ISABEL ORTIZ JARABA

Efecto de la selección de espermatozoides mediante centrifugación coloidal sobre la calidad de las dosis de semen de asno refrigeradas y congeladas

Effect of sperm selection using colloid centrifugation on sperm quality of cooled and frozen-thawed donkey semen

UNIVERSIDAD DE CÓRDOBA
FACULTAD DE VETERINARIA
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ISABEL ORTIZ JARABA

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"EFECTO DE LA SELECCIÓN DE ESPERMATOZOIDES MEDIANTE CENTRIFUGACIÓN COLOIDAL SOBRE LA CALIDAD DE LAS DOSIS DE SEMEN DE ASNO REFRIGERADAS Y CONGELADAS"

"Effect of Sperm Selection using Colloid Centrifugation on Sperm Quality of Cooled and Frozen-Thawed Donkey Semen"

Memoria para optar al grado de Doctor presentada por:

Isabel Ortiz Jaraba

Bajo la dirección de los Doctores:

Manuel Hidalgo Prieto

Jesús M. Dorado Martín

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TITULO: *Efecto de la selección de espermatozoides mediante centrifugación coloidal sobre la calidad de las dosis de semen de asno refrigeradas y congeladas*

AUTOR: *Isabel Ortiz Jaraba*

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5. **I. Ortiz**, J. Dorado, J. M. Morrell, J. Gosálvez, F. Crespo, J.M. Jiménez, M. Hidalgo. New approach to assess sperm DNA fragmentation dynamics: Fine-tuning mathematical models. Submitted to Journal of Animal Science and Biotechnology

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

INFORMAN:

Que el trabajo de tesis presentado por Dña. Isabel Ortiz Jaraba, titulado "*Efecto de la selección de espermatozoides mediante centrifugación coloidal sobre la calidad de las dosis de semen de asno refrigeradas y congeladas*" ha sido realizado bajo nuestra dirección y cumple con los artículos 24 y 35 de la norma reguladora de los Estudios de Doctorado de la Universidad de Córdoba para su presentación como compendio de publicaciones, así como para obtener la mención internacional.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 3 de octubre de 2016

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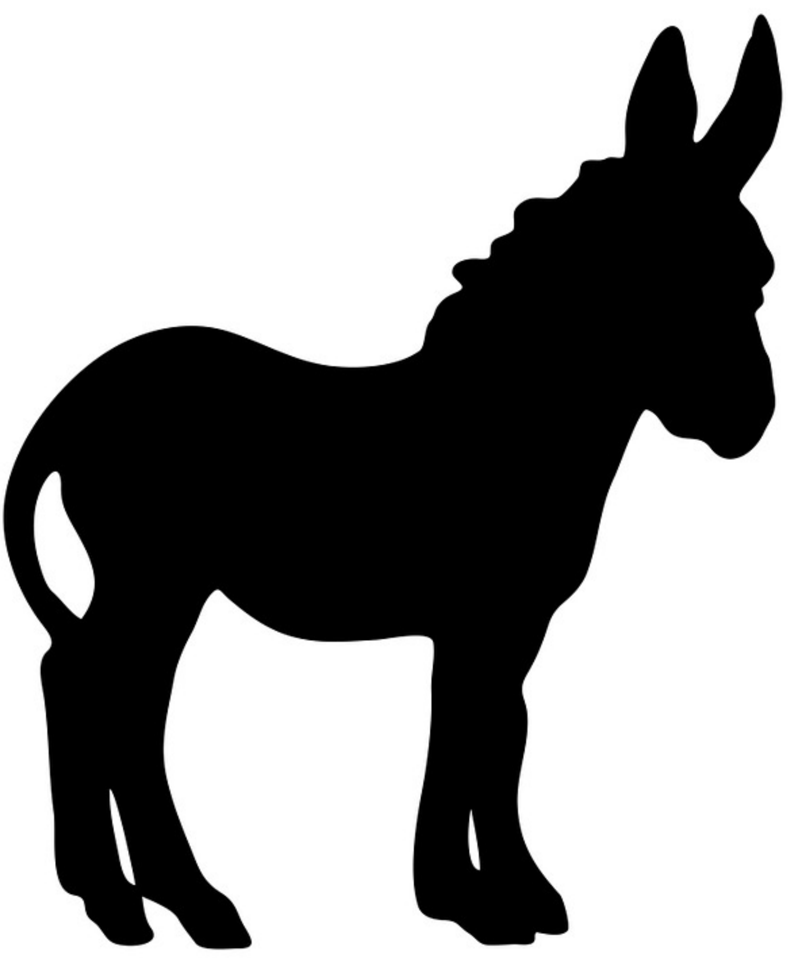

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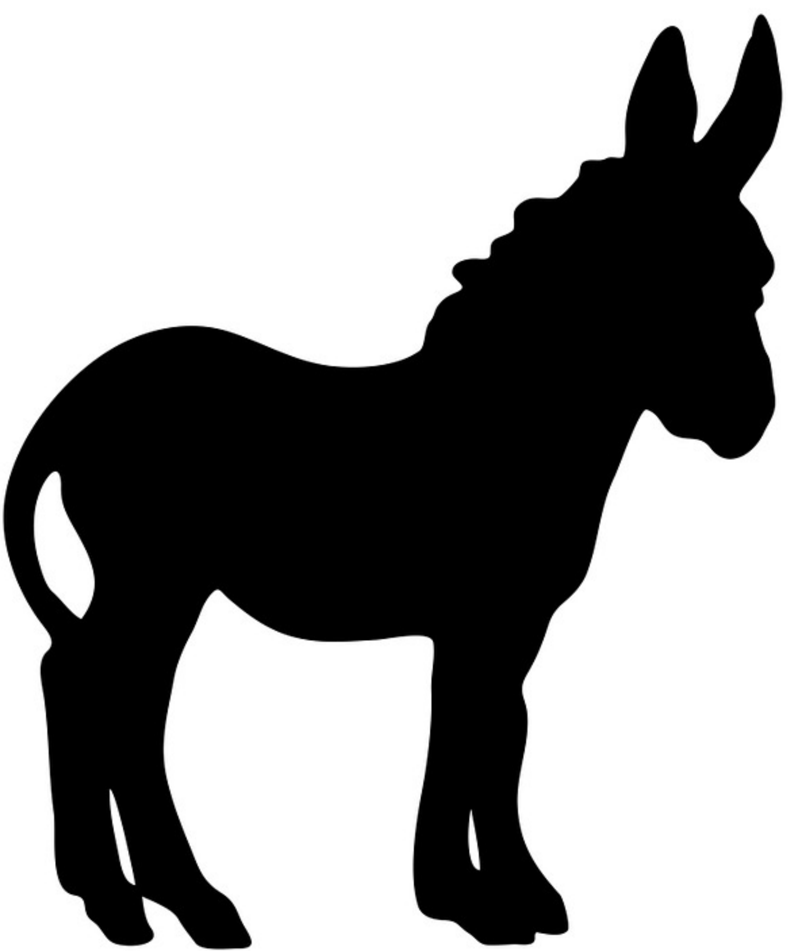

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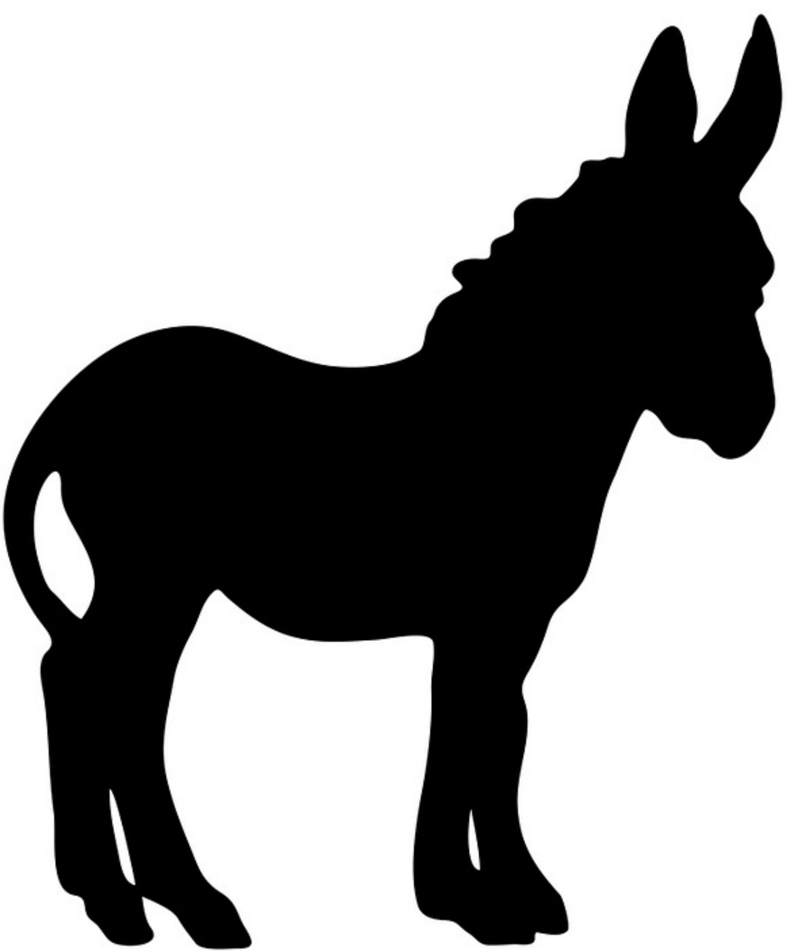
RESUMEN



RESUMEN

A día de hoy, los asnos han perdido su rol tradicional, lo que ha resultado en la inclusión de las razas de asnos ibéricas en el catálogo de especies amenazadas de la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO). En la actualidad, la importancia de los asnos está aumentando de nuevo y es esencial que los garañones con características genéticas más deseables puedan ser empleados para aumentar el número de individuos. La inseminación artificial con semen refrigerado o congelado es una de las estrategias más populares a la hora de enviar y preservar material genético de asnos valiosos. Sin embargo, estas técnicas dañan al espermatozoide, disminuyendo en consecuencia las tasas de gestación. En este sentido, la selección espermática mediante centrifugación coloidal de una sola capa (SLC) ha mostrado ser útil en la selección de espermatozoides con mejor calidad en distintas especies animales. Sin embargo, al inicio de la presente Tesis Doctoral, no había ningún estudio que evalúe el efecto de la SLC en semen de asno; además, no existe ningún coloide específicamente diseñado para semen de asno. Por lo tanto, es esencial valorar el efecto que diferentes coloides tienen en la calidad espermática de semen de asno refrigerado y congelado.

En la primera publicación se estudió el efecto de la centrifugación coloidal de una sola capa (SLC) con Androcoll-E-Large en los parámetros espermáticos de semen de asno tras 24 h de refrigeración. Para ello, se obtuvieron eyaculados de asnos de raza asnal Andaluza que se refrigeraron a 5°C. La SLC se llevó a cabo transcurridas 24 h de refrigeración usando el coloide Androcoll-E-Large. En el primer experimento, todos los parámetros espermáticos analizados (movimiento total y progresivo, vitalidad, morfología espermática y parámetros cinéticos) se compararon estadísticamente entre las muestras de semen antes y después de ser procesadas mediante SLC. Los parámetros evaluados obtuvieron valores significativamente más altos ($P<0,05$) en las muestras procesadas mediante SLC. En el segundo experimento, las muestras de semen se clasificaron en dos grupos en función del movimiento progresivo antes de la SLC. No se encontraron diferencias significativas entre grupos, lo que indica que la SLC mejoró los parámetros de calidad espermática de todo el conjunto de muestras procesadas, independientemente de su movimiento progresivo original. En conclusión, la SLC con Androcoll-E-Large puede ser utilizada en semen de asno, aumentando la calidad espermática de las dosis de semen tras 24 h de refrigeración.

El objetivo de la segunda publicación fue determinar si la SLC mejora la calidad del semen descongelado de asno y si esta mejora potencial está relacionada con la resistencia a la congelación del eyaculado. Así, se congeló semen de asnos de raza asnal Andaluza mediante un protocolo estándar. Se analizaron y compararon los parámetros espermáticos de muestras descongeladas antes y después de ser sometidas a SLC. La calidad espermática se estimó integrando en un único valor el movimiento espermático (valorado mediante análisis espermático computarizado), morfología y vitalidad (evaluadas respectivamente con microscopía de campo claro y fluorescencia). La resistencia a la congelación espermática se definió como la relación entre la calidad espermática obtenida antes y después de la congelación-descongelación. Los eyaculados se clasificaron en grupos de resistencia a la congelación baja, media y alta usando los percentiles 25 y 75 como límites. Todos los parámetros espermáticos fueron significativamente más altos ($P<0,01$) en las muestras seleccionadas con SLC que en las no seleccionadas, siendo algunos parámetros cinéticos incluso más altos que en el semen fresco. El incremento de los parámetros espermáticos tras la SLC estuvo correlacionado con la resistencia a la congelación del eyaculado, obteniéndose los valores más altos tras someter a SLC las muestras procedentes de eyaculados con baja resistencia a la congelación. Concluimos que, en base a los parámetros de calidad espermática evaluados, la SLC puede ser un procedimiento adecuado para mejorar la calidad espermática post-descongelación del semen criopreservado de asno, particularmente en aquellos eyaculados con baja resistencia a la congelación.

