J., Guerra, M., 2011. Effects of antioxidants and duration of pre-freezing equilibration on frozen-thawed ram semen. Theriogenology 76, 342-350.

Chatterjee, S., Gagnon, C., 2001. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol. Reprod. Dev. 59, 451-458.

Cummins, J.M., Jequier, A.M., Kan, R., 1994. Molecular biology of human male infertility: links with aging, mitochondrial genetics, and oxidative stress? Mol. Reprod. Dev. 37, 345-362.

de Lamirande, E., Jiang, H., Zini, A., Kodama, H., Gagnon, C., 1997. Reactive oxygen species and sperm physiology. Rev. Reprod. 2, 48-54.

Didion, B.A., Kasperson, K.M., Wixon, R.L., Evenson, D.P., 2009. Boar fertility and sperm chromatin structure status: a retrospective report. J. Androl. 30, 655-660.

Domínguez-Rebolledo, Á.E., Fernández-Santos, M.R., Bisbal, A., Ros-Santaella, J.L., Ramón, M., Carmona, M., Martínez-Pastor, F., Garde, J.J., 2010. Improving the effect of incubation and oxidative stress on thawed spermatozoa from red deer by using different antioxidant treatments. Reprod., Fertil. Dev. 22, 856-870.

Domínguez-Rebolledo, A., Fernández-Santos, M., García-Álvarez, O., Maroto-Morales, A., Garde, J., Martínez-Pastor, F., 2009. Washing increases the susceptibility to exogenous oxidative stress in red deer spermatozoa. Theriogenology 72, 1073-1084.

Evenson, D., Jost, L., 2000. Sperm chromatin structure assay is useful for fertility assessment. Meth. Cell Sci. 22, 169-189.

Evenson, D.P., LARSON, K.L., Jost, L.K., 2002. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J. Androl. 23, 25-43.

Evenson, D.P., Wixon, R., 2006. Clinical aspects of sperm DNA fragmentation detection and male infertility. Theriogenology 65, 979-991.

Fernandez-Santos, M.R., Martinez-Pastor, F., Garcia-Macias, V., Esteso, M.C., Soler, A.J., Paz, P., Anel, L., Garde, J.J., 2007. Sperm characteristics and DNA integrity of Iberian red deer (Cervus elaphus hispanicus) epididymal spermatozoa frozen in the presence of enzymatic and nonenzymatic antioxidants. J. Androl. 28, 294.

Fernández-Santos, M., Domínguez-Rebolledo, A., Esteso, M., Garde, J., Martínez-Pastor, F., 2009a. Refrigerated storage of red deer epididymal spermatozoa in the epididymis, diluted and with vitamin C supplementation. Reprod. Dom. Anim. 44, 212-220.

Fernandez-Santos, M., Esteso, M., Montoro, V., Soler, A., Garde, J., 2006. Cryopreservation of Iberian red deer (Cervus elaphus hispanicus) epididymal spermatozoa: effects of egg yolk, glycerol and cooling rate. Theriogenology 66, 1931-1942.

Fernández-Santos, M., Martínez-Pastor, F., Matias, D., Domínguez-Rebolledo, A., Esteso, M., Montoro, V., Garde, J., 2009b. Effects of long-term chilled storage of red deer epididymides on DNA integrity and motility of thawed spermatozoa. Anim. Reprod. Sci. 111, 93-104.

Foote, R.H., Brockett, C.C., Kaproth, M.T., 2002. Motility and fertility of bull sperm in whole milk extender containing antioxidants. Anim. Reprod. Sci. 71, 13-23.

Garde, J., Martínez-Pastor, F., Gomendio, M., Malo, A., Soler, A., Fernández-Santos, M., Esteso, M., García, A., Anel, L., Roldán, E., 2006. The application of reproductive technologies to natural populations of red deer. Reprod. Dom. Anim. 41, 93-102.

Lipovac, M.G.V., 2000. Antioxidative effect of melatonin on human spermatozoa. Sys. Biol.Reprod. Med. 44, 23-27.

Love, C.C., 2005. The sperm chromatin structure assay: a review of clinical applications. Anim. Reprod. Sci. 89, 39-45.

Malo, A.F., Garde, J.J., Soler, A.J., Garcia, A.J., Gomendio, M., Roldan, E.R., 2005. Male fertility in natural populations of red deer is determined by sperm velocity and the proportion of normal spermatozoa. Biol. Reprod. 72, 822-829.

Mara, L., Accardo, C., Pilichi, S., Dattena, M., Chessa, F., Chessa, B., Branca, A., Cappai, P., 2005. Benefits of TEMPOL on ram semen motility and in vitro fertility: a preliminary study. Theriogenology 63, 2243-2253.

Martínez, A., Martínez-Pastor, F., Álvarez, M., Fernández-Santos, M., Esteso, M., De Paz, P., Garde, J., Anel, L., 2008. Sperm parameters on Iberian

red deer: Electroejaculation and post-mortem collection. Theriogenology 70, 216-226.

Martinez-Pastor, F., Aisen, E., Fernandez-Santos, M.R., Esteso, M.C., Maroto-Morales, A., Garcia-Alvarez, O., Garde, J.J., 2009. Reactive oxygen species generators affect quality parameters and apoptosis markers differently in red deer spermatozoa. Reproduction 137, 225.

Martínez-Pastor, F., Fernández-Santos, M., Del Olmo, E., Domínguez-Rebolledo, A., Esteso, M., Montoro, V., Garde, J., 2008. Mitochondrial activity and forward scatter vary in necrotic, apoptotic and membrane-intact spermatozoan subpopulations. Reprod., Fertil. Dev. 20, 547-556.

Martinez-Pastor, F., Garcia-Macias, V., Alvarez, M., Chamorro, C., Herraez, P., Paz, P., Anel, L., 2006a. Comparison of two methods for obtaining spermatozoa from the cauda epididymis of Iberian red deer. Theriogenology 65, 471-485.

Martínez-Pastor, F., Martínez, F., García-Macías, V., Esteso, M.C., Anel, E., Fernández-Santos, M.R., Soler, A.J., de Paz, P., Garde, J., Anel, L., 2006b. A pilot study on post-thawing quality of Iberian red deer spermatozoa (epididymal and electroejaculated) depending on glycerol concentration and extender osmolality. Theriogenology 66, 1165-1172.

Mata-Campuzano, M., Alvarez-Rodríguez, M., Del Olmo, E., Fernández-Santos, M., Garde, J., Martínez-Pastor, F., 2012. Quality, oxidative markers and DNA damage (DNA) fragmentation of red deer thawed spermatozoa after incubation at 37 C in presence of several antioxidants. Theriogenology 78, 1005-1019.

Mickle, D.A., Weisel, R.D., 1993. Future directions of vitamin E and its analogues in minimizing myocardial ischemia-reperfusion injury. Can. J. Cardiol. 9, 89-93.

Morillo-Rodríguez, A., Macías-García, B., Tapia, J., Ortega-Ferrusola, C., Peña, F., 2012. Consequences of butylated hydroxytoluene in the freezing extender on post-thaw characteristics of stallion spermatozoa in vitro. Andrologia 44, 688-695.

Neagu, V., García, B.M., Sandoval, C.S., Rodríguez, A.M., Ferrusola, C.O., Fernández, L.G., Tapia, J., Peña, F., 2010. Freezing dog semen in presence of the antioxidant butylated hydroxytoluene improves postthaw sperm membrane integrity. Theriogenology 73, 645-650.

Ortega-Ferrusola, C., Sotillo-Galán, Y., Varela-Fernández, E., Gallardo-Bolaños, J., Muriel, A., González-Fernández, L., Tapia, J., Pena, F., 2008. Detection of "Apoptosis-Like" Changes during the Cryopreservation Process in Equine Sperm. J. Androl. 29, 213-221.