En la tercera publicación, los objetivos propuestos fueron determinar la calidad espermática de las muestras de semen de asno congeladas y descongeladas tras SLC usando Androcoll-E-Small en comparación con la centrifugación simple (SW) y la no centrifugación (UDC) y valorar si el efecto en la calidad espermática tras SLC o SW depende de la calidad de la muestra. Los eyaculados congelados y descongelados de asno de raza asnal Andaluza se dividieron en tres alícuotas y se procesaron usando tres técnicas diferentes tras la descongelación: el control diluido y no centrifugado (UDC), centrifugación simple (SW) y centrifugación coloidal (SLC). Después, el índice de calidad espermática se estimó integrando todos los parámetros (movimiento total y progresivo, integridad de la membrana y fragmentación del ADN) en un único valor. Se estudió la relación entre la calidad espermática de las muestras descongeladas y diluidas (UDC) y el efecto de SW y SLC en los parámetros espermáticos. El índice de calidad espermática fue significativamente superior ($P<0,001$) en las muestras procesadas mediante SLC ($0,8 \pm$

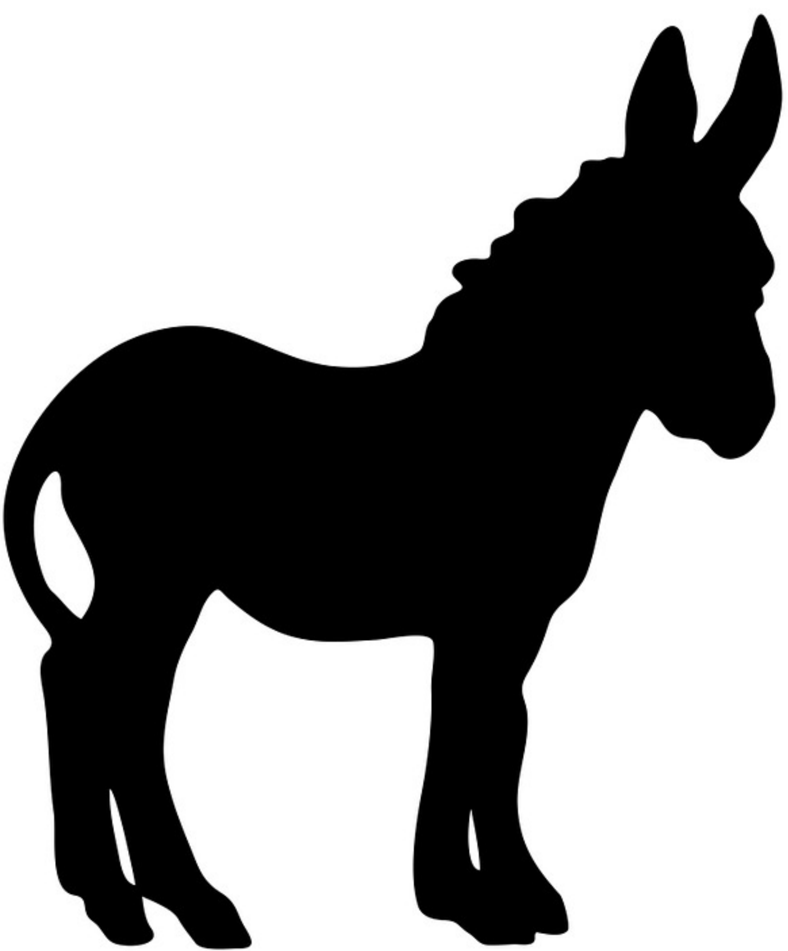
0,0) que en las UDC (0,6 ± 0,0) y SW (0,6 ± 0,0), independientemente del índice de calidad espermático de la muestra tras descongelación. En conclusión, la SLC en semen de asno congelado y descongelado usando Androcoll-E-Small puede ser un procedimiento adecuado para seleccionar espermatozoides con mejor calidad, en particular en aquellas muestras donde se necesita un aumento en el movimiento espermático.

En el cuarto estudio se compararon dos coloides (EquiPure y Androcoll) diseñados para semen de caballo, pero aptos también para la selección de espermatozoides de semen descongelado de asno, para valorar si hay alguna diferencia en su capacidad de seleccionar espermatozoides con buen movimiento, integridad de la membrana plasmática y mejor distribución de las subpoblaciones de movimiento espermático. Las muestras de semen se dividieron en los siguientes tratamientos: una alícuota antes de la congelación (Fresh) y cuatro alícuotas tras la descongelación: control diluido no centrifugado (UDC), centrifugación simple (SW), centrifugación coloidal usando EquiPure Bottom Layer (SLC-EquiPure) y Androcoll (SLC-Androcoll). Se compararon entre tratamientos el movimiento total (TM), progresivo (PM) y parámetros cinéticos (valorados con análisis de esperma computarizado), integridad de la membrana plasmática (IMS, evaluada mediante microscopía de fluorescencia), y distribución de las subpoblaciones de espermatozoides móviles (sP). SLC-EquiPure y SLC-Androcoll fueron los tratamientos que obtuvieron mayores TM y PM. SLC-Androcoll consiguió los valores medios más altos en la mayoría de los parámetros cinéticos, pero SLC-EquiPure mejoró la IMS. Se encontraron cuatro sP, SLC-Androcoll seleccionó un mayor porcentaje de espermatozoides pertenecientes a la sP4, es decir, los más rápidos y progresivos. En conclusión, la selección coloidal usando EquiPure o Androcoll tiene un efecto diferente en las características espermáticas. EquiPure selecciona espermatozoides con membranas intactas y movimiento progresivo pero relativamente lentos, mientras que Androcoll selecciona los espermatozoides más rápidos y progresivos.

El quinto estudio se centró en la fragmentación del ADN espermático en semen de asno. La fragmentación del ADN espermático (sDF) se considera un parámetro importante a la hora de predecir *in vitro* la fertilidad potencial de una muestra de semen. La centrifugación coloidal (SLC) podría ser una técnica adecuada para seleccionar los espermatozoides de asno más resistentes a la fragmentación del ADN tras la descongelación. Estudios previos han mostrado que, para evidenciar el daño latente en la molécula de ADN, sDF debe ser valorada de forma dinámica, donde la velocidad de fragmentación del ADN en cada tratamiento indica cómo de resistente es el ADN al daño

iatrogénico. La velocidad de fragmentación se calcula usando la pendiente de una ecuación de regresión lineal. Sin embargo, no se ha estudiado si las dinámicas de fragmentación del ADN espermático se ajustan a este modelo. Los objetivos de este estudio fueron por tanto evaluar el efecto de distintos tratamientos post-descongelación en la fragmentación del ADN espermático y elucidar el modelo matemático más preciso (regresión lineal, exponencial o polinómico) para la fragmentación del ADN a lo largo del tiempo en semen de asno congelado y descongelado. Una vez sometidas las muestras a dilución (UDC), centrifugación simple (SW) y centrifugación coloidal (SLC), se observó que los valores de sDF fueron significativamente menores tras 6 h de incubación en SLC que en UDC y SW. Los valores del coeficiente de determinación (R^2) fueron significativamente mayores para el modelo polinómico de segundo grado que para el lineal o el exponencial. Los valores más altos de aceleración de fragmentación (asDF) se obtuvieron en las muestras SW, seguidas de SLC y UDC. En conclusión, la SLC tras descongelación parece preservar durante más tiempo la longevidad del ADN en comparación con UDC y SW. Además, el ajuste de modelos ha mostrado que las dinámicas de fragmentación espermática en semen de asno congelado y descongelado se ajusta a un modelo polinómico de segundo grado, lo que implica que la velocidad de fragmentación no es una constante y que la aceleración de la fragmentación del ADN debe de ser tenida en cuenta a la hora de evidenciar daño oculto en la molécula del ADN.

SUMMARY



SUMMARY

Andalusian donkey has lost its traditional role, which has resulted in the inclusion of this breed in the United Nations Food and Agricultural Organization endangered species list. Nowadays, the importance of donkey is increasing again and it is essential that the jackasses with desirable genetic features can be used as sperm donors. Artificial insemination (AI) using cooled and frozen-thawed semen doses is one of the most popular strategies in order to ship and preserve genetic material from valuable individuals. However, these techniques cause major damage to spermatozoa, consequently decreasing the pregnancy rates. In this regard, sperm selection by single layer centrifugation (SLC) through silica colloids has shown to select those spermatozoa with better quality in several animal species. However, there is not any study which assesses the effect that SLC has on donkey sperm, besides, there is not any colloid specifically designed for donkey sperm. Therefore, it is of the utmost importance to evaluate the effect of different colloids on sperm quality in cooled and frozen-thawed donkey semen.

The first study aimed to determine the effect of single layer centrifugation (SLC) using Androcoll-E-Large on donkey sperm quality parameters after 24 h of cool-storage. Ejaculates were collected from Andalusian donkeys and then cooled at 5°C. SLC was carried out after 24 h of cool-storage using Androcoll-E-Large. In the first experiment, all sperm parameters assessed (total and progressive sperm motility, viability, sperm morphology and sperm kinematics VCL, VSL, VAP, LIN, STR, WOB, ALH, and BCF) were statistically compared between semen samples processed or not with Androcoll-E-Large. Significant differences ($P<0.05$) were found between SLC-selected and unselected semen samples for all parameters assessed, obtaining better results after SLC. In the second experiment, semen samples were classified in two groups according to their sperm progressive motility (PM) before SLC. Then, the increments obtained in semen quality parameters after SLC were compared between groups. No significant differences were found between groups, indicating that SLC improved the sperm quality parameters of the entire set of semen samples processed with independence to their original PM. In conclusion, SLC with Androcoll-E-Large can be used in donkeys, increasing the sperm quality of cooled-stored donkey semen doses after 24 h of cool storage.

The objective of the second study was to determine whether colloid SLC improves post-thaw donkey sperm quality and if it is related to ejaculate freezability. Semen from Andalusian donkeys was frozen following a standard protocol. SLC was performed on frozen-thawed semen and post-thaw sperm parameters were compared with uncentrifuged samples. Sperm quality was estimated by integrating in a single value sperm motility (assessed by computer-assisted sperm analysis), morphology and viability (evaluated under bright-field or fluorescence microscopy). Sperm freezability was calculated as the relationship between sperm quality obtained before freezing and after thawing. Ejaculates were classified into low, medium and high freezability groups using the 25th and 75th percentiles as thresholds. All sperm parameters were significantly ($P<0.01$) higher in SLC-selected samples in comparison to uncentrifuged frozen-thawed semen and several kinematic parameters were even higher than those obtained in fresh semen. The increment of sperm parameters after SLC selection was correlated with ejaculate freezability, obtaining the highest values after SLC in samples with low freezability. Based on the sperm quality parameters evaluated, SLC can be a suitable procedure to improve post-thaw sperm quality of cryopreserved donkey semen, in particular for those ejaculates with low freezability.

The aims of the third study were to determine the sperm quality of frozen-thawed donkey sperm samples after SLC using Androcoll-E-Small in comparison to sperm washing (SW) or no centrifugation and to determine if the effect on the sperm quality after SLC or SW depends on the quality of the sample. Frozen-thawed sperm samples from Andalusian donkeys were divided into three aliquots, and they were processed using three different techniques after thawing: uncentrifuged diluted control (UDC), SW and SLC. Afterward, sperm quality index was estimated by integrating all parameters (total and progressive sperm motility, membrane integrity, and DNA fragmentation) in a single value. The relationship between the sperm quality of thawed UDC samples and the effect on sperm parameters in SW and SLC-selected samples was assessed. Sperm quality index was significantly higher ($P<0.001$) in SLC (0.8 ± 0.0) samples than in UDC (0.6 ± 0.0) and SW (0.6 ± 0.0) samples, regardless of the sperm quality index after thawing of the sperm sample. In conclusion, SLC of frozen-thawed donkey spermatozoa using Androcoll-E-Small can be a suitable procedure for selecting frozen-thawed donkey sperm with better quality, in particular in those samples where an improvement in motility is needed.

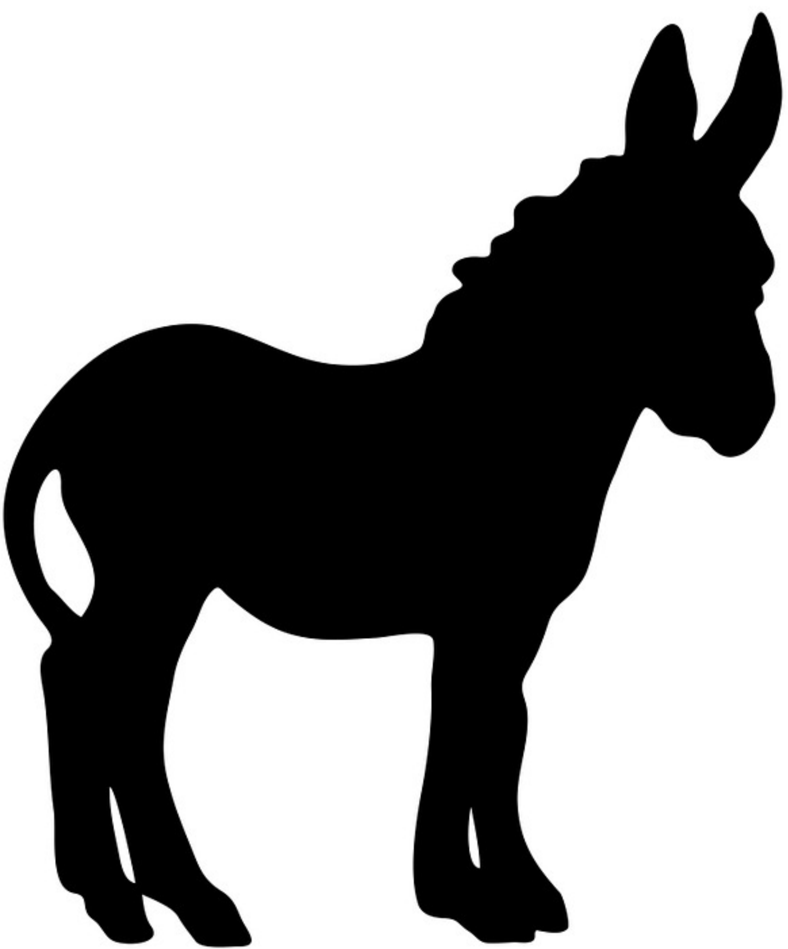
The fourth study compared two colloids developed for stallion semen (EquiPure and Androcoll) in order to assess if there was any difference in their ability to select sperm

with good motility, membrane integrity and a better distribution of the motile subpopulations. For this purpose, semen samples were divided between the following treatments: one aliquot prior to freezing (Fresh), and four after thawing: uncentrifuged diluted control, sperm washing, colloid centrifugation using EquiPure Bottom Layer (SLC-EquiPure) and Androcoll-E (SLC-Androcoll). Total (TM), progressive (PM) and sperm motility kinematics (assessed by computer-assisted sperm analysis); membrane integrity (IMS, evaluated under fluorescence microscopy); and distribution of motile sperm subpopulations (sP) were compared between treatments. SLC-EquiPure and SLC-Androcoll showed higher TM and PM than the other treatments. The highest mean values in most kinematics were obtained for SLC-Androcoll, but IMS was improved after SLC-EquiPure. Four sP were found, SLC-Androcoll selected more sperm in sP4, i. e. those with more progressive and faster sperm. In conclusion, colloid centrifugation using Androcoll or EquiPure has a different effect on sperm features. EquiPure selects sperm with intact membranes and progressive but relatively slow sperm, whereas Androcoll selects the fastest and most progressively motile sperm.

The fifth study focused on sperm DNA fragmentation (sDF). sDF has been proved to be an important parameter in order to predict in vitro the potential fertility of a semen sample. Colloid centrifugation could be a suitable technique to select those donkey sperm more resistant to DNA fragmentation after thawing. Previous studies have shown that to elucidate the latent damage of the DNA molecule, sDF should be assessed dynamically, where the rate of fragmentation between treatments indicate how resistant is DNA to iatrogenic damage. The rate of fragmentation is calculated using the slope of a linear regression equation. However, it has not been studied if sDF dynamics fits this model. The objectives of this study were to evaluate the effect of different after-thawing centrifugation protocols on sperm DNA fragmentation and elucidate the most accurate mathematical model (linear regression, exponential or polynomial) for DNA fragmentation over time in frozen-thawed donkey semen. After submitting post-thaw semen samples to no centrifugation (UDC), SW and SLC protocols, sDF values were significantly lower after 6 h of incubation in SLC samples than in SW and UDC. Coefficient of determination (R^2) values were significantly higher for a second order polynomial model than for linear or exponential. The highest values for acceleration of fragmentation (asDF) were obtained for SW, followed by SLC and UDC. In conclusion, SLC after thawing seems to preserve longer DNA longevity in comparison to UDC and SW. Moreover, the fine-tuning of models has shown that sDF dynamics in frozen-thawed

donkey semen fits a second order polynomial model, which implies that fragmentation rate is not constant and fragmentation acceleration must be taken into account to elucidate hidden damage in the DNA molecule.

INTRODUCTION



INTRODUCTION

The donkey's relationship with human populations is well documented (Rossel *et al.,* 2008). The earliest domesticated donkey bones identified archeologically date from 4600 to 4000 BC. Thenceforth, donkey has been used as a valuable pack animal. Nonetheless, in the last century, donkeys are losing their traditional role in industrialized countries and the number of animals is decreasing rapidly. For example, the number of animals from the Spanish donkey breeds (Andalusian, Balear, Catalonian, Encartaciones, Majorera and Zamorano-Leones) has decreased dramatically during recent years. As a result, all of them have been included in the UN Food and Agricultural Organization (FAO) list of domestic animas to be conserved (FAO, DAD-IS http://dad.fao.org). In 2014, the Andalusian donkey population was 793 individuals with only 100 breeding males. Nowadays, the role of these preserved animals is changing. In this sense, donkeys are used in the production of hypoallergenic milk, as pet therapy for human beings to treat several diseases such Alzheimer and as draught animals in those areas in which the used of machinery is banned. All this has increased the interest in donkey reproduction. Considering the importance of biodiversity and of preservation of domestic species resources, the optimization of sperm shipping and creation of genetic banks for these donkey breed is needed (Rota *et al.,* 2012).

Artificial insemination (AI) is a very important assisted reproductive technique to increase the number of individuals of many species by improving gene distribution and reducing inbreeding. AI can be carried out using fresh, cooled or frozen-thawed semen samples. Although fresh semen preserves better its properties and is a simpler technique than cooling or freezing, it is not suitable for shipping or storage. Regarding cooling, it is documented that the semen cooling process causes several changes in mammal spermatozoa known as a whole as "cold shock" (Watson 2000). It is particularly focused on changes in sperm membranes (Peña *et al.,* 2011), but it also alters cellular metabolism and organelles, decreases motility and induces irregularities in the sperm motility pattern (Sieme *et al.,* 2008). As a consequence, sperm quality decreases during the cooling process and so do pregnancy rates (Varner *et al.,* 1989; Vidament *et al.,* 2009). The deleterious effect of cold is even more dramatic when it comes to freezing and thawing. The most important factors causing cryoinjury are considered to be the osmotic stress caused by dehydration of the extender during freezing and thawing (Watson 2000; Morris *et al.,* 2007) and the toxicity caused by unequal distribution of cryoprotectants (Pukazhenthi *et*

al., 2014). Moreover, pregnancy rates after inseminations using frozen-thawed are even lower than when using frozen semen doses (Vidament *et al.,* 2009; Rota *et al.,* 2012).

In order to increase the sperm quality of semen samples, a number of sperm selection techniques have been developed (Morrell *et al.,* 2009b). Density-gradient centrifugation (DGC) through a silane-coated silica colloid has been previously used as a two-layer density gradient for separating equine spermatozoa (Morató *et al.,* 2013; Stoll *et al.,* 2013). DGC has been compared with a simpler colloid centrifugation procedure, called single layer centrifugation (SLC), which was developed to work with only one layer of colloid (Morrell *et al.,* 2009a; Morrell *et al.,* 2009b). In this method, spermatozoa are centrifuged through a column of glycidoxypropyltrimethoxylane-coated silica colloid in a species-specific formulation, resulting in the selection of motile, morphologically normal spermatozoa with intact membranes and good chromatin integrity (Morrell *et al.,* 2009a; Morrell *et al.,* 2009d). For that purpose, different commercial products have been developed for use in stallions by modifying the non-silica portion of the gradient medium (Edmond *et al.,* 2012). SLC using Androcoll has been successfully used for sperm selection in different animal species (Morrell *et al.,* 2009e; Thys *et al.,* 2009; Chatdarong *et al.,* 2010). The formulation for stallions is Androcoll-E (Johannisson *et al.,* 2009). This formulation has been used to improve the quality of fresh (Morrell *et al.,* 2009a), frozen (Macias Garcia *et al.,* 2009a; Macias Garcia *et al.,* 2009b; Hoogewijs *et al.,* 2011) and cooled-stored (Morrell *et al.,* 2009d; Bergqvist *et al.,* 2011) stallion sperm samples with a shorter preparation time and less complicated process than the conventional DGC (Morrell *et al.,* 2009b). However, a specific formulation for donkey semen has not been developed yet. Moreover, this procedure was developed to process small volumes of semen, which could be useful for example, to increase the sperm quality of frozen-thawed samples. Nevertheless, this technique is unsuitable to prepare cool semen doses for equine AI, where large volumes of semen are required. In order to solve this problem, a new presentation of Androcoll-E has been developed for large volumes of stallion semen: Androcoll-E-Large (Morrell *et al.,* 2009c). Using this new formulation, up to 15-18 ml of semen can be processed easily and quickly. Sperm quality parameters are also improved in SLC-selected stallion semen samples using Androcoll-E-Large (Morrell *et al.,* 2011a). However, despite common belief, the transfer of knowledge and procedures from horses to donkeys often achieves poor results (Contri *et al.,* 2010); so additional studies should be performed to evaluate if SLC previously used with great success in stallion semen (Morrell *et al.,* 2011a; Morrell *et al.,* 2011b) is suitable for donkey semen samples as well.

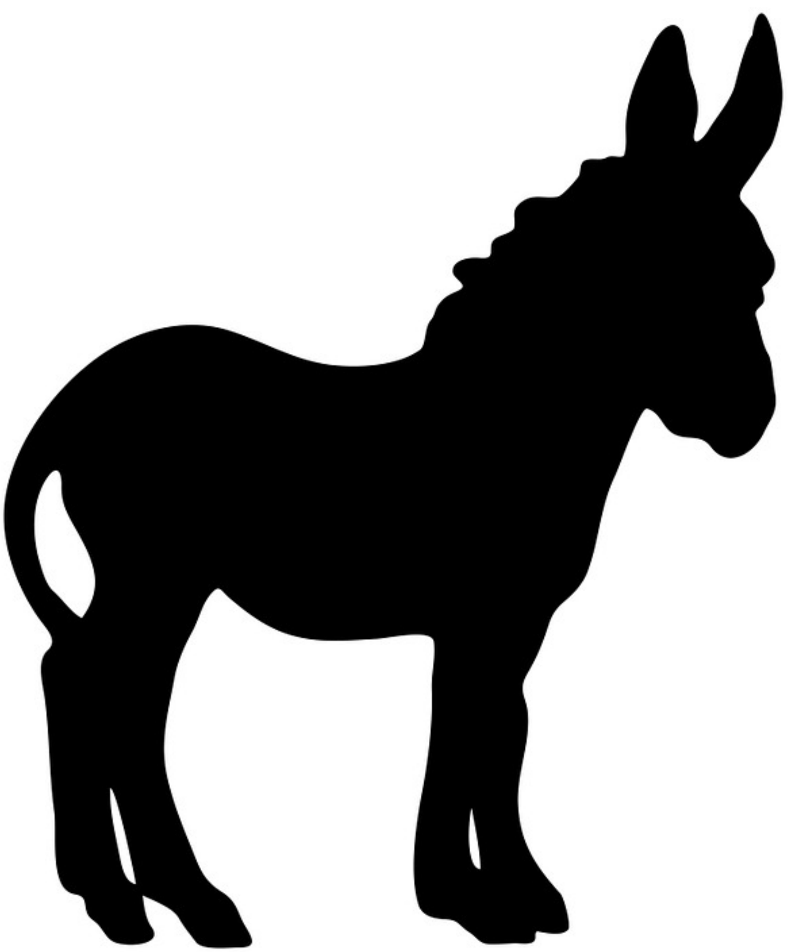
Regarding cryopreservation, current sperm cryopreservation procedures show variable responses between animals in equine species. Post-thaw semen quality and sperm freezability seem to vary consistently among stallions. The high variability between stallions for tolerating cryopreservation is a further complicating factor (Loomis and Graham 2008). Due to this fact, stallions have been classified as good, average or poor freezers according to the ability of their spermatozoa to withstand the freezing and thawing process. The sperm freezability of valuable genetic individuals is a key factor in reproductive strategies to conserve endangered species, such as the Andalusian donkey. It would be useful to obtain a genetic reservoir from a valuable jack with low sperm freezability or to improve post-thaw sperm quality after thawing. For that purpose, SLC could be applied to improve the sperm quality of certain individuals or semen samples. However, to our knowledge, the relationship between the freezability of donkey ejaculates and the improvement of sperm quality obtained after SLC has not been determined yet.

Different strategies have been proposed to improve post-thaw sperm quality, including dilution or removing freezing extenders by simple sperm washing (SW) to reduce the concentration of cryoprotectant in the semen sample. It has been hypothesized that glycerol, the cryoprotectant most commonly used for horse semen cryopreservation, may be toxic for donkey jack sperm (Trimeche *et al.,* 1998) or exert a negative effect on donkey jenny fertility (Vidament *et al.,* 2009). These facts could be responsible for the disappointing results of AI with frozen-thawed donkey sperm (Rota *et al.,* 2012). However, when several studies investigated this phenomenon, glycerol could not be confirmed as responsible for either toxicity of donkey sperm (Oliveira *et al.,* 2006b) or female uterus inflammation (Katila 2005; Vidament *et al.,* 2009). Thus, that hypothesis currently remains unclear. Additionally, replacement of freezing extenders with seminal plasma by dilution or simple SW after thawing has recently been proposed for processing donkey sperm straws before AI (Rota *et al.,* 2012). However, in that study, better post-thaw sperm motility was shown when frozen-thawed donkey sperm was reextended with semen extender rather than with seminal plasma. An alternative approach to improve the quality of frozen-thawed sperm doses and to remove the cryoprotectant used would be to select those spermatozoa that are most likely to achieve fertilization from the rest of the semen sample using SLC (Rodriguez Martinez and Morrel 2009). However, no studies comparing redilution, SLC, and SW have been performed in donkey frozen-thawed sperm samples.

SLC has been shown useful to improve sperm quality and the distribution of sperm subpopulations in different animal species (Stoll *et al.,* 2008; Dorado *et al.,* 2013c; Martinez-Alborcia *et al.,* 2013; Urbano *et al.,* 2013; Anel-López *et al.,* 2015). However, the effect of colloid centrifugation on the structure of the motile subpopulations in donkey sperm has not been assessed yet. On the other hand, the colloids used to select donkey sperm (EquiPure and Androcoll-E) are developed for stallion sperm separation, and although comparisons of different products and protocols for sperm selection have been carried out in some animal species (Makkar *et al.,* 1999; Samardzija *et al.,* 2006; Sabatini *et al.,* 2014b), such comparison has not been carried out with donkey semen.

Last but not least, the importance of the assessment of sperm chromatin to predict the potential fertility is well proved in humans and animals (Evenson 2016). The crucial role that sperm DNA fragmentation (sDF) plays in sperm analysis is due to its relationship with infertility problems after obtaining apparently normal values for routine sperm parameters such as motility, morphology or integrity of sperm membranes (Oleszczuk *et al.,* 2013). The assessment of this parameter is even more critical when sperm quality is limited or compromised, as it happens in some subfertile males, cool-shipped or frozen-thawed semen samples (Brinsko *et al.,* 2003). Therefore, it becomes of the utmost importance to select spermatozoa with intact DNA in order to achieve a higher success in pregnancy rates (Morrell *et al.,* 2008). Previous studies have concluded that, although sperm quality was improved when colloid centrifugation was performed, this procedure did not select intact DNA spermatozoa performing a static analysis of sDF (baseline value). Nevertheless, it has been shown in several studies (Lopez-Fernandez *et al.,* 2007; Gosalvez *et al.,* 2011a) that a dynamic assessment of sDF is more accurate to simulate *ex vivo* sperm maintenance and to evaluate latent chromatin damage than only considering baseline values. These studies submitted semen samples to thermal stress and recorded sDF values at different times. Then, a linear regression equation was calculated and the rates of fragmentation (the slope of the linear regression equation, SDF%/time) of the treatments were compared. This approach solved the issue of the encrypted DNA damage, however, another question arose: Do DNA dynamics fit to a linear regression model? Although linear regression is the simplest model, it entails that DNA damage is then a simple process with constant speed. Before accepting this statement as an actual fact, a fine-tuning of mathematical models for DNA fragmentation over time should be carried out.

OBJETIVOS



OBJETIVOS

En la presente Tesis Doctoral se plantearon diversos objetivos específicos, desarrollados en cada uno de las cinco publicaciones incluidas en el compendio:

Objetivo 1. (**Primera publicación:** "Effect of single layer centrifugation using Androcoll-E-Large on the sperm quality parameters of cooled-stored donkey semen doses". *I. Ortiz, J. Dorado, L. Ramírez, J. M. Morrell, D. Acha, M. Urbano, M. J. Gálvez, J. J. Carrasco, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo.* Animal (2014), 8:2, 308-315):

> Determinar si los parámetros de calidad espermática de las muestras de semen refrigerado de asno pueden ser mejoradas con centrifugación coloidal de una sola capa usando el coloide Androcoll-E-Large.

Objetivo 2. (**Segunda publicación:** "Colloid single-layer centrifugation improves post-thaw donkey (Equus asinus) sperm quality and is related to ejaculate freezability". *I. Ortiz, J. Dorado, D. Acha, M.J. Gálvez, M. Urbano, M. Hidalgo.* Reproduction, Fertily and Development (2015), 27, 332-340):

> Valorar el efecto de la centrifugación coloidal de una sola capa en semen descongelado de asno en los parámetros de calidad espermática y evaluar la relación entre la resistencia a la congelación espermática y la mejora de los parámetros espermáticos tras la centrifugación coloidal.

Objetivo 3. (**Tercera publicación:** "Effect of single-layer centrifugation or washing on frozen-thawed donkey semen quality: Do they have the same effect regardless of the quality of the sample?" *I. Ortiz, J. Dorado, J. M. Morrell, F. Crespo, J. Gosálvez, M.J. Gálvez, D. Acha, M. Hidalgo.* Theriogenology (2015), 84, 294-300):

> Determinar si la centrifugación coloidal de una sola capa usando Androcoll-E en semen congelado y descongelado de asno podría seleccionar espermatozoides de buena calidad en comparación con la centrifugación simple o la no centrifugación; y determinar si el efecto en la calidad espermática tras la centrifugación coloidal depende de la calidad inicial de la muestra.