Peña, F., Johannisson, A., Wallgren, M., Rodriguez Martinez, H., 2003. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Anim. Reprod. Sci. 78, 85-98.

Peña, F., Rodríguez Martínez, H., Tapia, J., Ortega Ferrusola, C., Gonzalez Fernandez, L., Macías García, B., 2009. Mitochondria in mammalian sperm physiology and pathology: a review. Reprod. Dom. Anim. 44, 345-349.

Rath, D., Moench-Tegeder, G., Taylor, U., Johnson, L., 2009. Improved quality of sex-sorted sperm: A prerequisite for wider commercial application. Theriogenology 71, 22-29.

Roca, J., Gil, M.A., Hernandez, M., Parrilla, I., Vazquez, J.M., Martinez, E.A., 2004. Survival and Fertility of Boar Spermatozoa After Freeze-Thawing in Extender Supplemented With Butylated Hydroxytoluene. J. Androl. 25, 397-405.

Ronald, L., Wu, X., Schaich, K., 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J. Agric. Food Chem. 53, 4290-4302.

Silva, S., Soares, A., Batista, A., Almeida, F., Nunes, J., Peixoto, C., Guerra, M., 2011. In Vitro and In Vivo Evaluation of Ram Sperm Frozen in Tris Egg-yolk and Supplemented with Superoxide Dismutase and Reduced Glutathione. Reprod. Dom. Anim. 46, 874-881.

Storey, B.T., 1997. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. Mol. Hum. Reprod. 3, 203-213.

Tuncer, P.B., Bucak, M.N., Büyükleblebici, S., Sarıözkan, S., Yeni, D., Eken, A., Akalın, P.P., Kinet, H., Avdatek, F., Fidan, A.F., 2010. The effect of cysteine and glutathione on sperm and oxidative stress parameters of post-thawed bull semen. Cryobiology 61, 303-307.

**Table 1**

Physiological effects on red deer sperm after thawing (Time 0) and after 2 h of incubation at 37 ºC (Time 2) supplemented with reduced glutathione (GSH) and Trolox (TRX) antioxidants at two different concentrations (1 and 5 mM); Samples without antioxidants were treated as Controls[1, 2]

| | AOX | %VIAB | %APOP | %MITO | %PNA | %DFI |
|---|---|---|---|---|---|---|
| 0 | Control | 43.5 ± 2.0$^{A}$ | 28.5 ± 1.7$^{A}$ | 38.7 ± 2.6$^{A}$ | 67.1 ± 2.2$^{A}$ | 4.3 ± 0.3 |
| 2 | Control | 28.9 ± 3.1$^{B}$ | 16.9 ± 1.2$^{B;a}$ | 10.6 ± 2.2$^{B;a}$ | 39.1 ± 3.6$^{B}$ | 4.2 ± 0.3 |
| | GSH1 | 28.0 ± 2.2 | 15.2 ± 1.0$^{a}$ | 15.2 ± 2.4$^{b}$ | 42.5 ± 3.4 | 4.1 ± 0.3 |
| | GSH5 | 32.0 ± 2.8 | 14.6 ± 1.1$^{a,b}$ | 14.6 ± 2.4$^{b}$ | 42.5 ± 3.3 | 3.5 ± 0.3 |
| | TRX1 | 29.7 ± 3.1 | 12.4 ± 1.1$^{b,c}$ | 12.4± 1.7$^{a}$ | 34.7 ± 3.7 | 4.1 ± 0.3 |
| | TRX5 | 29.1 ± 3.3 | 11.7 ± 0.9$^{c}$ | 11.7 ± 1.9$^{a}$ | 37.0 ± 4.0 | 3.7 ± 0.3 |

$^{A-B}$For each semen variable, different superscripts indicate differences ($P$<0.001) between time 0 and 2 h for Control samples

$^{a-c}$For each semen trait, different superscript means indicate differences ($P$<0.001) among treatments after 2 h of incubation

Variable description: VIAB = viable sperm (YO-PRO-1-/PI-; %); APOP = apoptotic sperm (YO-PRO-1+/PI-); MITO = viable sperm with greater mitochondrial membrane potential (MT+/YO-PRO-1-; %); PNA = viable sperm with intact acrosome (PNA-/PI-) DFI = sperm with medium-to-high DNA fragmentation (%)

**Figure legend.**

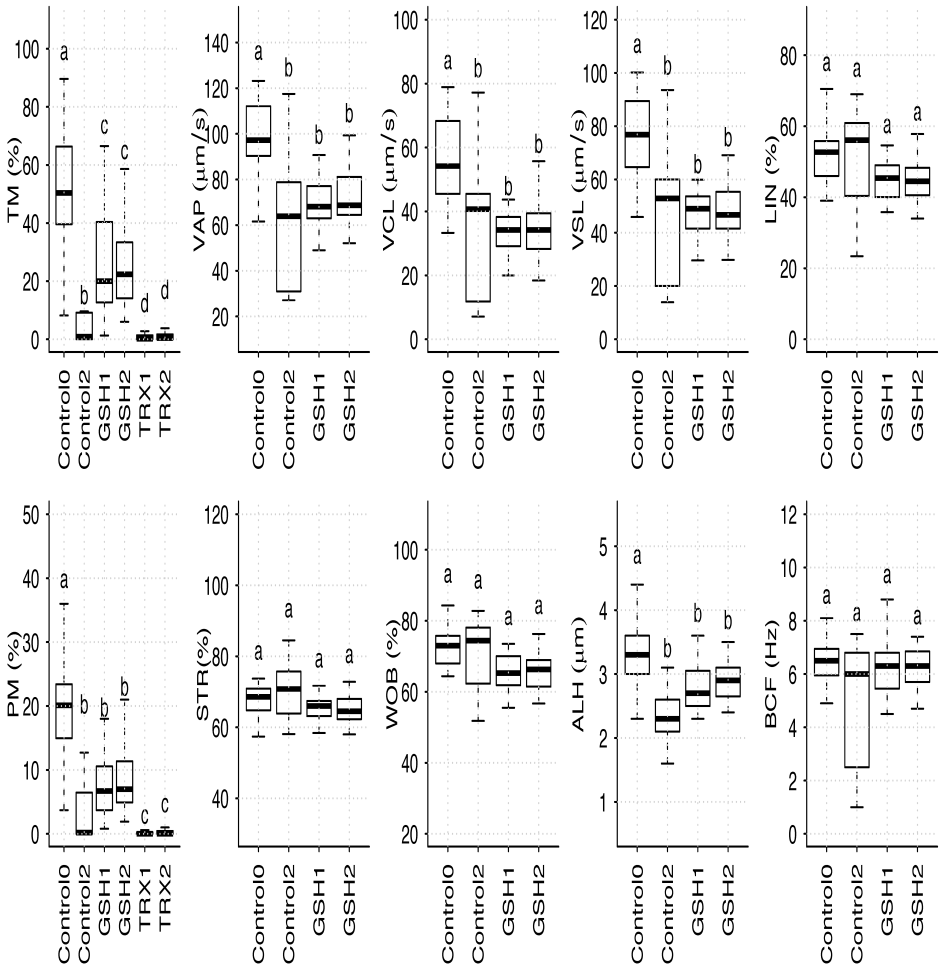
**Fig. 1.**

Effects of GSH on sperm motility variables assessed by Computer Assisted Sperm Analysis (CASA) after thawing (Control0) and after 2 h of incubation (39 ºC) (Control2, Glutathione reduced (GSH 1 mM and GSH 5 mM)). Only TM and PM are included for the Trolox treatment to depict the absence of movement by sperm cells in those samples

[a-d] For each semen variable, different superscripts indicate differences (*P*<0.001) among treatments

Variable description: TM = total motile (%); PM = progressive motile (%); VCL= curvilinear velocity path (μm/s); VAP = average path velocity (μm/s); VSL = straight line velocity (μm/s); LIN = Linearity (%); STR= straightness (%); WOB = wobble (%); ALH = lateral head displacement (μm); BCF = beat cross frequency (Hz)

**Figure 1**


TM (%)
VAP (µm/s)
VCL (µm/s)
VSL (µm/s)
LIN (%)
PM (%)
STR(%)
WOB (%)
ALH (µm)
BCF (Hz)
Control0
Control2
GSH1
GSH2
TRX1
TRX2

# DISCUSIÓN GENERAL

La comercialización de dosis espermáticas sexadas es ya una realidad en especies como el vacuno de leche o el ganado equino. Sin embargo, para otras especies, como el ciervo rojo, con una significancia económica menor dentro del cómputo general de la ganadería no aún no está desarrollada.