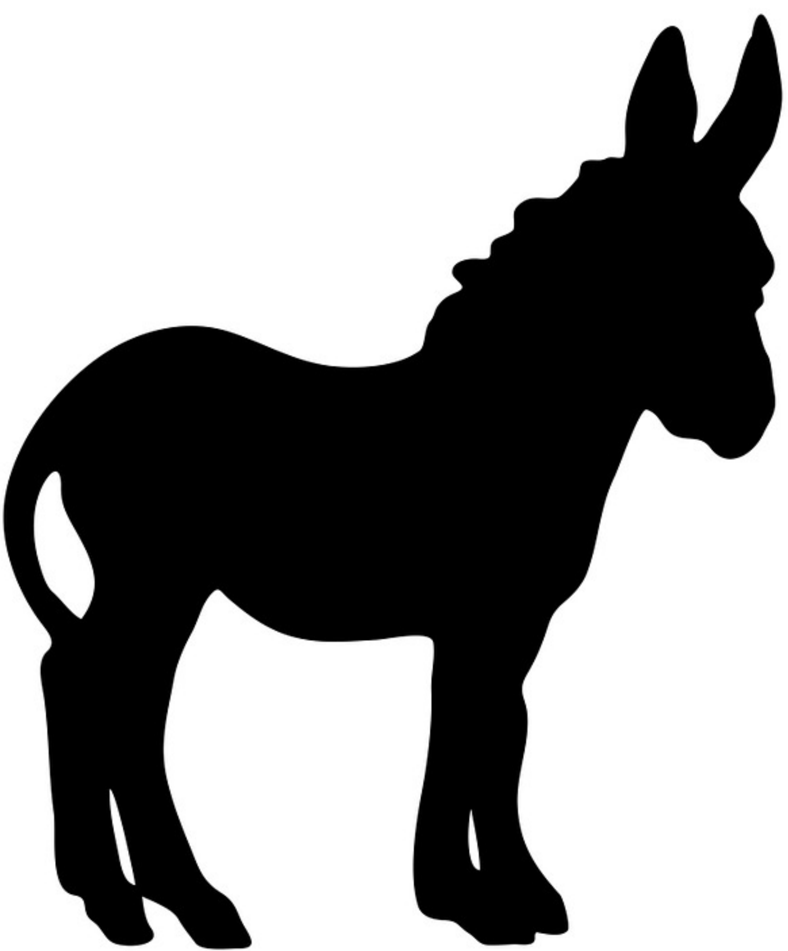
Objetivo 4. **(Cuarta publicación:** "Comparison of EquiPure and Androcoll-E for donkey sperm selection after thawing: effect of sperm motility, membrane integrity and motile sperm subpopulations". *I. Ortiz, J. Dorado, J. M. Morrell, M. J. Gálvez, D. Acha, M. Hidalgo.* Enviado a Animal Journal):

Comparar dos coloides comerciales para semen de caballo adecuados para semen de asno para evaluar si uno de ellos es mejor seleccionando espermatozoides con buen movimiento, integridad de la membrana y distribución de las subpoblaciones de espermatozoides móviles tras descongelación usando la centrifugación simple y la dilución como controles.

Objetivo 5. **(Quinta publicación:** "New approach to assess sperm DNA fragmentation dynamics: Fine-tuning mathematical models". *I. Ortiz, J. Dorado, J. M. Morrell, J. Gosálvez, F. Crespo, J. M. Jiménez, M. Hidalgo.* Enviado a Journal of Animal Science and Biotechnology):

Valorar el efecto de diferentes protocolos de centrifugación post-descongelación en la fragmentación del ADN espermático y elucidar el modelo matemático más preciso para la fragmentación del ADN en el tiempo en semen de asno congelado y descongelado.

OBJECTIVES



OBJECTIVES

In the present Doctoral Thesis several objectives were set in each publication as follows:

Objective 1. (First publication: "Effect of single layer centrifugation using Androcoll-E-Large on the sperm quality parameters of cooled-stored donkey semen doses." *I. Ortiz, J. Dorado, L. Ramírez, J. M. Morrell, D. Acha, M. Urbano, M. J. Gálvez, J. J. Carrasco, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo.* Animal (2014), 8:2, 308-315):

> To determine if the sperm quality parameters of cooled-stored donkey semen samples can be improved after single layer centrifugation (SLC) using Androcoll-E-Large.

Objective 2. (Second publication: "Colloid single-layer centrifugation improves post-thaw donkey (Equus asinus) sperm quality and is related to ejaculate freezability". *I. Ortiz, J. Dorado, D. Acha, M.J. Gálvez, M. Urbano, M. Hidalgo.* Reproduction, Fertily and Development (2015), 27, 332-340):

> To assess the effect of colloid single-layer centrifugation on post-thaw donkey sperm quality parameters and to evaluate the relationship between sperm freezability and improvement of sperm parameters after SLC selection.

Objective 3. (Third publication: "Effect of single-layer centrifugation or washing on frozen-thawed donkey semen quality: Do they have the same effect regardless of the quality of the sample?" *I. Ortiz, J. Dorado, J. M. Morrell, F. Crespo, J. Gosálvez, M.J. Gálvez, D. Acha, M. Hidalgo.* Theriogenology (2015), 84, 294-300):

> To determine if SLC using Androcoll-E on frozen-thawed donkey sperm samples could select high-quality sperm in comparison to sperm washing (SW) or no centrifugation and if the effect on the sperm quality after SLC or SW depends on the quality of the sample.

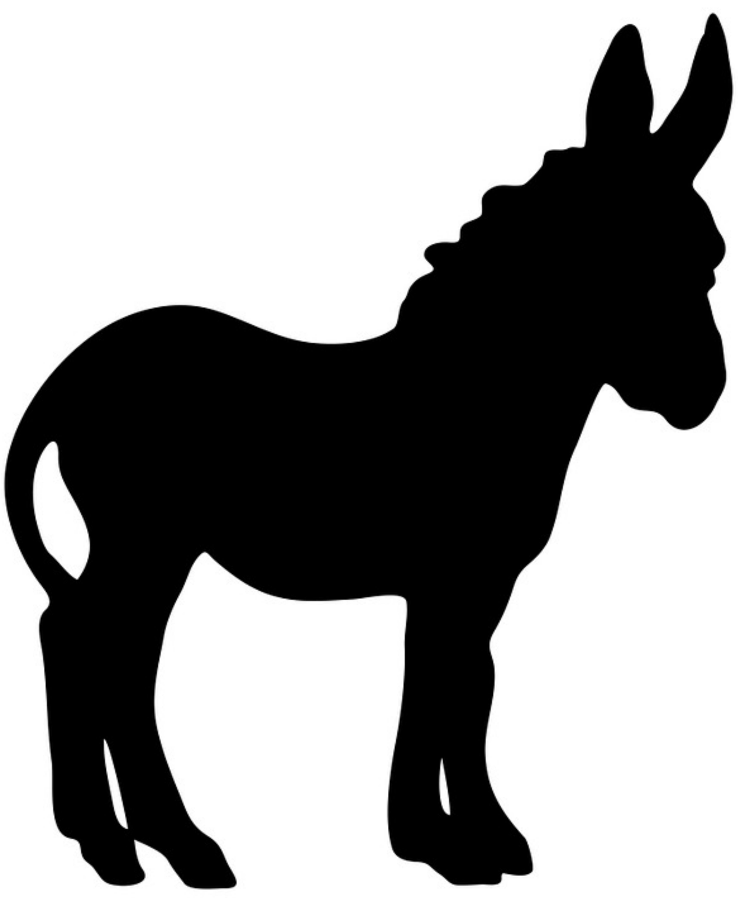
Objective 4. (Fourth publication: "Comparison of EquiPure and Androcoll-E for donkey sperm selection after thawing: effect of sperm motility, membrane integrity and motile sperm subpopulations". *Submitted to Animal Journal):*

To compare two commercial stallion colloids suitable for donkey sperm in order to assess if one of them is better at selecting sperm with good motility, membrane integrity and distribution of the motile subpopulations after thawing using dilution and sperm washing as controls.

Objective 5. (Fifth publication: "New approach to assess sperm DNA fragmentation dynamics: Fine-tuning mathematical models". *I. Ortiz, J. Dorado, J. M. Morrell, J. Gosálvez, F. Crespo, J. M. Jiménez, M. Hidalgo.* Submitted to Journal of Animal Science and Biotechnology):

To evaluate the effect of different after-thawing centrifugation protocols on sperm DNA fragmentation and to elucidate the most accurate mathematical model for DNA fragmentation over time in frozen-thawed donkey semen.

CHAPTERS



CHAPTER 1

“Effect of single layer centrifugation using Androcoll-E-Large on the sperm quality parameters of cooled-stored donkey semen doses”

I Ortiz, J Dorado, L Ramírez, JM Morrell, D Acha, M Urbano, MJ Gálvez, JJ Carrasco, V Gómez-Arrones, R Calero-Carretero, M Hidalgo. *Animal (2014), 8 (2): 308-315.*

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Effect of single layer centrifugation using Androcoll-E-Large on the sperm quality parameters of cooled-stored donkey semen doses

I. Ortiz[1], J. Dorado[1], L. Ramírez[1], J. M. Morrell[2], D. Acha[1], M. Urbano[1], M. J. Gálvez[1], J. J. Carrasco[3], V. Gómez-Arrones[3], R. Calero-Carretero[3] and M. Hidalgo[1†]

[1]Animal Reproduction Group, Department of Animal Medicine and Surgery, University of Cordoba, 14071 Cordoba, Spain; [2]Department of Clinical Sciences, Swedish University of Agricultural Sciences (SLU), Box 7054, SE-75007, Uppsala, Sweden; [3]Equine Center for Assisted Reproduction Services, CENSYRA-Extremadura Government, 06007 Badajoz, Spain

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The aim of this study was to determine the effect of single layer centrifugation (SLC) using Androcoll-E-Large on donkey sperm quality parameters after 24 h of cool-storage. Ejaculates were collected from Andalusian donkeys and then cooled at 5°C. SLC was carried out after 24 h of cool-storage using Androcoll-E-Large. In the first experiment, all sperm parameters assessed (total and progressive sperm motility, viability, sperm morphology and sperm kinematics VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF) were statistically compared between semen samples processed or not with Androcoll-E-Large. Significant differences ($P < 0.05$*) were found between SLC-selected and unselected semen samples for all parameters assessed, obtaining better results after SLC. In the second experiment, semen samples were classified in two groups according to their sperm progressive motility (PM) before SLC. Then, the increments obtained in semen quality parameters after SLC were compared between groups. No significant differences were found between groups, indicating that SLC improved the sperm quality parameters of entire set of semen samples processed with independence to their original PM. In conclusion, SLC with Androcoll-E-Large can be used in donkeys, increasing the sperm quality of cooled-stored donkey semen doses after 24 h of cool storage.*

Keywords: single layer centrifugation, donkey semen, sperm cooling, Androcoll-E-Large

Implications

Andalusian donkey has lost its traditional role, which has resulted in the inclusion of this breed in the UN Food and Agricultural Organization endangered species list. Nowadays, the importance of donkey is increasing again and it is essential that the jackasses with desirable genetic features have as good sperm quality as possible. Sperm selection by single layer centrifugation (SLC) through silica colloids has shown to improve the sperm quality in other animal species. Recently, Androcoll-E-Large has been developed as a colloid suitable for processing large volumes of semen; however, there are no studies that substantiate the potentially beneficial effects of SLC on donkey semen doses.

† Present address: Department of Animal Medicine and Surgery, Faculty of Veterinary Sciences, University of Cordoba, Campus de Rabanales (Edif. Hospital Clínico Veterinario), Ctra. Madrid-Cádiz, km 396, 14071, Córdoba, Spain. E-mail: mhidalgo@uco.es

Introduction

The donkey's relationship with human populations is well-documented (Rossel *et al*., 2008). The earliest domesticated donkey bones identified archeologically date to 4600 to 4000 BC. Thenceforth, donkey has been used as a valuable pack animal. Nonetheless, in the last century, in industrialized countries donkeys are losing their traditional role and the number of animals is decreasing rapidly. For example, the number of animals from the Spanish donkey breeds (Andalusian, Balear, Catalonian, Encartaciones, Majorera and Zamorano-Leonés) has decreased dramatically during recent years. As a result, all of them have been included in the UN Food and Agricultural Organization (FAO) list of domestic animals to be conserved (FAO, DAD-IS http://dad.fao.org/). In 2011, the Andalusian donkey population was 760 individuals with only 101 breeding males. Nowadays, the role of these preserved animals is changing. In this sense, donkeys are used in the production of hypoallergenic milk, as

pet therapy for human beings to treat several diseases such Alzheimer and as draught animals in those areas in which the use of machines is banned. All this has increased the interest in donkey reproduction.

Artificial insemination (AI) with cooled-stored semen doses is considered to be one of the most important assisted reproductive technique to increase the number of individuals of many species, in order to improve gene distribution and reduce inbreeding. Suboptimal pregnancy rates (45%) after AI using cooled donkey sperm was obtained in a previous study (Vidament *et al*., 2009). It may result from breeding with low quality or not adequately processed for shipment semen. The quality of sperm samples is crucial when cooled-stored semen is used for AI. It is documented that the semen cooling process causes several changes in mammal spermatozoa known as a whole as 'cold shock' (Watson, 2000). It is particularly focused on changes in sperm membranes (Peña *et al*., 2011); however, this negative effect alters cellular metabolism and organelles, decreases motility and induces irregularities in the sperm motility pattern (Sieme *et al*., 2008). As a consequence, sperm quality decreases during the cooling process and so do pregnancy rates (Varner *et al*., 1989).

In order to increase the sperm quality of semen samples, a number of sperm selection techniques have been developed (Morrell, 2012). Recently, single layer centrifugation (SLC) using Androcoll^{TM} (SLU, Uppsala, Sweden) has been successfully used for sperm selection in different animal species (Morrell *et al*., 2009e; Thys *et al*., 2009; Chatdarong *et al*., 2010). In this SLC technique, spermatozoa are centrifuged through a column (single layer) of silane-coated silica colloid in a species-specific formulation. The formulation for stallions is Androcoll-E (Johannisson *et al*., 2009) and has been recently commercialized by Minitüb GmbH (Tiefenbach, Germany). Androcoll-E^{TM} has been used to select robust spermatozoa in terms of motile and morphologically normal sperm, with intact membranes and good chromatin integrity (Morrell *et al*., 2009c). On the basis of all this sperm parameters to perform the sperm quality analysis, Androcoll-E has improved the quality of fresh (Morrell *et al*., 2009a), frozen (Macias Garcia *et al*., 2009a; Macias Garcia *et al*., 2009b; Hoogewijs *et al*., 2011) and cooled-stored (Morrell *et al*., 2009d; Bergqvist *et al*., 2011) stallion sperm samples with a shorter preparation time and less complicated process than the conventional density gradient centrifugation (Morrell *et al*., 2009b). However, a specific formulation for donkey semen has not been developed yet. Moreover, this procedure was developed to process small volumes of semen, which could be useful for example, to increase the sperm quality of frozen-thawed stallion semen samples. However, this technique is unsuitable to prepare cool semen doses for equine AI, where large volumes of semen are required. In order to solve this problem, recently a new presentation of Androcoll-E has been developed for large volumes of stallion semen: Androcoll-E-Large. Using this new formulation, up to 15 to 18 ml of semen can be processed easily and quickly. Sperm quality parameters, such as sperm motility, morphology and chromatin integrity are also improved in SLC-selected stallion semen samples using Androcoll-E-Large (Morrell *et al*., 2011a). However, to our knowledge, only preliminary results of the use of SLC to improve sperm motility in cooled donkey semen samples have been published by our research group (Ortiz *et al*., 2012). Despite common belief, the transfer of knowledge and procedures from horses to donkeys often achieves poor results (Contri *et al*., 2010b); so additional studies should be performed to evaluate if SLC previously used with great success in stallion semen (Morrell *et al*., 2011b) is suitable for donkey semen samples as well.