En un primer estudio en cérvidos, Gao *et al.* (2010) realizó con éxito inseminaciones artificiales con muestras espermáticas sexadas a baja concentración espermática (1 y 2 x $10^6$) y descongeladas de Sika (*Cervus nippon*) en hembras de ciervo rojo (*Cervus elaphus*). Sin embargo, la fertilidad obtenida para las dosis sexadas a baja concentración fue mucho menor que la fertilidad obtenida para el control con muestras comerciales estándar a 1 x $10^7$. Además, no se sabe si dicha diferencia en la tasa de fertilidad pudo ser debida a la diferencia de concentración de las dosis espermáticas, a un posible daño subletal de las muestras sometidas al proceso de sex-sorting o a una combinación de ambas.

Por tanto, el objetivo principal del capítulo 1 de la presente tesis doctoral fue evaluar los efectos del proceso de sexado sobre la calidad espermática de muestras de ciervo rojo, obtenidas mediante electroeyaculación y sometidas al proceso de sorting pero no separadas (BSS), muestras con alta pureza de espermatozoides X (XSS) y muestras con alta pureza de espermatozoides Y (YSS) tras someterlas a un proceso de congelación-descongelación, además de comparar la fertilidad entre muestras YSS contra muestras BSS también sometidas a un proceso de congelación descongelación. En dicho estudio llevado a cabo en el capítulo 1 de la presente tesis doctoral, los espermatozoides de ciervo rojo fueron capaces de sobrevivir a un proceso completo de sex-sorting y congelación-descongelación a pesar del prolongado tiempo del proceso y de la gran cantidad de puntos críticos del proceso para la viabilidad de los espermatozoides. Se pudieron observar diferencias de calidad en función del tipo de espermatozoide y del punto de muestreo (justo tras la descongelación, y tras someter las muestras a una incubación a 37ºC durante 2 horas). De esta forma los espermatozoides en pureza X (XSS) fueron los que peores valores de motilidad mostraron tras la descongelación frente al resto de grupos experimentales. Sin embargo dichas diferencias en motilidad desaparecieron tras la incubación para la mayoría de los paramentos cinéticos

analizados. Los resultados obtenidos por otros autores en diferentes especies en el apartado de movilidad son muy variables y en ocasiones contradictorios. De este modo algunos autores como (Lindsey *et al.* 2003) en equino o (Suh y Schenk 2003) en bovino, mostraron que el proceso de sexado reducía algunos parámetros cinéticos como la VCL o la ALH. Por el contrario, encontró, en porcino, un aumento de los parámetros anteriormente citados tras el proceso de sorting. En cambio, otros autores como Brogliatti *et al.* (2004) no encontraron diferencias entre muestras sexadas y no sexadas en las características de movilidad de muestras espermáticas de bovino. Esta cantidad de diferencias entre los resultados obtenidos en los diferentes estudios nos hacen pensar que puedan ser debidas a la gran cantidad de tratamientos y pasos de los que esta formado el proceso de sexado además de las posibles diferencias entre especies. Se ha demostrado que especies como la ovina tienen una alta resistencia al proceso de sorting mostrando mejores valores de motilidad en espermatozoides sometidos al proceso de sorting respecto a la muestra original (Graaf y Evans 2007). En nuestro estudio tanto la VCL como la ALH no cambiaron a lo largo de la incubación post-descongelación, lo que nos sugiere la alta resistencia de los espermatozoides de ciervo a dicho proceso.

Tras la descongelación el porcentaje de espermatozoides en estado de apoptosis mostro un aumento significativo para la muestras XSS respecto a las muestras NS, BSS y YSS. Además las muestras sometidas al proceso de sorting (BSS, XSS y YSS) mostraron valores superiores de apoptosis respecto a la muestra control (NS) tras la incubación. Por el contrario, el porcentaje de espermatozoides vivos con acrosoma intacto y el porcentaje de espermatozoides con alto potencial de membrana mitocondrial no mostraron diferencias entre las muestras sometidas al proceso de sorting (BSS, XSS y YSS) y el control (NS) aunque en todos los casos disminuyeron tras la incubación respecto a la descongelación. Este resultado contrasta con el obtenido por da Silva *et al.* (2013) en equino, donde encontró que el porcentaje de espermatozoides con la membrana intacta se redujo tras el proceso de sorting mientras que el porcentaje de espermatozoides con cambios tempranos de apoptosis aumentó.

En el apartado de integridad del DNA, las muestras en pureza Y (YSS) mostraron un porcentaje menor dé %DFI respecto al resto de grupos experimentales incluido el control no sexado (NS) tras la incubación. Este resultado está en concordancia con el obtenido por Kjelland *et al.* (2011) en ciervo de cola blanca. En dicho estudio se sugirió que la población de espermatozoides obtenidos después del proceso de sexado tuvo una estabilidad del DNA mayor que las muestras espermáticas convencionales no sexadas.

A pesar de que la población de espermatozoides Y (YSS) obtuvo mejores resultados de calidad en el análisis de fragmentación de la cromatina que el resto de grupos experimentales y no hubo diferencias en otros parámetros como viabilidad, estado acrosomal o actividad mitocondrial, tras realizar un estudio de fertilidad entre muestras espermáticas BSS e YSS los resultados de fertilidad fueron peores para el grupo YSS respecto al BSS. Este hecho nos sugiere que hay otro tipo de parámetros o vías que no han sido evaluadas en nuestro estudio, que probablemente sean esenciales para llevar a cabo la fertilización.

En el segundo capítulo de la presente tesis doctoral tuvimos como objetivo evaluar la susceptibilidad del estrés oxidativo inducido a muestras espermáticas de ciervo rojo obtenidas mediante electroeyaculación y sometidas a un proceso de sorting (BSS) respecto a muestras espermáticas convencionales obtenidas también mediante electroeyaculación (NS) tras un proceso de congelación-descongelación y determinar si el uso de 2 antioxidantes (Glutatión reducido (GSH) y Trolox (TRX)) podía proteger las muestras espermáticas de dicho estrés oxidativo.

Ambos grupos experimentales fueron susceptibles al estrés oxidativo como evidenciaron los resultados obtenidos en motilidad espermática. Un estudio previo de nuestro grupo (Martinez-Pastor *et al.* 2009) evidencio que concentraciones entre 0.1 y 1 de $H_2O_2$ mM eran perjudiciales para espermatozoides epididimarios de ciervo rojo, produciendo una inhibición de la motilidad tras 1 h de incubación. No se sabe con exactitud el mecanismo por el cual el $H_2O_2$ produce la inhibición de la motilidad espermática, aunque si se

sabe que inhibe enzimas esenciales como la glucose-6-fosfato deshidrogenasa (Maneesh y Jayalekshmi 2006).

La viabilidad en cambio mostro un resultado inesperado, ya que no solo no produjo un descenso significativo de la viabilidad sino que en las muestras NS a la concentración de H100 mantuvo tras la incubación de 2 h de forma significativa valores mas altos de viabilidad respecto al control sin oxidante. Estos resultados son compatibles con el trabajo realizado por Leahy *et al.* (2010) donde obtuvieron unos resultados similares cuando reportaron que la proporción de espermatozoides viables se mantuvo mas alta en el grupo experimental con una concentración de $H_2O_2$ de 45 µM en muestras espermáticas descongeladas de ovino tras una incubación de 3 h a 37 ºC. De hecho, sus autores reportaron que no tenían constancia de ningún estudio previo en el cual la adición de $H_2O_2$ hubiera tenido un efecto beneficioso sobre la viabilidad espermática.

Por su parte el daño en DNA mostro unos resultados muy interesantes, donde este se incremento de forma significativa para las muestras espermáticas convencionales (NS) suplementadas con $H_2O_2$ tras la incubación, pero no mostrando incremento alguno para las muestras sometidas al proceso de sorting (BSS). Este resultado nos indicó que los espermatozoides sometidos al proceso de sorting mostraron una mayor resistencia al estrés oxidativo en su cromatina. Este efecto puede ser debido a uno de los pasos que el proceso de sorting tiene per se, en el cual se descartan los espermatozoides muertos. Boe-

Hansen *et al.* (2005) descubrieron diferencias significativas en el porcentaje de fragmentación del DNA entre muestras espermáticas convencionales y sometidas al proceso de sorting en toro, donde demostraron que la población de espermatozoides obtenida tras el proceso de sorting presentaba una mejora en la integridad de la cromatina. Además estos autores sugirieron que muy probablemente esta mejora estaría ligada a la eliminación de los espermatozoides muertos y mal orientados durante el proceso de sorting. Mas tarde este hecho fue confirmado por Gosálvez *et al.* (2011) quienes demostraron los espermatozoides con cromatina mas dañada se acumulaban en la población de espermatozoides muertos separados por el sorter.

El ultimo objetivo de este segundo capitulo se centró en evaluar si la adición de 2 sustancias antioxidantes (glutatión reducido y Trolox) podía contrarrestar el efecto de la oxidación inducida y preservar la calidad espermática tras someter las muestras a una incubación de 2 h a 37 ºC. La suplementación con GSH, a pesar de no mostrar diferencias en el porcentaje de viabilidad, si mostro una importante mejora de la movilidad espermática respecto al Trolox y al control sin suplementar en ambos tipos de muestras espermáticas (NS y BSS) en presencia de oxidante ($H_2O_2$). También ayudó a mantener valores mas bajos de fragmentación de la cromatina en las muestras convencionales (NS) tras la incubación en presencia de oxidante respecto al control sin antioxidante. Por el contrario este efecto no fue observado en las muestras sometidas al proceso de sorting (BSS), si bien es cierto que dichas muestras espermáticas no mostraron un incremento en el porcentaje de espermatozoides con fragmentación del DNA. Los buenos resultados del GSH obtenidos en movilidad espermática están el la misma línea que los obtenidos en un estudio previo (Anel-López *et al.* 2012) realizado en espermatozoides epididimarios obtenidos post-mortem donde el GSH fue utilizado como aditivo en el extender antes de someterlos a la congelación descongelación. Tras la descongelación, aquellas muestras suplementadas con GSH mostraron mejores valores de movilidad tras someter las muestras a una incubación de 6 h a 39 ºC. Del mismo modo, el capitulo cuarto de la presente tesis doctorar, que derivó en la publicación (Anel-lópez *et al.* 2015), evaluó el efecto del GSH en muestras espermáticas obtenidas mediante electroeyaculación y sometidas a un proceso de congelación descongelación como paso previo para evaluar la idoneidad de este antioxidante para su posterior aplicación en muestras sometidas a un proceso de sorting obteniendo también buenos resultados, donde el GSH mantuvo valores mas altos de calidad espermática respecto de aquellas muestras no suplementadas, tras una incubación de 2 h a 39 ºC.

Sin embargo la adición de Trolox no reporto buenos resultados. A priori los resultados obtenidos en ciervo rojo en estudios anteriores habían reportado todo tipo de resultados, en unos casos muy beneficiosos pero en otros casos no. Por un lado, en el estudio llevado a cabo por Domínguez-Rebolledo *et al.* (2009) el Trolox disminuyó la susceptibilidad al estrés oxidativo de

espermatozoides de ciervo rojo epididimarios tras ser sometidos a un proceso de congelación-descongelación y a un lavado. Además, en otro estudio de nuestro grupo el Trolox había mostrado una gran capacidad para eliminar radicales libres de oxigeno en ciervo rojo a una concentración de 10 µM (Martinez-Pastor *et al.* 2009). Por el contrario, el presente estudio demostró que el uso de Trolox en el rango de 1 a 2 mM no fue una buena opción para la conservación de la calidad espermática en muestras convencionales (NS) ni en muestras sometidas a un proceso de sorting (BSS). De hecho su principal acción fue inhibir en gran medida la motilidad. Estos resultados negativos obtenidos en motilidad, concuerdan con algunos obtenidos con anterioridad en muestras epididimarias de ciervo rojo ( Fernandez-Santos *et al.* 2007; Anel-López *et al.* 2012) y en muestras eyaculadas obtenidas por electroeyaculación (Anel-lópez *et al.* 2015). Si bien es cierto, nuestro trabajo realizado en 2012 (Anel-López *et al.* 2012), a través de un estudio de la producción de malondialdehido (MDA), nos permitió averiguar que el Trolox llevó a cabo una excesiva eliminación de ROS y así pudimos hipotetizar que probablemente ese exceso en la eliminación de ROX produjo un desequilibrio redox causando esta inhibición de la motilidad.

Como conclusión de este segundo capitulo, podemos decir que el hallazgo principal fue la aparición de un aumento en el porcentaje de espermatozoides en estado de apoptosis en aquellas muestras sometidas al proceso de sorting y a un proceso de congelación descongelación, lo que podría sugerir que dicho proceso produce algún tipo de daño subletal a dichos espermatozoides. Pero al mismo tiempo el proceso de sorting selecciono una población con una cromatina mas resistente al estrés oxidativo respecto a las muestras convencionales. Además, el GSH a una concentración de 1 mM se postulo como un buen aditivo para mantener la calidad espermática, no siendo así para el Trolox que a concentraciones de 1 y 2 mM mostro un impacto muy negativo en la motilidad espermática.

El objetivo principal del tercer capitulo fue evaluar el efecto de someter muestras espermáticas de ciervo rojo, obtenidas mediante electroeyaculación o post-mortem, a un gradiente de selección monocapa (Androcoll-S) tras la descongelación. El uso de técnicas de selección espermática para mejorar la

calidad, podria convertirse en una herramienta importante para la preparación y el manejo de muestras espermáticas con diferentes fines. El uso de gradientes monocapa, en este caso Androcoll-S, es especialmente interesante debido a que su utilización es mucho mas sencilla de llevar a cabo que en el caso de otros sistemas de selección espermática tales como el swim up o gradientes de densidad bicapa.

En nuestro estudio, la selección espermática llevada a cabo tras la descongelación con Androcoll-S mejoró de forma significativa la mayoría de los parámetros analizados tanto en muestras obtenidas mediante electroeyaculación, como en muestras epididimarias obtenidas post-mortem, además de mantener dicha mejora respecto a las mismas muestras no seleccionadas, tras someter las muestras a una incubación de 2 h a 37 ºC. La mejora en la calidad de la motilidad espermática fue muy marcada para ambos tipos de muestras espermáticas. De esta manera, estos resultados concuerdan con trabajos llevados a cabo en las especies canina (Dorado *et al.* 2013), equina (García *et al.* 2009) o caprina (Jiménez-Rabadán *et al.* 2012). Además otros autores (Martinez-Alborcia *et al.* 2013), observaron una mejora en la motilidad espermática de verraco tras la descongelación, cuando procesaron el semen con Androcoll antes de realizar la congelación.