Thus, the aim of this study was to determine if the sperm quality parameters of cooled-stored donkey semen samples can be improved after SLC using Androcoll-E-Large.

Material and methods

Animals

Four healthy, mature, Andalusian donkeys, aged from 6 to 15, were used as semen donors. One of the jackasses was owned by 'Donkey's House Foundation' (Rute, Córdoba, Spain) and was housed in individual paddocks placed at the Veterinary Teaching Hospital (VTH) of the University of Córdoba (Spain). The feeding consisted of alfalfa hay and water *ad libitum*. The other three donkeys were housed at the Equine Center for Assisted Reproduction Services (CENSYRA) in Badajoz (Spain), where they usually live, and they were fed with a mixture of grasses hay.

Semen collection

Semen was collected using a Missouri artificial vagina with an in-line gel filter (Minitüb) in the presence of a jenny in natural or induced estrus to stimulate copulatory activity. Three to four ejaculates per animal were collected twice a week obtaining a total number of 13 ejaculates. Total and progressive sperm motility was evaluated from fresh semen by CASA. Gel free volume (ml) was measured in a collector. Sperm concentration ($\times 10^6$ spermatozoa/ml) was assessed with a sperm photometer (Spermacue®; Minitüb).

Semen processing

Immediately after collection, an aliquot of raw semen was extended with INRA96 (IMV, l'Aigle, France) at 37°C until a final concentration of 100×10^6 sperm/ml . Extended semen was maintained at room temperature (~22°C) for 15 min in a 50 ml corning tube. Semen samples were slowly cooled (0.3°C/min) for 2 h in an equitainer at 5°C. After that, 20 ml of each semen sample were loaded in syringes previously cooled at 5°C in a fridge. Syringes were then placed in a Styrofoam box at 5°C (Minitüb) previously loaded with two cold packs. Cooled-stored semen doses were shipped to the Animal Reproduction Laboratory if the ejaculates were collected at CENSYRA or cooled and stored following the same methodology if semen was collected at the VTH. All semen samples were evaluated after 24 h of cool storage at 5°C in a shipping box for the following sperm quality parameters.

Computer-assisted sperm motility analysis
Sperm motility was objectively evaluated using the Sperm Class Analyzer (SCA 2011 v.5.0.1; Microptic S.L., Barcelona, Spain). This system consists of an optical phase-contrast microscope (Eclipse 50i; Nikon, Tokyo, Japan), a warm plate at 37°C (OK 51-512, Osaka, Digifred SL, Barcelona, Spain) and a high-speed digital camera (A312fc, Basler™ AG, Ahrensburg, Germany), which captures a total number of 25 consecutive digitalized frames in 1 s per captured field (image-capture rate, one photograph every 40 ms and a PC (Intel Inside®, Pentium 4®, Intel Labs, Barcelona, Spain) to analyze and save data. CASA settings were as follows: cell size from 15 to 75 μm^2; connectivity 12; progressive spermatozoa >75% of the straightness coefficient (STR). An aliquot of each semen sample was extended with INRA96 (IMV Technologies, L'Aigle, France) to a final concentration of 25×10^6 sperm/ml and then incubated at 38°C for 10 min. After that, 5 µl of each diluted semen sample were placed in a Mackler counting chamber (Sefi-Medical Instruments Ltd., Haifa, Israel). Three drops, with two randomly microscopic fields per drop, were analyzed in each semen sample. The trajectory of each individual sperm was determined by the SCA software obtaining CASA sperm kinematic parameters: total motility (TM), progressive motility (PM), sperm curvilinear velocity (VCL, µm/s), sperm linear velocity (VSL, µm/s), average path velocity (VAP, µm/s), linear coefficient (LIN, VSL/VCL × 100), straightness coefficient (STR, VSL/VAP × 100), wobble coefficient (WOB, VAP/VCL × 100), amplitude of lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz) were assessed.

Sperm morphology
Sperm morphology was performed by visual examination on slides stained with Diff-Quick® (Baxter DADE AG 3186, Düdingen, Switzerland) as described previously (Hidalgo *et al.*, 2006). At least 200 sperm were evaluated from each semen sample and the percentage of normal and abnormal forms was recorded.

Sperm viability
Sperm viability was assessed using a supravital stain (Cortes-Gutierrez *et al.*, 2008) based on the red/green emission of two fluorescent dyes: acridine orange and propidium iodide, respectively (Duo-Vital Kit; Halotech DNA SL, Madrid, Spain). At least 200 sperm were counted, considering green spermatozoa as 'live sperm', and red or red-green as 'dead sperm'.

SLC with Androcoll-E-Large
Before SLC, both cooled-stored semen samples and colloid were allowed to equilibrate at room temperature (about 22°C) for 30 min to avoid temperature fluctuations. Fifteen milliliters of cooled semen were carefully layered on top of 15 ml Androcoll-E-Large located in 50 ml corning tubes, taking care not to disrupt the interface. The suspension was centrifuged at 300 × **g** for 20 min without brake (Eppendorf, 5702 RH; Eppendorf AG, Hamburg, Germany). The supernatant (semen extender, seminal plasma and colloid) was removed and the sperm pellet recovered and transferred to a clean tube containing INRA96. According to the protocol described by Morrell *et al.* (2011a). Concentration of the sperm pellets was measured using the SCA system. After that, semen samples were adjusted to a final concentration of 25 million sperm/ml and then sperm motility, morphology and viability were analyzed as described above. The yield of selected spermatozoa was calculated according to the following formula:

$$\text{Yield} = (\text{number of spermatozoa in sperm pellet}/\text{number of spermatozoa in initial load}) \times 100$$

Experimental design
Experiment 1. Effect of sperm selection using Androcoll-E-Large in cooled-stored donkey semen doses for 24 h at 5°C. Two aliquots of each semen sample were taken. One of them was immediately evaluated for sperm quality parameters following the methodology described above. The other one was subjected to SLC with Androcoll-E-Large as described previously and then evaluated. The results of the sperm quality parameters assessed in uncentrifuged semen samples (unselected) were compared with those obtained after SLC centrifugation with Androcoll-E-Large (SLC-selected).

Experiment 2. Relationship between PM of uncentrifuged samples and improvement of sperm parameters in SLC-selected samples. Semen samples were divided into two groups according to the original sperm PM of unselected samples (Group 1: PM $\leqslant$ 43.7%; Group 2: PM $\geqslant$ 43.8%). Increment obtained in each semen quality parameter after SLC-selection was compared between groups.

Statistical analysis
Data analyses were performed using SPSS v20.0 for Mac OS X (IBM, SPSS Statistics, Armonk, NY, USA) and SAS v9.0 for Windows (SAS Institute Inc, Cary, NC, USA). Analysis of the data was carried out using a general linear model (GLM), with animals, treatments and ejaculates as fixed effects. Differences between treatments in each animal were also assessed using GLM, with the fixed effects being ejaculates and treatment. A two-step cluster procedure was performed to classify the cooled semen samples according to their initial progressive sperm motility (before SLC-treatment). Comparisons between groups were performed using a one-way ANOVA. Normality of the data distributions and variance homogeneity were checked by the Kolmogorov–Smirnov and Cochran tests, respectively. Values were expressed as mean and root mean square error (RMSE). Statistical significance was set at $P < 0.05$.

Results

Sperm parameters from all the ejaculates assessed in fresh semen aliquots were between the ranges considered as physiologic when evaluating donkey sperm (Table 1). The sperm yield (%) of total sperm obtained after SLC with Androcoll-E-Large was 25.4.

Table 1 *Sperm parameters immediately after collection and yield when SLC was carried out after storage of extended semen at 5°C for 24 h from all animals*

	Donkey 1 ($n=3$)		Donkey 2 ($n=3$)		Donkey 3 ($n=3$)		Donkey 4 ($n=4$)		Mean values ($n=13$)	
Fresh semen parameters	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Gel-free volume (ml)	55.9	4.9	85.0	41.4	44.0	2.1	44.0	2.1	58.7	24.0
Sperm concentration ($\times 10^6$ per ml)	288.7	43.3	409.7	203.2	405.3	22.9	405.3	22.9	353.2	101.9
Total sperm count ($\times 10^9$)	15.9	1.3	29.9	6.9	17.8	0.9	17.8	0.9	19.8	6.7
TM (%)	95.3	1.5	90.3	6.1	94.3	3.1	94.3	3.1	94.6	4.0
PM (%)	73.0	3.0	70.7	4.7	67.3	4.9	67.3	4.9	75.9	9.6
Yield after SLC (%)	26.5	2.7	30.7	6.1	24.4	3.5	24.4	3.5	25.4	5.0

n = number of ejaculates; TM = total motility; PM = progressive motility; SLC = single layer centrifugation.
Values are expressed as mean and s.d. (standard deviation).

Table 2 *Parameters of semen quality for uncentrifuged (n = 13) and SLC-selected (n = 13) donkey sperm samples stored at 5°C for 24 h*

	Treatments			
Parameters	Uncentrifuged	SLC-selected	RMSE	Statistics (*P*-value)
TM (%)	69.0	79.9	9.0	<0.001
PM (%)	46.6	63.1	13.4	<0.01
Live (%)	66.7	71.1	5.8	<0.01
Normal (%)	79.6	87.0	6.9	<0.05

SLC = single layer centrifugation; RMSE = root mean square error; TM = total motility; PM = progressive motility; Live = live sperm; Normal = normal forms.
Values are expressed as mean and RMSE.

Comparison of sperm quality parameters between uncentrifuged and SLC-selected samples after 24 h of cool storage

All sperm parameters assessed (motility, viability and morphology) were higher in the SLC-selected samples compared with uncentrifuged controls (Table 2).

Mean total sperm motility was significantly higher ($P < 0.001$) in SLC-selected samples in comparison to unselected samples (79.9% *v.* 69.0%), which means an increment of 10.9%. Moreover, sperm PM was also significantly ($P < 0.01$) higher in SLC-selected samples (63.1% *v.* 46.6%). This parameter increased 16.5% when compared with uncentrifuged samples (Table 2).

The statistical analysis performed to assess viability showed significantly increased values ($P < 0.01$) in the live sperm percentage from SLC-selected aliquots (71.1% *v.* 66.7%). In this case SLC-selected samples had an increment of 4.4% in viability values (Table 2).

Normal sperm morphology in SLC-selected samples was also significantly higher ($P < 0.05$) than uncentrifuged semen (87.0% *v.* 79.6%), increasing by 7.4 the percentage of normal forms (Table 2).

Most of the sperm kinematics parameters assessed (VCL, VSL, VAP, LIN, STR, WOB and BCF) was significantly improved ($P < 0.001$) in the SLC samples compared with the uncentrifuged controls (Table 3).

Relationship between PM of uncentrifuged samples and improvement of sperm parameters in SLC-selected samples

Significant differences ($P < 0.001$) were found between the progressive sperm motility of the two groups obtained (Table 4); however, no significant differences in the improvement of sperm quality parameters after SLC were seen between groups (Table 5).

Discussion

The objective of the present study was to determine if sperm quality parameters from cooled donkey semen doses stored for up to 24 h could be improved after SLC using Androcoll-E-Large, which has been used successfully in previous studies to process large volumes of stallion semen (Morrell *et al.*, 2011a).

According to the results obtained in this study, SLC using Androcoll-E-Large significantly improved total and PM, vitality and normal sperm morphology in donkey sperm doses after 24 h of cool-storage. Kinematic sperm parameters were also improved.

These results are in agreement with previous studies which supported SLC using Androcoll-E-Large is an effective method to select motile sperm in stallions (Morrell *et al.*, 2008, 2009d; Johannisson *et al.*, 2009). Preliminary results of the use of SLC in donkey semen samples have been published by our research group (Ortiz *et al.*, 2012); however, to our knowledge, this is the first full research article in which the effect of colloid centrifugation is tested in cooled-stored donkey sperm doses.

Total and PM are traditionally considered as essential indicators to evaluate the quality of a sperm semen sample (Love, 2011). In this study, both parameters were lower in the unselected samples than those obtained in previous

Table 3 *Kinematic parameters for uncentrifuged (n = 13) and SLC-selected (n = 13) donkey sperm samples when SLC was carried out after storage of extended semen at 5°C for 24 h*

	Treatments			
Parameters	Uncentrifuged	SLC-selected	RMSE	Statistics (*P*-value)
VCL (μm/s)	108.1	113.4	58.3	<0.001
VSL (μm/s)	73.7	86.1	55.1	<0.001
VAP (μm/s)	92.7	99.5	56.1	<0.001
LIN (%)	58.5	65.7	28.7	<0.001
STR (%)	72.4	77.5	26.3	<0.001
WOB (%)	76.5	80.3	19.0	<0.001
ALH (μm)	2.7	2.6	1.2	<0.001
BCF (Hz)	7.7	8.6	4.4	<0.001

SLC = single layer centrifugation; RMSE = root mean square error; VCL = curvilinear velocity; VSL = linear velocity; VAP = average path velocity; LIN = linear coefficient; STR = straightness coefficient; WOB = Wobble coefficient; ALH = mean lateral head displacement; BCF = frequency of head displacement.
Values are expressed as mean and RMSE.

Table 4 *Groups obtained according to the original sperm progressive motility of cooled-stored semen samples before SLC (uncentrifuged samples)*

		Progressive sperm motility before SLC (%)	
Group	*n*	Mean	Range
G1	7	27.7^{A}	0–43.7
G2	6	68.5^{B}	43.8–100

n = number of semen samples; SLC = single layer centrifugation.
$^{A-B}$Indicate significant differences ($P < 0.001$).