El proceso de congelación-descongelación es uno de los procesos mas utilizados por razones logísticas a la hora de manejar muestras espermáticas, y a la vez es uno de los procesos que mas daño causa sobre el espermatozoide, especialmente en sus membranas, no solo en ciervo rojo (Esteso *et al.* 2003), sino también en otras especies como por ejemplo la especie porcina (Peña *et al.* 2003). Los resultados obtenidos en nuestro estudio demostraron que la selección llevada a cabo mediante el uso de Androcoll-S tras la descongelación, mejoro el porcentaje de espermatozoides vivos con la membrana intacta y redijo el porcentaje de espermatozoides apoptóticos en ambos tipos de muestras espermática (electroeyaculadas y epididimarias) en comparación con las mismas muestras no seleccionadas. Además el porcentaje de espermatozoides viables sin marcadores apoptóticos mantuvo en valores mas altos para las muestras seleccionadas respecto a las no seleccionadas tras ser sometidas a una incubación de 2 h a 37ºC. García *et al.*

(2009) encontró unos resultados similares, donde la proporción de espermatozoides vivos aumentó y la proporción de espermatozoides apoptóticos disminuyó después realizar una selección con Androcoll en muestras espermáticas descongeladas de caballo.

Por su parte, la mitocondria espermática debe ser considerada como una de las estructuras mas sensibles del espermatozoide al shock frio (Ortega‐Ferrusola *et al.* 2008). En nuestro estudio, tras la selección con Androcoll llevada a cabo justo después de la descongelación, el porcentaje de espermatozoide con actividad mitocondrial alta aumento de forma muy notable respecto a las muestras no seleccionadas, y mantuvo dicha diferencia tras la incubación. Esta mejora es muy importante debido a la gran importancia de la mitocondrial dentro del funcionamiento del espermatozoide. Algunos estudios han demostrado que los ribosomas de las mitocondrias están muy relacionados con la traducción de proteínas en el espermatozoide (Gur y Breitbart 2006). Además, la inhibición de la traducción de proteínas podria reducir de forma significativa funciones del espermatozoide tales como la motilidad o la capacitación, produciendo en consecuencia una reducción de la fertilidad. También se ha demostrado que una actividad mitocondrial alta del espermatozoide puede relacionarse con altos valores de fertilidad en humanos (Gallon *et al.* 2006). Estas mejoras, tanto en la motilidad como en la actividad mitocondrial pueden estar relacionadas teniendo en cuenta la estrecha relación que guardan como Paoli *et al.* (2011) demostró.

El porcentaje de espermatozoides con alta fragmentación de su DNA mostro valores muy bajos tanto para las muestras obtenidas mediante electroeyaculación, como para aquellas obtenidas post-mortem. No se encontraron diferencias ni entre muestras seleccionadas y no seleccionadas tanto a la descongelación, como tras la incubación. Jiménez-Rabadán *et al.* (2012) obtuvieron resultados muy similares. Dicho estudio tampoco encontró ninguna diferencia en el porcentaje de fragmentación del DNA cuando compararon sus muestras seleccionadas con las no seleccionadas. El grado de compactación de la cromatina en espermatozoides de ciervo rojo es muy alto, y por este motivo no esperábamos altos porcentajes de fragmentación del DNA. Además varios estudios anteriores (Didion *et al.* 2009; Evenson y Wixon 2006;

Love 2005) demostraron que los umbrales de daño en la cromatina se sitúan en valores muy superiores a los obtenido en nuestro estudio. Es debido a ese grado tan bajo de fragmentación del DNA que no hemos podido observar ningún efecto con el uso de Androcoll respecto a las muestras no seleccionadas.

La conclusión a la que podemos llegar es que la selección mediante centrifugación con Androcoll-S, en muestras descongeladas de ciervo rojo, obtenidas tanto por electroeyaculación como post-mortem, es una técnica idónea que nos permite mejorar de forma notable la calidad espermática en ambos tipos de muestras.

En el capitulo 4 tratamos de evaluar los efectos del uso de 2 antioxidantes (glutatión reducido y trolox) en muestras descongeladas de ciervo rojo obtenidas mediante electroeyaculación. El uso de antioxidantes como aditivos a los medios de manejo espermático ha despertado gran interés en los últimos años. Sin embargo, los resultados obtenidos a menudo han sido muy variables e incluso en ocasiones contradictorios debido a las diferencias entre especies y entre antioxidantes. Un ejemplo es el hidroxitolueno butilado (BHT) que ha demostrado un efecto beneficioso en espermatozoides de perro (Neagu *et al.* 2010) o cerdo (Roca *et al.* 2004), no siendo así en espermatozoides de caballo (Morillo‐Rodríguez *et al.* 2012).

El proceso de congelación-descongelación de muestras espermáticas, es un proceso ampliamente extendido y necesario por razones logísticas, que puede inducir la generación de radicales libres de oxigeno y en consecuencia potenciar los efectos producidos por el shock frio y empeorar el proceso de criopreservación (Chatterjee y Gagnon 2001). Se sabe que los espermatozoides son altamente sensibles al la peroxidación lipídica, pero este efecto se ha demostrado que puede ser prevenido mediante el uso de antioxidantes o plasma seminal (Storey 1997). El uso de antioxidantes como aditivo de los medios de manejo, debe ser considerado como una herramienta que nos permita mejorar la calidad espermática para el aumento de los rendimientos de otros procesos tales como el sex-sorting espermático o la fecundación in vitro. El sex-sorting espermático, es una tecnología especialmente interesante en especies como el ciervo reojo donde el valor de

la producción se encuentra estrechamente ligado al sexo de los individuos. Como hemos podido describir en los capítulos 1 y 2 de la presente tesis doctoral es un proceso complejo donde la calidad de las muestras con las que se va a trabajar juega un papel critico a la hora de obtener un buen rendimiento, además de todos los pasos lesivos a los que son sometidos los espermatozoides durante el propio proceso; altas tasas de dilución, tinción con sondas fluorescentes, procesos mecánicos a lo largo del citómetro de flujo, la aplicación de cargas eléctricas a las gotas donde se encuentran sumergidos los mismos en el momento de la separación, o la centrifugación necesaria para reconcentrar las muestras. Por estos motivos, creemos que la adición de antioxidantes a los medios de trabajo podria ayudar a prevenir dichos efectos negativos.

Estudios previos con muestras espermáticas epididimarias en ciervo rojo mostraron al trolox en el rango µM como un buen aditivo para la suplementación de los medios de manejo (Martinez-Pastor *et al.* 2009; Martínez-Pastor *et al.* 2008). Además, Domínguez-Rebolledo *et al.* (2009) encontró efectos beneficiosos usando trolox a 1 mM en muestras epididimarias descongeladas de ciervo rojo tras someterlas a un proceso de lavado. Por el contrario, un estudio llevado a cabo en espermatozoides epididimarios de ciervo rojo, donde el diluyente de congelación se suplemento con trolox en el rango mM, mostro un efecto negativo tras la descongelación y tras someter las muestras a una incubación de 6 h a 39 ºC, especialmente en la motilidad (Anel-López *et al.* 2012). Sin embargo, en dicho estudio se pudo comprobar que el trolox produjo una fuerte inhibición de la peroxidación lipídica respecto a las muestras control y a las suplementadas con GSH. Por tanto la hipótesis que se planteo fue que a esas concentraciones en ese tipo de muestras el exceso de eliminación de ROS tuvo un efecto negativo en la calidad espermática. A pesar de las diferencias entre dicho estudio y el que representa este capitulo cuarto de la presente tesis doctoral (Origen espermático y momento de aplicación del antioxidante) obtuvimos resultados similares, donde la inhibición de la motilidad fue la principal acción llevada a cabo por el trolox. Por lo tanto, estos resultados nos sugieren que en espermatozoides de ciervo, estas concentraciones de trolox producen una ruptura del equilibrio redox hacia un déficit de ROS que

produce este efecto negativo en la calidad espermática. Hay que tener en cuenta que los Ros juegan un papel importante en algunas de las rutas metabólicas involucradas en la motilidad, capacitación espermática o reacción acrosómica (de Lamirande *et al.* 1997).