Table 5 *Comparison of the increment of sperm parameters of cooled-stored semen after SLC between semen samples grouped on the basis of their original progressive motility*

	Semen sample group according to original PM		
Increment of sperm parameters after SLC	Group 1 (PM = 27.7)	Group 2 (PM = 68.5)	RMSE
TM-I (%)	13.5	8.3	6.4
PM-I (%)	20.1	12.3	9.1
L-I (%)	4.2	4.6	7.6
NF-I (%)	9.1	5.2	6.7
Yield (%)	24.5	26.5	5.1

SLC = single layer centrifugation; RMSE = root mean square error; TM = total motility; PM = progressive motility; L = live sperm; NF = normal forms
No significant differences were found between groups ($P > 0.05$).
Values are expressed as mean and RMSE.
TM-I (TM increment) = (TM% SLC-selected)–(TM% uncentrifuged).
PM-I (PM increment) = (PM% SLC-selected)–(PM% uncentrifuged).
L-I (L increment) = (L% SLC-selected)–(L% uncentrifuged).
NF-I (NF increment) = (NF% SLC-selected)–(NF% uncentrifuged).

studies (Mello *et al*., 2000; Rota *et al*., 2008; Contri *et al*., 2010a), however, total and PM were significantly enhanced when comparing unselected with SLC-selected samples. These results agree with studies performed with cooled stallion semen doses (Morrell *et al*., 2011a). In this way, the capacity of SLC with Androcoll-E-Large to increase total and progressive stallion sperm motility after 24 h of cool storage compared to uncentrifuged semen samples has also been shown.

Sperm viability represents the integrity of sperm plasma membrane. It is supposed that all motile sperm should be alive; however, these parameters are not always related (Love *et al*., 2003). Our results showed a lower percentage

of live sperm than motile sperm. This fact is explained in a previous study by Cortes-Gutierrez *et al.* (2008) in which they discovered that some live sperm remained unstained with Duo-Vital® staining, becoming 'invisible' to the evaluator and the final percentage of live sperm is lower than the true value. Nevertheless, sperm viability percentage was significantly higher in the samples centrifuged with Androcoll-E-Large. Another study has also reported that SLC using this colloid improves cooled stallion semen doses (Morrell *et al.*, 2009b). This is quite an interesting finding bearing in mind that the temperature drop triggers several changes in the spermatozoa known as cold shock (Watson, 2000), mainly in the acrosomal and plasma membrane decreasing sperm quality because of death or shortening of sperm life (Petrunkina *et al.*, 2005; Peña *et al.*, 2012). Consequently, pregnancy rates after insemination of cool semen decrease (Heckenbichler *et al.*, 2011) implying this last motive a good reason to consider colloid centrifugation as an option in order to improve sperm viability of cooled semen samples.

Sperm morphology was also improved after SLC, being the proportion of normal forms higher in SLC-selected samples in comparison to uncentrifuged cooled-stored semen doses. These results correspond to those from other studies in stallions (Morrell *et al.*, 2011a). Percentage of normal forms has been related to pregnancy rates (Morrell *et al.*, 2008). This could suggest that samples processed with Androcoll-E-Large which presented better morphology would be more fertile than unprocessed ones.

Mean sperm yield obtained was 25.4% being the range in stallions from 20% to 69%. Although the recovery rate is small compared with those obtained in stallions (Morrell *et al.*, 2009c, 2011a), these differences can be explained by this colloid formulation, which has been previously used with stallions and not with donkeys. This fact can be explained attending to previous studies performed with horses (Morrell *et al.*, 2009b), where it is described that volume and concentration must be adjusted to a specific colloid. If this requirement is not fulfilled significant differences in the yield are obtained. Further studies are needed in order to develop more accurate protocols to use in donkeys (testing different sperm concentrations, volumes of sperm and colloid, centrifugation times and densities) or a specific colloid for donkey sperm to increase the yield obtained.

On the other hand, previous studies have calculated AI donkey semen doses based on sperm concentration excluding other parameters. These authors concluded that a sperm dose of 400 millions of total sperm in 10 ml was followed with acceptable pregnancy rates (Vidament *et al.*, 2009). This number of sperm per dose could be decreased if sperm parameters like motility, viability or morphology are taken into consideration. In our study, the mean yield obtained was ~381 million of sperm in each semen sample processed (15 ml sperm $\times$ 100 million sperm per ml $\times$ recovery rate/100). However, donkey sperm concentration of raw semen is much larger than horse sperm concentration (Miró *et al.*, 2009). If we scaled-up the yield to the number of sperm in the whole ejaculate, we could obtain around 5000 million of SLC-selected sperm. It makes possible to prepare more than 12 cooled doses for AI with 400 million of sperm per dose.

Sperm velocities, mainly VCL and VSL are the most important kinematic parameters related to potential fertility (Olds-Clarke, 1989). Results obtained in this study revealed a highly significant ($P < 0.001$) enhancement of both parameters after SLC. In the same way, the remaining kinematic parameters assessed were also significantly higher ($P < 0.001$) after SLC except for ALH, which was significantly smaller. Irregular trajectories are mainly induced by two causes: (1) low linearity (LIN = VSL/VCL $\times$ 100) or (2) a high degree of lateral deviation of the head (ALH), both related to very low VSL and very high values of VAP (Mortimer, 2000). In our study, VSL and VAP increases in SLC-selected samples accompanied with lower mean values of ALH. This implies that SLC excludes sperm with an irregular trajectory. Since differences between treatments in each animal have shown different results, individual factors should be considered when processing samples by SLC. Previous studies performed in stallions reported an improvement of all sperm kinematics parameters in frozen-thawed semen, obtaining similar results to fresh semen evaluation prior freezing (Macias Garcia *et al.*, 2009a). To conclude, SLC with Androcoll-E-Large improves both general and kinematic motility parameters in donkey sperm samples, indicating better sperm motility.

In experiment 2, Androcoll-E-Large improved every sperm quality parameter regardless of the PM of the original sample, and there were no differences in the yield between groups. This result suggests that cooled semen samples are suitable for processing with Androcoll-E-Large. Furthermore, even semen samples with higher progressive sperm motility after cool storage are capable of being improved using Androcoll-E-Large. However, as mean values for the increment of different sperm quality parameters and yield were different, maybe a larger number of ejaculates and animals could show significant differences between groups.

In short, this general improvement of sperm quality in donkey cooled semen doses based on the enhancement of sperm parameters could be related to selection of the most robust 'fertile spermatozoa' from the entire sperm population. Nevertheless, we could think that a number of some good spermatozoa that still remained some potential fertility ability may be lost during the SLC process. Consequently, it could also affect fertility after SLC. However, it is important to highlight the fact that SLC not only selected the robust 'fertile' sperm but also removed dead and immotile sperm. Removing dead or immotile sperm from a semen sample means that sources of reactive oxygen species are also removed and that should improve fertility of AI doses (Morrell *et al.*, 2013). Since previous studies have obtained moderate pregnancy rates (45%) in jennies inseminated with unselected cooled semen (Vidament *et al.*, 2009), SLC could be used to try to increase this percentage. The relationship between SLC-selected semen and fertility has been reported before in stallions (Morrell *et al.*, 2011b). However,

no experiences have been performed in donkeys. Once SLC has been shown to be an effective method to improve sperm quality parameters of cooled-stored donkey semen doses *in vitro*, further studies are needed to relate sperm quality enhancement of donkey semen doses to pregnancy rates after AI.

In conclusion, SLC with Androcoll-E-Large improved total and PM, viability, morphology as well as most of sperm kinematics parameters assessed over the entire set of donkey semen doses processed after 24 h of cool storage.

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CHAPTER 2

"Colloid single-layer centrifugation improves post-thaw donkey (Equus asinus) sperm quality and is related to ejaculate freezability"

I Ortiz, J Dorado, D Acha, MJ Gálvez, M Urbano, M Hidalgo.

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Colloid single-layer centrifugation improves post-thaw donkey (*Equus asinus*) sperm quality and is related to ejaculate freezability

I. Ortiz[A], *J. Dorado*[A], *D. Acha*[A], *M. J. Gálvez*[A], *M. Urbano*[A] *and M. Hidalgo*[A,B]

[A]Animal Reproduction Group, Department of Animal Medicine and Surgery, University of Cordoba, 14071 Córdoba, Spain.
[B]Corresponding author. Email: mhidalgo@uco.es

Abstract. The aim of this study was to determine whether colloid single-layer centrifugation (SLC) improves post-thaw donkey sperm quality and if this potential enhancement is related to ejaculate freezability. Semen from Andalusian donkeys was frozen following a standard protocol. SLC was performed on frozen–thawed semen and post-thaw sperm parameters were compared with uncentrifuged samples. Sperm quality was estimated by integrating in a single value sperm motility (assessed by computer-assisted sperm analysis), morphology and viability (evaluated under brightfield or fluorescence microscopy). Sperm freezability was calculated as the relationship between sperm quality obtained before freezing and after thawing. Ejaculates were classified into low, medium and high freezability groups using the 25th and 75th percentiles as thresholds. All sperm parameters were significantly ($P < 0.01$) higher in SLC-selected samples in comparison to uncentrifuged frozen–thawed semen and several kinematic parameters were even higher than those obtained in fresh semen. The increment of sperm parameters after SLC selection was correlated with ejaculate freezability, obtaining the highest values after SLC in semen samples with low freezability. We concluded that, based on the sperm-quality parameters evaluated, SLC can be a suitable procedure to improve post-thaw sperm quality of cryopreserved donkey semen, in particular for those ejaculates with low freezability.

Additional keywords: cryopreservation, sperm freezability.

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Introduction

According to the Food and Agricultural Organisation (FAO), Spanish donkey breeds, including Andalusian donkey (*Equus asinus*), are in danger of disappearing because of their small population size and serious risks of inbreeding (Vidament *et al.* 2009). Considering the importance of biodiversity and of preservation of domestic species resources, the creation of genetic banks for these donkey breeds is needed (Rota *et al.* 2012). However, the scant information available regarding the assessment of sperm cryopreservation in Spanish breeds of donkeys is not consistent with the risk of the extinction of these populations (Dorado *et al.* 2013).

Cryopreservation causes major damage to equine spermatozoa (Watson 2000), which may result in low sperm quality after thawing and a reduction in pregnancy rates obtained after artificial insemination (AI). Taking into account the osmotic stress produced by cryopreservation on the sperm cell (González-Fernández *et al.* 2012), several strategies have been developed to improve freezing and thawing procedures in equine species (Salazar *et al.* 2011; Contri *et al.* 2012; Legha and Pal 2012) including the evaluation of different extenders (Alvarenga *et al.* 2005; Morillo Rodríguez *et al.* 2011; González-Fernández *et al.* 2012; Legha and Pal 2012), cryoprotectants (Alvarenga *et al.* 2005; Hoffmann *et al.* 2011), centrifugation protocols (Hoogewijs *et al.* 2010; Bliss *et al.* 2012; Edmond *et al.* 2012; Ferrer *et al.* 2012) and cooling, freezing and thawing rates (Bradford and Buhr 2002; Clulow *et al.* 2008). Additionally, several methods have been developed for improving the quality of semen samples by selecting those spermatozoa that are most likely to achieve fertilisation from the rest of the sperm population (Morrell *et al.* 2008*b*). Separation techniques used include sephadex column separation, glass wool and several density-gradient centrifugation protocols. Density-gradient centrifugation (DGC) through a silane-coated silica colloid has been previously used as a two-layer density gradient for separating equine spermatozoa (Morató *et al.* 2013; Stoll *et al.* 2013). DGC has been compared with a simpler colloid centrifugation procedure, so-called single-layer centrifugation (SLC), which was developed to work with only one layer of colloid (Morrell *et al.* 2008*a*). In this method, spermatozoa are centrifuged through a column of glycidoxypropyltrimethoxysilane-coated silica colloid in a species-specific formulation, resulting in the selection of motile, morphologically normal spermatozoa with intact membranes

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and good chromatin integrity (Morrell *et al.* 2009*a*, 2009*b*). For that purpose, different commercial products have been developed for use in stallions by modifying the non-silica portion of the gradient medium (Edmond *et al.* 2012). Several studies have examined the use of colloid centrifugation in frozen–thawed stallion spermatozoa (Macías García *et al.* 2009). However, to our knowledge, in spite of species-specific differences between stallions and donkeys, no studies have been performed in donkey frozen–thawed semen samples.

Current sperm cryopreservation procedures show variable responses between animals in equine species. Post-thaw semen quality and sperm freezability seem to vary consistently among stallions. The high variability between stallions for tolerating cryopreservation is a further complicating factor (Loomis and Graham 2008). Due to this fact, stallions have been classified as good, average or poor freezers according to their ability of their spermatozoa to withstand the freezing and thawing process (Loomis and Graham 2008). The sperm freezability of valuable genetic individuals is a key factor in reproductive strategies to conserve endangered species, such as the Andalusian donkey. It would be useful to obtain a genetic reservoir from a valuable jack with low sperm freezability or to improve post-thaw sperm quality of sperm banks already created with poor results after thawing. For that purpose, SLC could be applied to improve the sperm quality of certain individuals or semen samples. However, to our knowledge, the relationship between the freezability of donkey ejaculates and the improvement of sperm quality obtained after SLC selection has not yet been determined.

The aims of this study were: (1) to assess the effect of colloid single-layer centrifugation on post-thaw donkey sperm quality parameters and (2) to evaluate the relationship between sperm freezability and improvement of sperm parameters after SLC selection.

Materials and methods

Animals

Four Andalusian donkeys, aged from 6 to 15 years and clinically healthy, were used. This study was conducted in collaboration with 'Donkey's House' Foundation (Rute, Córdoba, Spain). Animals were housed during the experiments in individual paddocks placed at the Veterinary Teaching Hospital (VTH) of the University of Córdoba (Spain). The feeding consisted of alfalfa hay and water *ad libitum*.

Semen collection and evaluation

Semen was collected from each donkey on a regular basis of two ejaculates per week using an artificial vagina with an in-line gel filter (Minitüb, Tiefenbach, Germany) in the presence of a jenny in natural or prostaglandin-induced oestrus (luprostiol 7.5 mg intra-muscular, Prosolvin; Intervet International B.V., Boxmeer, The Netherlands) to stimulate libido. A total number of twelve ejaculates were obtained (three ejaculates per animal).

Immediately after collection, the gel-free volume was measured in a graduated collector (mL). Sperm concentration was calculated with a photometer (SpermaCue; Minitüb) as described by Vidament *et al.* (2009). The photometer was frequently calibrated using a control cuvette provided by the manufacturer. Before the assessment of sperm motility, morphology and viability, a semen aliquot was diluted with a skimmed-milk extender (EquiPro; Minitüb) to reach a sperm concentration of $\sim 25 \times 10^6$ spermatozoa mL^{-1}, incubated at 37°C for 10 min and then assessed as described below.

Computer-assisted sperm motility analysis (CASA)

Sperm motility was objectively evaluated by CASA using the Sperm Class Analyser (SCA) (Microptic S.L., Barcelona, Spain). This system consists of an optical phase-contrast microscope at 100× magnification (Eclipse 50i; Nikon, Tokyo, Japan) with a prewarmed stage at 37°C and a high-speed digital camera (A312fc; Basler AG, Ahrensburg, Germany) connected to a personal computer with the SCA motility software (Version 5.0.1). A total number of 25 consecutive digitalised frames in one second were obtained from a single field. Images were taken in a time lapse of 1 s, corresponding to a velocity of image-capturing of 1 photograph every 40 ms. The settings parameters of the software analysis were as follows: cell size from 15 to 75 μm^2, connectivity 12, progressive spermatozoa >75% of the straightness coefficient and average path velocity $>90\ \mu m\ s^{-1}$. For each evaluation, 5 µL of each diluted semen sample was placed in a Mackler counting chamber for analysis (Sefi-Medical Instruments Ltd, Haifa, Israel). Three drops, with two random microscopic fields per drop, were evaluated in each semen sample, including a minimum of 200 spermatozoa. The trajectory of each individual spermatozoon was determined by the SCA software obtaining the following sperm kinematic parameters: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, $\mu m\ s^{-1}$), straight-line velocity (VSL, $\mu m\ s^{-1}$), average path velocity (VAP, $\mu m\ s^{-1}$), linearity (LIN, ratio VSL/VCL × 100), straightness (STR, VSL/VAP × 100), wobble (WOB, ratio VAP/VCL × 100), amplitude of lateral head displacement (ALH, µm) and beat-cross frequency (BCF, Hz).

Sperm morphology

Sperm morphology was examined by light microscopy visual evaluation of slides stained with Diff-Quick (Baxter DADE AG 3186, Düdingen, Switzerland) as described previously (Hidalgo *et al.* 2006). At least 200 spermatozoa per slide were counted and the percentage of normal forms was recorded (NF, %).

Sperm viability

Sperm viability was assessed using a supravital stain (Cortés-Gutiérrez *et al.* 2008) based on the red–green emission of two fluorescent dyes: acridine orange and propidium iodide, respectively (Duo-Vital Kit; Halotech DNA SL, Madrid, Spain). The staining was performed following the manufacturer's instructions. At least 200 spermatozoa were counted per slide using fluorescence microscopy (Olympus BX40; Olympus, Tokyo, Japan) and a U-ND25–2 filter (a 460 to 490 nm excitation filter). Two staining patterns were discerned: viable spermatozoa (green emission) and dead spermatozoa (red or red–green emission). The percentage of viable spermatozoa (VS, %) was recorded.

Semen freezing and thawing

Semen samples were frozen following a standard protocol described for donkeys (Serres-Dalmau 2003) with modifications. Briefly, fresh semen was diluted in a ratio of 1 : 1 (v : v)

with EquiPro skimmed milk extender and then centrifuged 7 min at 400*g* at 22°C. The supernatant was removed and the sperm pellet was re-extended in semen freezing medium with glycerol (Gent; Minitüb) to a final concentration of 200×10^6 spermatozoa mL^{-1}. Spermatozoa were slowly cooled to 5°C within 2 h and then loaded in 0.5-mL plastic straws. The straws were frozen horizontally in racks placed 2.5 cm above the surface of liquid nitrogen (LN_2) for 5 min and placed into LN_2 tanks. After at least 1 week of storage, straws were thawed by immersion in a 37°C water bath for 30s.

Colloid single-layer centrifugation

Sperm selection was performed on frozen–thawed semen samples using EquiPure, purchased from Nidacon International AB (Gothenburg, Sweden). EquiPure consists of a commercial double-layer colloid density gradient based on silane-coated silica particles. Colloid single-layer centrifugation (SLC) was performed using the highest-density layer (EquiPure Bottom Layer). Before SLC, the colloid was allowed to equilibrate at room temperature (~22°C). Briefly, 1.5 mL of frozen–thawed semen were carefully layered on top of 4 mL EquiPure Bottom Layer located in a 15-mL corning tube, taking care not to disrupt the interface. The suspension was centrifuged at 300*g* for 20 min at 22°C. The supernatant (semen extender and colloid) was removed and the sperm pellet recovered was transferred to a clean tube containing 1 mL of skimmed milk extender. Concentration of the sperm pellets was measured using the SCA system. After that, semen samples were adjusted to a final concentration of 25×10^6 spermatozoa mL^{-1} and then sperm motility, morphology and viability were analysed as described above. The yield of selected spermatozoa was calculated according to the following formulae:

$$\text{Yield}\ (\%) = (\text{number of spermatozoa in sperm pellet} / \text{number of spermatozoa in initial load}) \times 100$$

$$\text{Yield of motile spermatozoa}\ (\%) = (\text{number of motile spermatozoa in sperm pellet} / \text{number of motile spermatozoa in initial load}) \times 100$$

Experiment 1: effect of colloid single-layer centrifugation (SLC) on post-thaw sperm parameters

SLC with EquiPure Bottom Layer was performed on frozen–thawed donkey semen and post-thaw sperm parameters compared with control samples. For that purpose, two aliquots of each frozen–thawed semen sample were taken. One of them was directly evaluated for sperm quality parameters following the methodology described above (uncentrifuged control samples). The other one was submitted to SLC-selection with EquiPure Bottom Layer and then evaluated. Post-thaw sperm parameters, including the sperm-quality index (SQi) were compared between uncentrifuged (control) and SLC-selected frozen–thawed semen. The SQi was calculated as the score of the sperm-quality parameters assessed according to the following formula: $SQi = \sum Sp/100n$, where Sp is a sperm parameter (independent variable) and n the number of parameters. The sperm parameters included in this formula were: total motility, progressive motility, viability and normal forms. Values for SQi ranged between 0 and 1, where SQ = 1 represents the maximum level of sperm quality.

Experiment 2: relationship between sperm freezability and the increment of sperm parameters after SLC

The relationship between the increment of sperm-quality parameters after SLC and the freezability of each semen sample was evaluated. The SQi was used to calculate the sperm-freezability index (SFi) of each ejaculate as follows: SFi = SQib/SQia, where SQia is the sperm quality of ejaculates before freezing and SQib is the sperm quality after thawing. Values for SFi ranged between 0 and 1, where SFi = 1 represents the maximum level of sperm freezability. Thereafter, ejaculates were classified according to their sperm freezability into low, medium and high groups using the 25th and 75th percentiles as thresholds. Ejaculates with SFi values below the 25th percentile were considered as low freezability, values between the 25th and 75th as medium freezability and values higher than the 75th percentile as high freezability. The improvement of each sperm parameter after SLC was calculated and compared between freezability groups. The increment of each post-thaw sperm parameter after SLC-treatment was calculated as the difference of its value obtained before and after SLC-treatment according to the formula: P increment (%) = (P% SLC-selected) – (P% uncentrifuged), where P is the value of each sperm parameter.

Statistical analysis

Statistical analysis of the sperm parameters assessed was performed on the sample means and the values were expressed as mean ± standard error of the mean (s.e.m.). Data analysis was performed using Statistical Analysis Systems software (SAS version 9.0; SAS Institute Inc., Cary, NC, USA). The effect of SLC on post-thaw sperm parameters was carried out using a general linear model (PROC MIXED) with animals, treatments and ejaculates as fixed effects.

Differences in post-thaw sperm parameters between groups of ejaculates classified according to their sperm freezability were evaluated by one-way ANOVA. Thereafter, differences in the increment of each sperm-quality parameter after SLC-treatment were also evaluated among low, medium and high freezability ejaculate groups by one-way ANOVA. The Duncan test was used for *post hoc* analyses. Correlations between the increment of each sperm parameter after SLC-treatment and sperm freezability were analysed using the Pearson coefficient (*r* value). Normality of the data distributions and variance homogeneity were checked by the Kolmogorov–Smirnov and Cochran tests, respectively. Scatter plots presented visual images of the data.

Results

Experiment 1: effect of colloid single-layer centrifugation (SLC) on post-thaw sperm parameters

Comparison between mean parameters of fresh semen and frozen–thawed samples are shown in Table 1. In general, cryopreservation induced a significant decrease in the mean

Table 1. Comparison of sperm parameters assessed between fresh and frozen–thawed donkey semen (unselected and SLC-selected) across the entire set of semen samples ($n = 12$)

VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency. ^{a,b,c} Superscripts indicate significant differences ($P < 0.001$). ^{A,B} Superscripts indicate significant differences ($P < 0.01$)

Sperm parameter	Fresh semen	Frozen–thawed semen samples	
		Unselected	SLC-selected
Total motility (%)	96.25 ± 0.86^{a}	66.12 ± 5.59^{c}	83.49 ± 13.89^{b}
Progressive motility (%)	83.04 ± 1.88^{a}	51.02 ± 5.39^{c}	70.83 ± 5.58^{b}
Viability (%)	83.64 ± 2.60^{a}	64.44 ± 2.15^{c}	73.62 ± 2.97^{b}
Normal forms (%)	83.65 ± 1.85^{A}	78.15 ± 1.64^{B}	82.88 ± 1.70^{A}
Sperm-quality index (0–1)	0.87 ± 0.12^{a}	0.65 ± 0.30^{c}	0.78 ± 0.29^{b}
VCL ($\mu m\ s^{-1}$)	171.60 ± 0.54^{a}	133.00 ± 0.99^{c}	137.50 ± 0.74^{b}
VSL ($\mu m\ s^{-1}$)	106.90 ± 0.51^{a}	104.31 ± 0.96^{b}	102.89 ± 0.69^{b}
VAP ($\mu m\ s^{-1}$)	143.32 ± 0.50^{a}	121.50 ± 0.97^{b}	123.20 ± 0.71^{b}
LIN (%)	58.81 ± 0.21^{c}	68.23 ± 0.41^{b}	69.43 ± 0.31^{a}
STR (%)	70.13 ± 0.20^{c}	76.90 ± 0.37^{b}	78.44 ± 0.28^{a}
WOB (%)	80.60 ± 0.12^{c}	83.91 ± 0.26^{b}	85.06 ± 0.18^{a}
ALH (μm)	4.09 ± 0.14^{a}	2.44 ± 0.15^{c}	2.75 ± 0.14^{b}
BCF (Hz)	7.53 ± 0.28^{c}	8.53 ± 0.65^{b}	9.12 ± 0.50^{a}

values of TM, PM, VS, SQi, VCL, VSL, VAP, ALH ($P < 0.001$) and NF ($P < 0.01$) of frozen–thawed unselected control samples in comparison to fresh semen. Centrifugation of frozen–thawed donkey spermatozoa through a single-layer colloid significantly ($P < 0.001$) improved all the sperm parameters assessed except VSL and VAP, which were statistically equal to uncentrifuged samples. Additionally, no significant differences were found between values of NF obtained in fresh semen and SLC-selected frozen–thawed samples. Moreover, mean values of LIN, STR, WOB and BCF were even higher in SLC-selected samples in comparison to fresh semen and uncentrifuged samples.

The sperm yield of the SLC procedure varied from 18.21% to 54.00% (mean sperm recovery rate of 30.30 ± 2.99%). The yield of motile spermatozoa after colloid centrifugation varied from 20.95% to 78.41% (mean motile sperm recovery rate of 41.59 ± 5.56%).

Experiment 2: relationship between sperm freezability and the increment of sperm parameters after SLC

Mean and range values of the SFi obtained after classification of donkey ejaculates into low, medium and high freezability groups using the 25th and 75th percentiles as thresholds are shown in Table 2. This classification produced significant differences between groups for most of the sperm parameters assessed (Table 3). The SQi, which includes all the sperm quality parameters assessed, were significantly different between the three groups ($P < 0.01$) with greater values in the high freezability group, intermediate values in the medium freezability group and lower values in the low freezability group. TM ($P < 0.01$) and NF ($P < 0.05$) were significantly greater in the intermediate and high freezability groups

Table 2. Classification of donkey ejaculates ($n = 12$) according to their sperm freezability index into low, medium and high freezability groups using the 25th and 75th percentiles as thresholds

Percentile	Ejaculate group	Sperm freezability index (0–1)	
		Range	Mean value
<25th	Low	<0.72	0.60
25th – 75th	Medium	0.72 – 0.82	0.76
>75th	High	>0.82	0.86

Table 3. Comparisons of post-thaw sperm parameters between ejaculate groups classified according to the sperm freezability index ($n = 12$)

Values are mean ± s.e.m. Different letters between treatments indicates significant differences: *$P < 0.05$, **$P < 0.01$

Post-thaw sperm parameters	Ejaculate freezability		
	Low	Medium	High
Sperm-quality index (0–1)**	0.53 ± 0.47^{c}	0.65 ± 0.26^{b}	0.76 ± 0.05^{a}
Total motility (%)**	41.80 ± 11.05^{b}	69.18 ± 4.33^{a}	84.33 ± 2.01^{a}
Progressive motility (%)**	31.77 ± 8.57^{b}	$49.62 \pm 5.13^{a,b}$	73.10 ± 1.62^{a}
Viability (%)	67.13 ± 7.23^{a}	62.07 ± 2.75^{a}	66.50 ± 0.80^{a}
Normal forms (%)*	70.96 ± 3.53^{b}	80.09 ± 1.42^{a}	81.47 ± 1.74^{a}

in comparison to the low freezability group. Significant differences ($P < 0.01$) were also found between the high and low freezability groups for PM. No differences were found between groups for viable spermatozoa.

The increments of each sperm parameter after SLC selection according to the ejaculate freezability group are shown in Fig. 1. The increments of almost all the sperm parameters assessed were significantly higher ($P < 0.05$) after SLC selection in the low freezability ejaculates in comparison to the medium and high freezability groups. The medium and high freezability groups were not different. Only the viable spermatozoa parameter was not different between any of the ejaculate groups.

Correlations between the increment of post-thaw sperm parameters after SLC and the sperm freezability index are shown in Table 4 and Fig. 2. A negative correlation was found between SFi and TM-I ($r = -0.76$, $P < 0.001$), PM-I ($r = -0.59$, $P < 0.001$), NF-I ($r = -0.43$, $P = 0.01$) and SQ-I ($r = -0.63$, $P < 0.001$). SFi was not correlated with VS ($r = 0.40$; $P > 0.05$) or yield ($r = 0.01$; $P > 0.05$). These results confirm those obtained in Fig. 1, which means the spermatozoa with the lowest freezability had the highest increment of sperm parameters after SLC selection of frozen–thawed semen samples.