Por el contrario, el uso de GSH ha cumplido su objetivo, manteniendo la calidad espermática mas alta que las muestras control tras la incubación. Esta mejora, especialmente marcada en parámetros de movilidad, no solo ha mejorado los valores de movilidad total, sino también los de movilidad progresiva. Esta mejora en el mantenimiento de buenos valores de movilidad es importante dada la importancia de la velocidad espermática en la fertilidad en ciervo rojo (Malo *et al.* 2005). En la misma línea el GSH tras la incubación, mantuvo valores mas altos de actividad mitocondrial respecto al control sin aditivos y a las muestras suplementadas con trolox. Este efecto protector sobre la actividad mitocondrial durante la incubación es especialmente importante dada la importancia que esta estructura juega no solo en el mantenimiento energético de la célula, sino también por estar involucrada en otras rutas metabólicas relacionadas con la motilidad , capacitación o apoptosis (Peña *et al.* 2009).

Por su parte y como hemos observado en la mayoría de los capítulos de la presente tesis doctora, el % de espermatozoides con alta tasa de fragmentación del DNA fue mínimo., por lo que no se encontraron diferencias ni respecto al tiempo de incubación, ni entre los diferentes tratamientos con antioxidantes. Como ya se ha demostrado la cromatina de espermatozoides de ciervo rojo es muy resistente a ser dañada incluso después de largos periodos de almacenamiento (Fernández-Santos *et al.* 2009). Además, se sabe que el umbral, en el porcentaje de espermatozoides con un alto contenido de DNA fragmentado, para producir un impacto negativo en la fertilidad de diferentes especies (Didion *et al.* 2009; Evenson y Wixon 2006; Love 2005), se encuentra muy por encima los valores obtenidos en el presente estudio.

Los resultados de la presente tesis doctoral ponen de manifiesto que el proceso de sex-sorting es una técnica de reproducción asistida viable en el ciervo rojo que puede ser de gran valor para mejorar la producción de dicha especie. Además, el uso del gradiente de selección espermática mediante

centrifugación con Androcoll-S y la adición de antioxidantes (glutatión reducido) a los medios de manipulación espermática podrian ayudarnos a mejorar el rendimiento del proceso de sex-sorting espermático en ciervo rojo.

# BIBLIOGRAFÍA

## Bibliografía

Aitken, R.J., 1995. Free radicals, lipid peroxidation and sperm function. Reprod. Fertil. Dev. 7, 659–668.

Aitken, R.J., Jones, K.T., Robertson, S.A., 2012. Reactive oxygen species and sperm function--in sickness and in health. J. Androl. 33, 1096–1106.

Anel-López, L., Álvarez-Rodríguez, M., García-Álvarez, O., Álvarez, M., Maroto-Morales, A., Anel, L., de Paz, P., Garde, J.J., Martínez-Pastor, F., 2012. Reduced glutathione and Trolox (vitamin E) as extender supplements in cryopreservation of red deer epididymal spermatozoa. Anim. Reprod. Sci. 135, 37–46. doi:10.1016/j.anireprosci.2012.09.001

Anel-lópez, L., Garcia-Alvarez, O., Maroto-Morales, A., Iniesta-Cuerda, M., L. Anel-Lópeza O. Garcia-Alvareza, A.M.-M.M.I.-C.M.R.A.J.S.M.R.F.-S.J.J.G., Ramón, M., Soler, A.J., Fernández-Santos, M.R., Garde, J.J., 2015. Reduced glutathione addition improves both the kinematics and physiological quality of post-thawed red deer sperm. Anim. Reprod. Sci. 162, 73–79. doi:10.1016/j.anireprosci.2015.09.012

Asher, G. W., Berg, D. K., Evans, G., 2000. Storage of semen and artificial insemination in deer. Anim. Reprod. Sci. 62, 195–211. doi:10.1016/S0378-4320(00)00159-7

Asher, G.W., Monfort, S.L., Wemmer, C., 1999. Comparative reproductive function in cervids: implications for management of farm and zoo populations. J. Reprod. Fertil. 54, 143–156.

Atmaca, G., 2004. Antioxidant effects of sulfur-containing amino acids. Yonsei Med. J. 45, 776–788.

Baker, M.A., Aitken, R.J., 2004. The importance of redox regulated pathways in sperm cell biology. Mol. Cell. Endocrinol. 216, 47–54. doi:10.1016/j.mce.2003.10.068

Ball, B.A., Vo, A.T., Baumber, J., 2001. Generation of reactive oxygen species by equine spermatozoa. Am. J. Vet. Res. 62, 508–515. doi:10.2460/ajvr.2001.62.508

Boe-Hansen, G.B., Morris, I.D., Ersbøll, A.K., Greve, T., Christensen, P., 2005. DNA integrity in sexed bull sperm assessed by neutral Comet assay and sperm chromatin structure assay. Theriogenology 63, 1789–1802. doi:10.1016/j.theriogenology.2004.08.004

Brogliatti, G., Barreiro, G., Larraburu, G., Laborde, A., 2004. 10 CASA EVALUATION OF SEXED AND NON-SEXED FROZEN BULL SEMEN. Reprod. Fertil. Dev. 16, 127–128. doi:10.1071/RDv16n1Ab10

Chatterjee, S., Gagnon, C., 2001. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol. Reprod. Dev. 59, 451–458.

da Silva, C.M.B., Ferrusola, C.O., Rodriguez, A.M., Bolaños, J.M.G., Dávila, M.P., Morrell, J.M., Martínez, H.R., Tapia, J. a., Aparicio, I.M., Peña, F.J., Balao da Silva, C.M., Ortega Ferrusola, C., Morillo Rodriguez, a.,

Gallardo Bolaños, J.M., Plaza Dávila, M., Morrell, J.M., Rodriguez Martínez, H., Tapia, J. a., Aparicio, I.M., Peña, F.J., 2013. Sex sorting increases the permeability of the membrane of stallion spermatozoa. Anim. Reprod. Sci. 138, 241–251. doi:10.1016/j.anireprosci.2013.02.021

de Lamirande, E., Jiang, H., Zini, A., Kodama, H., Gagnon, C., 1997. Reactive oxygen species and sperm physiology. Rev. Reprod. 2, 48–54.

Didion, B.A., Kasperson, K.M., Wixon, R.L., Evenson, D.P., 2009. Boar fertility and sperm chromatin structure status: a retrospective report. J. Androl. 30, 655–660.

Domínguez-Rebolledo, Á.E., Fernández-Santos, M.R., Bisbal, A., Ros-Santaella, J.L., Ramón, M., Carmona, M., Martínez-Pastor, F., Garde, J.J., 2010. Improving the effect of incubation and oxidative stress on thawed spermatozoa from red deer by using different antioxidant treatments. Reprod. Fertil. Dev. 22, 856–870.

Domínguez-Rebolledo, A.E., Fernández-Santos, M.R., García-Álvarez, O., Maroto-Morales, A., Garde, J.J., Martínez-Pastor, F., 2009. Washing increases the susceptibility to exogenous oxidative stress in red deer spermatozoa. Theriogenology 72, 1073–1084.

Dorado, J., Gálvez, M.J., Morrell, J.M., Alcaráz, L., Hidalgo, M., 2013. Use of single-layer centrifugation with Androcoll-C to enhance sperm quality in frozen-thawed dog semen. Theriogenology 80, 955–962.

Esteso, M.C., Fernández-Santos, M.R., Soler, A.J., Garde, J.J., 2003. Head dimensions of cryopreserved red deer spermatozoa are affected by thawing procedure. CryoLetters 24, 260–267.

Evenson, D.P., Wixon, R., 2006. Clinical aspects of sperm DNA fragmentation detection and male infertility. Theriogenology 65, 979–991.

Fernandez-Santos, M.R., Martinez-Pastor, F., Garcia-Macias, V., Esteso, M.C., Soler, A.J., Paz, P., Anel, L., Garde, J.J., 2007. Sperm characteristics and DNA integrity of Iberian red deer (Cervus elaphus hispanicus) epididymal spermatozoa frozen in the presence of enzymatic and nonenzymatic antioxidants. J. Androl. 28, 294.

Fernández-Santos, M.R., Martínez-Pastor, F., Matias, D., Domínguez-Rebolledo, A.E., Esteso, M.C., Montoro, V., Garde, J.J., 2009. Effects of long-term chilled storage of red deer epididymides on DNA integrity and motility of thawed spermatozoa. Anim. Reprod. Sci. 111, 93–104.

Fernández-Santos, M.R., Domínguez-Rebolledo, A.E., Esteso, M.C., Garde, J.J., Martínez-Pastor, F., 2009. Refrigerated storage of red deer epididymal spermatozoa in the epididymis, diluted and with vitamin C supplementation. Reprod. Domest. Anim. 44, 212–220.

Gallon, F., Marchetti, C., Jouy, N., Marchetti, P., 2006. The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability. Fertil. Steril. 86, 1526–1530.

Gao, Q.H., Wang, H.E., Zeng, W.B., Wei, H.J., Han, C.M., Du, H.Z., Zhang, Z.G., Li, X.M., 2011. Embryo transfer and sex determination following

superovulated hinds inseminated with frozen–thawed sex-sorted Y sperm or unsorted semen in Wapiti (Cervus elaphus songaricus). Anim. Reprod. Sci. 126, 245–250. doi:10.1016/j.anireprosci.2011.05.006

Gao, Q.H., Wei, H.J., Han, C.M., Du, H.Z., Zhang, Z.G., Zhao, W.G., Zhang, Y., Li, S., 2010. Successful low dose insemination of flow cytometrically sorted Sika (Cervus nippon) sperm in Wapiti (Cervus elaphus). Anim. Reprod. Sci. 118, 89–93.

García, B.M., Morrell, J.M., Ortega-Ferrusola, C., González-Fernández, L., Tapia, J.A., Rodriguez-Martinez, H., Peña, F.J., 2009. Centrifugation on a single layer of colloid selects improved quality spermatozoa from frozen-thawed stallion semen. Anim. Reprod. Sci. 114, 193–202.

Gosálvez, J., Ramirez, M.A., López-Fernández, C., Crespo, F., Evans, K.M., Kjelland, M.E., Moreno, J.F., 2011. Sex-sorted bovine spermatozoa and DNA damage: I. Static features. Theriogenology 75, 197–205. doi:10.1016/j.theriogenology.2010.08.006

Graaf, S. De, Evans, G., 2007. Successful low dose insemination of flow cytometrically sorted ram spermatozoa in sheep. … Domest. Anim.

Gur, Y., Breitbart, H., 2006. Mammalian sperm translate nuclear-encoded proteins by mitochondrial-type ribosomes. Genes Dev. 20, 411–416.

Hollinshead, F.K., Evans, G., Evans, K.M., Catt, S.L., Maxwell, W.M., O'Brien, J.K., 2004. Birth of lambs of a pre-determined sex after in vitro production of embryos using frozen-thawed sex-sorted and re-frozen-thawed ram spermatozoa. Reproduction 127, 557–568.

Jabbour, H.N., Hayssen, V., Bruford, M.W., 1997. Conservation of deer: contributions from molecular biology, evolutionary ecology, and reproductive physiology. J. Zool. 243, 461–484.

Jiménez-Rabadán, P., Morrell, J.M., Johannisson, A., Ramón, M., García-Álvarez, O., Maroto-Morales, A., Álvaro-García, P.J., Pérez-Guzmán, M.D., Fernández-Santos, M.R., Garde, J.J., 2012. Single layer centrifugation (SLC) improves sperm quality of cryopreserved Blanca-Celtibérica buck semen. Anim. Reprod. Sci. 136, 47–54.

Kjelland, M.E., González-Marín, C., Gosálvez, J., López-Fernández, C., Lenz, R.W., Evans, K.M., Moreno, J.F., 2011. DNA fragmentation kinetics and postthaw motility of flow cytometric-sorted white-tailed deer sperm. J. Anim. Sci. 89, 3996–4006. doi:10.2527/jas.2011-4014

Leahy, T., Celi, P., Bathgate, R., Evans, G., Maxwell, W.M.C., Marti, J.I., 2010. Flow-sorted ram spermatozoa are highly susceptible to hydrogen peroxide damage but are protected by seminal plasma and catalase. Reprod. Fertil. Dev. 22, 1131–1140.

Lindsey, A., Muckle, L., Squires, E., 2003. Effects of caffeine stimulation on stallion sperm motion characteristics following 18-h storage, flow sorting, and cryopreservation. Theriogenology.

Lipovac, M.G. V, Gavella, M., Lipovac, V., 2000. Antioxidative effect of melatonin on human spermatozoa. Syst. Biol. Reprod. Med. 44, 23–27.

Lopes M.Sc., S., Sun Ph.D., J.-G., Jurisicova Ph.D., A., Meriano B.Sc., J., Casper M.D., R.F., D, J.-G.S.P., D, A.J.P., Sc, J.M.B., MD, R.F.C., 1998. Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. Fertil. Steril. 69, 528–532. doi:10.1016/S0015-0282(97)00536-0

Love, C.C., 2005. The sperm chromatin structure assay: a review of clinical applications. Anim. Reprod. Sci. 89, 39–45.

Malo, A.F., Garde, J.J., Soler, A.J., Garcia, A.J., Gomendio, M., Roldan, E.R., 2005. Male fertility in natural populations of red deer is determined by sperm velocity and the proportion of normal spermatozoa. Biol. Reprod. 72, 822–829.

Maneesh, M., Jayalekshmi, H., 2006. Role of reactive oxygen species and antioxidants on pathophysiology of male reproduction. Indian J. Clin. Biochem. 21, 80–89.

Martinez-Alborcia, M.J., Morrell, J.M., Gil, M.A., Barranco, I., Maside, C., Alkmin, D. V, Parrilla, I., Martinez, E.A., Roca, J., 2013. Suitability and effectiveness of single layer centrifugation using Androcoll-P in the cryopreservation protocol for boar spermatozoa. Anim. Reprod. Sci. 140, 173–179.

Martinez-Pastor, F., Aisen, E., Fernandez-Santos, M.R., Esteso, M.C., Maroto-Morales, A., Garcia-Alvarez, O., Garde, J.J., 2009. Reactive oxygen species generators affect quality parameters and apoptosis markers differently in red deer spermatozoa. Reproduction 137, 225.

Martínez-Pastor, F., Fernández-Santos, M.R., Olmo, E. Del, Domínguez-Rebolledo, A.E., Esteso, M.C., Montoro, V., Garde, J.J., 2008. Mitochondrial activity and forward scatter vary in necrotic, apoptotic and membrane-intact spermatozoan subpopulations. Reprod. Fertil. Dev. 20, 547–556.

Mickle, D.A., Weisel, R.D., 1993. Future directions of vitamin E and its analogues in minimizing myocardial ischemia-reperfusion injury. Can. J. Cardiol. 9, 89–93.

Morillo-Rodríguez, A., Macías-García, B., Tapia, J.A., Ortega-Ferrusola, C., Peña, F.J., 2012. Consequences of butylated hydroxytoluene in the freezing extender on post-thaw characteristics of stallion spermatozoa in vitro. Andrologia 44, 688–695.

Morton, K.M., Rowe, A.M., Maxwell, W.M.C., Evans, G., 2006. In vitro and in vivo survival of bisected sheep embryos derived from frozen-thawed unsorted, and frozen-thawed sex-sorted and refrozen-thawed ram spermatozoa. Theriogenology 65, 1333–1345.

Neagu, V.R., García, B.M., Sandoval, C.S., Rodríguez, A.M., Ferrusola, C.O., Fernández, L.G., Tapia, J.A., Peña, F.J., 2010. Freezing dog semen in presence of the antioxidant butylated hydroxytoluene improves postthaw sperm membrane integrity. Theriogenology 73, 645–650.

Ortega-Ferrusola, C., Sotillo-Galán, Y., Varela-Fernández, E., Gallardo-Bolaños, J.M., Muriel, A., González-Fernández, L., Tapia, J.A., Pena, F.J., 2008. Detection of "Apoptosis-Like" Changes During the Cryopreservation Process in Equine Sperm. J. Androl. 29, 213–221.

Paoli, D., Gallo, M., Rizzo, F., Baldi, E., Francavilla, S., Lenzi, A., Lombardo, F., Gandini, L., 2011. Mitochondrial membrane potential profile and its correlation with increasing sperm motility. Fertil. Steril. 95, 2315–2319.

Peña, F.J., Johannisson, A., Wallgren, M., Rodríguez-Martínez, H., 2003. Assessment of fresh and frozen–thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity. Theriogenology 60, 677–689.

Peña, F.J., Martínez, H.R., Tapia, J.A., Ferrusola, C.O., Fernandez, L.G., García, B.M., 2009. Mitochondria in mammalian sperm physiology and pathology: a review. Reprod. Domest. Anim. 44, 345–349.

Peña, F.J.J., Johannisson, A., Wallgren, M., Martinez, H.R., Peña, F.J.J., Johannisson, A., Wallgren, M., Martinez, H.R., Rodriguez Martinez, H., 2003. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Anim. Reprod. Sci. 78, 85–98. doi:10.1016/S0378-4320(03)00049-6

Prior, R.L., Wu, X., Schaich, K., Ronald, L., Wu, X., Schaich, K., 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J. Agric. Food Chem. 53, 4290–4302.

Pukazhenthi, B.S., Wildt, D.E., 2003. Which reproductive technologies are most relevant to studying, managing and conserving wildlife? Reprod. Fertil. Dev. 16, 33–46.

Rath, D., Moench-Tegeder, G., Taylor, U., Johnson, L.A., 2009. Improved quality of sex-sorted sperm: A prerequisite for wider commercial application. Theriogenology 71, 22–29.

Roca, J., Gil, M.A., Hernandez, M., Parrilla, I., Vazquez, J.M., Martinez, E.A., 2004. Survival and Fertility of Boar Spermatozoa After Freeze-Thawing in Extender Supplemented With Butylated Hydroxytoluene. J. Androl. 25, 397–405.

Storey, B.T., 1997. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. Mol. Hum. Reprod. 3, 203–213.

Suh, T., Schenk, J., 2003. Pressure during flow sorting of bull sperm affects post-thaw motility characteristics. Theriogenology.

Thys, M., Vandaele, L., Morrell, J.M., Mestach, J., Soom, A. Van, Hoogewijs, M., Rodriguez-Martinez, H., 2009. In vitro Fertilizing Capacity of Frozen-thawed Bull Spermatozoa Selected by Single-layer (Glycidoxypropyltrimethoxysilane) Silane-coated Silica Colloidal Centrifugation. Reprod. Domest. Anim. 44, 390–394.

# CONCLUSIONES

1. Las muestras espermáticas de ciervo rojo pueden ser sexadas en pureza de espermatozoides –Y con éxito, obteniendo dichas muestras mejor calidad espermática *in-vitro* que las muestras en pureza –X e incluso que las muestras espermáticas convencionales tras ser sometidas a un proceso de congelación-descongelación. Sin embargo, las muestras de espermatozoides –Y obtuvieron peores resultados de fertilidad *in-vivo* que las muestras sometidas al proceso de sorting sin ser separadas en pureza

2. El proceso de sorting produce un efecto subletal sobre los espermatozoides de ciervo rojo incrementando el porcentaje de células con marcadores apoptóticos que se manifiesta tras un proceso de congelación-descongelación, pero al mismo tiempo selecciona una población de espermatozoides con una cromatina menos susceptible al estrés oxidativo que las muestras espermáticas convencionales

3. La selección espermática mediante la centrifugación de muestras espermáticas de ciervo rojo obtenidas por electroeyaculación o post-mortem con un coloide monocapa (Androcoll-S) es una técnica idónea que nos permite mejorar la calidad espermática tras la descongelación en ambos tipos de muestras y en consecuencia nos permitiría mejorar otro tipo de técnicas de reproducción asistida.

4. El uso de glutatión reducido como aditivo de los diluyentes espermáticos de ciervo rojo en el rango milimolar (1, 2 and 5 mM), por sus efectos beneficiosos en la calidad espermática, podría ser una herramienta importante para la mejora de los medios tanto para el proceso de sex-sorting, como para otro tipo de técnicas tales como la fecundación in vitro.

5. El uso de trolox (análogo de la vitamina E) en el rango milimolar (1, 2 y 5 mM) no es un aditivo idóneo para la suplementación de muestras espermáticas de ciervo rojo obtenidas por electroeyaculación debido a si efecto negativo sobre la motilidad espermática y la ausencia de efecto para la mejora de otros parámetros fisiológicos como la viabilidad, la integridad de membranas o la actividad mitocondrial tras someter dichas muestras a un proceso de congelación-descongelación.

# CONCLUSIONS

1. Red deer sperm can be sex-sorted successfully for high purity Y- sperm, showing these samples better quality than X- sperm, even sometimes higher than conventional samples after a freezing-thawing process but, reporting worse fertility results than the bulk sorted sperm

2. The Sorting process performs a subletal effect, which increased the percentage of apoptotic cells, but at the same time the sorting process selects a sperm population with a more resistant chromatin to be injured by oxidative stress than conventional sperm samples after incubation

3. The sperm selection by single layer centrifugation with Androcoll-S after thawing for red deer sperm obtained by electroejaculation or post mortem is a suitable technique that can allow us to improve the sperm quality after thawing in both kinds of sperm samples and therefore to improve other assisted reproductive techniques

4. The use of reduced glutathione to supplement semen extenders in the millimolar range (1, 2 and 5 mM), because of its beneficial effect on the sperm quality, might be useful tool for improving the media, either for sperm sex sorting or other techniques such as in vitro fertilization in red deer sperm samples.

5. The use of trolox in the millimolar range (1, 2 and 5 mM) is not suitable as a supplement for electroejaculated red deer sperm because of its negative effect on sperm motility and the absence of any effect for improving physiological parameters such as viability, membrane integrity or mitochondrial activity

# ANEXO

Los trabajos llevados a cabo en la presente tesis doctoral que componen los capítulos 1 a 4 han dado lugar a 4 artículos científicos:

1. ***Effect of sex sorting and cryopreservation on sperm quality of Iberian red deer thawed spermatozoa***. Ha sido enviado a evaluar a la revista Theriogenology
2. ***The impact of oxidative stress on thawed bulk sorted red deer sperm***. Ha sido enviado a evaluar a la revista Reproduction in Domestic Animals.
3. ***Use of Androcoll-S after thawing improves the quality of electroejaculated and epididymal sperm samples from red deer***. Ha sido publicado en la revista Animal Reproduction Science.
4. ***Reduced glutathione addition improves both the kinematics and physiological quality of post-thawed red deer sperm***. Ha sido publicado en la revista Animal Reproduction Science.