Discussion

In this study, colloid single-layer centrifugation (SLC) was performed on frozen–thawed donkey semen. To our knowledge, this is the first attempt to separate cryopreserved donkey semen by SLC, based on preliminary results obtained by this research group (Ortiz *et al.* 2013). According to our results, processing of cryopreserved sperm samples by SLC resulted in higher sperm quality (motility, viability and normal forms) compared with

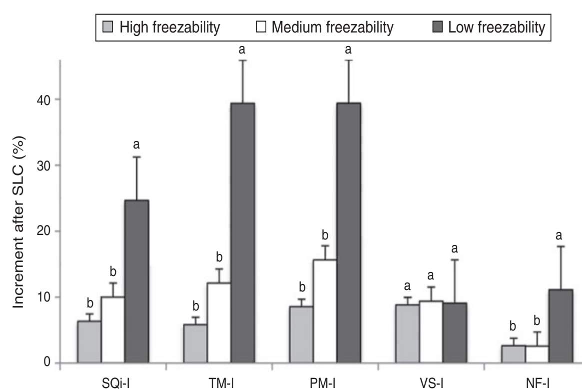

Fig. 1. Comparisons of the increments of each sperm parameter (mean ± s.e.m.) after colloid single-layer centrifugation (SLC) between ejaculate freezability groups (low, medium and high) across the entire set of semen samples ($n = 12$). Different letters between treatments indicates significant differences ($P < 0.05$). SQi-I, sperm quality index increment; TM-I, total motility increment; PM-I, progressive motility increment; VS-I, viable sperm increment; NF-I, normal forms increment.

Table 4. Correlations between the increment of post-thaw sperm parameters after SLC and the sperm freezability index ($n = 12$)
TM-I, total motility increment; PM-I, progressive motility increment; VS-I, viability sperm increment; NF-I, normal forms increment; SQi-I, sperm-quality index increment. Significant differences were set at $P < 0.05$

Increment of sperm parameters after SLC	Sperm freezability index (SFi)	
	Correlation (*r* value)	Statistics (*P* value)
TM-I	−0.76	<0.001
PM-I	−0.59	<0.001
VS-I	0.40	>0.05
NF-I	−0.43	=0.01
SQi-I	−0.63	<0.001
Yield after SLC	0.01	>0.05

unselected frozen–thawed control samples. Morphologically normal spermatozoa and some sperm kinetics parameters were similar or even higher in SLC-selected samples in comparison to fresh semen. In addition, the increment of sperm parameters after SLC was related to ejaculate freezability obtaining the highest values after SLC in semen samples with low freezability. These results confirm and extend previous reports that indicate that SLC through a colloidal matrix can select a population of spermatozoa that is positively enhanced in most of the sperm parameters usually measured in assessing sperm quality (Macías García *et al.* 2009; Stoll *et al.* 2013).

It is well known that current cryopreservation procedures induce a detrimental effect on post-thaw sperm-quality parameters (Ball 2008). Sperm selection after freezing and thawing is usually performed to improve the quality of cryopreserved semen. It has been shown that selection of the robust spermatozoa from a semen sample reduces the risk of using non-functional spermatozoa that would be unable to reach the site of fertilisation, and thus optimising the results of assisted reproductive technologies (Underwood *et al.* 2009). In this study, several sperm parameters were assessed to evaluate the sperm quality of semen samples before and after SLC. Additionally, the sperm-quality index (SQi) was calculated to integrate the different values of total and progressive sperm motility, viability and normal forms in a single value of SQi ranging from 0 to 1 (Cortés-Gutiérrez *et al.* 2008). The results obtained showed that the SQi was higher in SLC-selected semen in comparison to unselected frozen–thawed control samples. This general improvement in sperm quality after SLC also corresponds to higher values of post-thaw sperm motility, viability and normal forms in SLC-selected semen. These results are in agreement with previous studies where SLC has been carried out on frozen–thawed semen samples from different animal species (Clulow *et al.* 2008; Jiménez-Rabadán *et al.* 2012), including stallions (García *et al.* 2009; Macías García 2009; Stoll *et al.* 2013). In this study, SLC has been shown to separate sperm subpopulations after freezing and thawing according to differences in sperm motility, density or membrane characteristics that allow normal, motile and viable spermatozoa to migrate through the colloidal medium. It was reflected in an improvement of all the sperm parameters assessed after SLC and these results are correlated with the SQi, which integrates all of them into a single value. These results agree with previous findings in stallions and confirm that sperm-quality parameters do not represent independent variables to separate spermatozoa, as all of them were improved and successfully integrated in the SQi (Stoll *et al.* 2013).

The majority of the CASA motion parameters used in the present study have been previously identified as reliable markers of sperm quality and associated with fertility. In this study, VCL and ALH showed higher values in SLC-selected samples in comparison to uncentrifuged frozen–thawed semen. Previous studies agree on defining VCL as the most important velocity

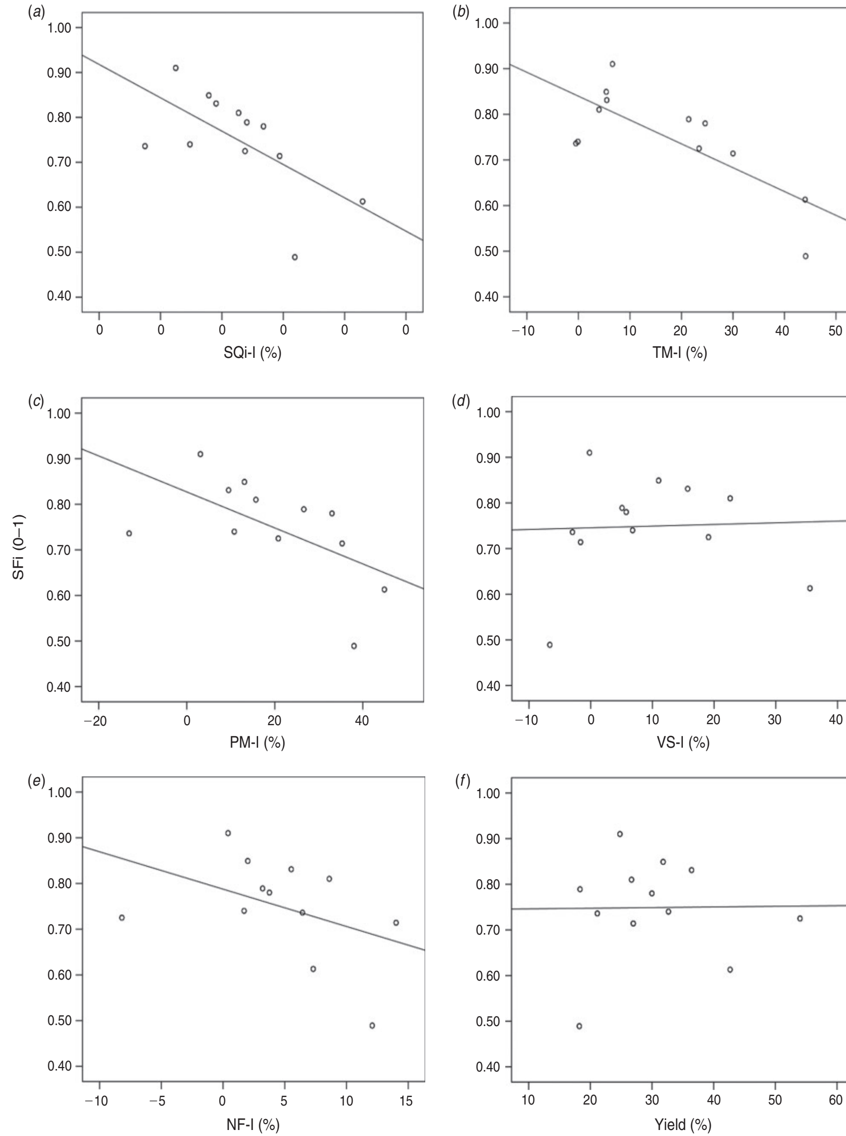

Fig. 2. Relationship between ejaculate freezability (SFi; 0–1) and the increments of each of the following sperm parameters after colloid single-layer centrifugation across the entire set of semen samples ($n = 12$): (*a*) SQi-I (%), sperm-quality increment, (*b*) TM-I (%), total motility increment, (*c*) PM-I (%), progressive motility increment, (*d*) VS-I (%), viability sperm increment, (*e*) NF-I (%), normal forms increment and (*f*) percentage sperm yield after single-layer centrifugation.

correlated with fertility values in different species. VCL appears to be critical for the formation of the sperm reservoir and the penetration of the zona pellucida; it is of key importance for formation of the sperm reservoir at the utero–tubal junction in mice (Olds-Clarke 1996) and is also inked to the ability of ram spermatozoa to penetrate cervical mucus (Robayo *et al.* 2008). Interestingly, some others kinetic parameters evaluated, such as LIN, STR, WOB and BCF were even higher in cryopreserved SLC-selected samples than in fresh semen. LIN, STR and WOB are considered to be motility quality indicators and BCF and ALH have been described as measures of sperm vigour (Cancel *et al.* 2000). These findings are in agreement with previous studies in stallions (García *et al.* 2009) and dogs (Dorado *et al.* 2011), which reported the predominance of a high-speed or progressive sperm subpopulation after centrifugation through a single-layer colloid. Therefore, in terms of sperm motility, the spermatozoa harvested after centrifugation through the SLC are significantly faster, more progressive and also more vigorous than the uncentrifuged spermatozoa.

In stallions, previous studies have been performed using DGC and SLC as two layers or single-layer centrifugation through the commercial colloid EquiPure to obtain good-quality sperm samples from cryopreserved ejaculates (Morató *et al.* 2013; Stoll *et al.* 2013). In this study, SLC through EquiPure Bottom Layer has been used for the first time in donkey spermatozoa to produce sperm preparations of equivalent quality as DGC or SLC previously reported in stallions using frozen–thawed semen. In this regard, from a clinical point of view, SLC with EquiPure offers an alternative, more practical method than discontinuous DGC for selection of good-quality donkey spermatozoa, thus saving time and effort in preparation. However, the sperm recovery rates obtained using this matrix of colloid were lower in comparison to other centrifugation protocols, as previously described in stallions (Bliss *et al.* 2012; Edmond *et al.* 2012).

Most sperm-selection systems result in loss of spermatozoa and, therefore, it may be that the proportion of selected functional cells is a critical factor of these techniques. In this study, SLC has been shown to be effective in improving sperm quality of donkey semen samples, besides reaching sperm recovery yields of 30%. The sperm yield obtained after SLC of cryopreserved stallion semen ranged from 20 to 40% (Macías García *et al.* 2009; Stoll *et al.* 2013). Although the sperm recovery obtained is close to values obtained in stallions, it is still low in comparison to other methods. As previously performed in several research papers in stallions, further studies are needed for testing different volumes of spermatozoa and colloid, centrifugation times and densities to obtain more accurate protocols to increase the yield obtained. Species-specific colloid formulation should be considered due to physical dynamics that may differently affect sperm recovery with different methods or species (Nicolas *et al.* 2012).

Some previous studies have reported that the sperm dose for AI in the uterine body of the jenny should be around 800×10^6 frozen–thawed spermatozoa (Vidament *et al.* 2009). From a practical point of view, it means around eight straws per AI, depending on the final concentration of each straw. In our study, the mean yield obtained was $\sim 30 \times 10^6$ of selected spermatozoa in each semen straw (100 million spermatozoa per straw $\times$ recovery rate/100). Having in mind the reference above, the number of straws to achieve this final number of spermatozoa should be increased when using SLC. However, elimination of undesirable spermatozoa from frozen–thawed semen with SLC enables semen doses enriched in specific sperm characteristics to be prepared for intrauterine AI and thus to obtain the highest pregnancy rates. Additionally, low-dose insemination by hysteroscopic or transrectally-guided deep-horn uterine insemination in equine species allows reduction of the number of spermatozoa per dose by as much as 5 million motile spermatozoa (Brinsko *et al.* 2003). According to the yield and the total sperm motility obtained after SLC, the number of motile spermatozoa recovered per straw was $\sim 26 \times 10^6$. Therefore, one single straw processed by SLC should be enough to inseminate approximately five jennies. However, further studies are needed in donkeys using low-dose insemination techniques with SLC-selected frozen–thawed semen samples.

Differences in the outcome of cryopreservation of stallion spermatozoa have been previously reported (Bradford and Buhr 2002). The chemical composition of the sperm membrane, which affects the membrane fluidity and permeability and the sperm osmotic tolerance, has been supposed to explain this variability (Graham 1996). Sperm cryo-resistance has been usually evaluated using several sperm parameters after thawing, i.e. post-thaw sperm motility $\geq 35\%$ (Loomis and Graham 2008), but its value as a predictor of fertilising ability remains unclear (Colenbrander *et al.* 2003). In this study, the sperm freezability index (SFi) was used to assess variability between ejaculates, integrating all the sperm parameters assessed instead of evaluating independent sperm parameters after thawing. For that purpose, we estimated the SQi of semen samples before freezing and after thawing and this resulted in a value of SFi ranging from 0 to 1, according to the ability of ejaculates to be frozen successfully. The SFi informs about the loss of sperm quality during the freezing and thawing process. Ejaculates were classified into low, medium and high freezability groups using the 25th and 75th percentiles as thresholds Additionally, this classification produced differences in post-thaw sperm-quality parameters between all three groups obtained (Martinez-Alborcia *et al.* 2012).

In this study, the increment of sperm parameters after SLC was different according to the ejaculate freezability. The improvement of sperm quality after SLC selection was significantly higher in ejaculates with low freezability. Ejaculates with medium and high freezability showed a similar increment in sperm quality after SLC; however, the tendency observed in our results was that the higher improvement of sperm quality after SLC selection occurred in the group with lower sperm cryo-resistance. It might be possible that with a larger number of ejaculates significant differences would also be observed between the medium and the high freezability groups. This means SLC improves all the semen samples assessed but this increment is higher if the freezability of the ejaculates is lower. SLC selection through a colloid matrix is usually performed as a tool to improve the sperm quality of poor semen samples. In this sense, from a practical point of view, the improvement of semen samples with poor sperm quality after freezing and thawing is

more interesting. Moreover, these findings offer additional possibilities to improve donkey sperm cryopreservation procedures. First, the sperm freezability of valuable genetic individuals is a key factor in the reproductive strategies to conserve endangered species, such as the Andalusian donkey. Variability between donkeys or ejaculates for tolerating cryopreservation is complicated and it may have a difficult solution. In the equine industry, a standard procedure for assessing suitability of commercial frozen semen production is to perform one or more split-ejaculate 'test-freezes' using different cryopreservation procedures. Several studies have reported that 20–40% of stallions could be considered as poor freezers on the basis of unacceptable sperm quality after cryopreservation (Vidament *et al.* 1997). Stallions with poor initial sperm quality or not producing acceptable post-thaw quality with any of the freezing techniques used are generally excluded from commercial semen cryopreservation (Loomis and Graham 2008). Unfortunately, it is not an option when semen must be frozen from endangered species with a limited number of genetically valuable males. Few studies have addressed variability in the freezability of jack semen (Taberner *et al.* 2010). Recently, Contri *et al.* (2012) supported this hypothesis finding variability in semen cryoresistance between donkeys. The present study was focussed on the evaluation of variability in sperm freezability between ejaculates and their response to SLC selection for improving sperm quality after thawing. According to our results, SLC can be performed in frozen–thawed semen samples of valuable donkeys with low sperm freezability to improve post-thaw sperm quality of cryopreserved samples. Additionally, SLC could also be applied to sperm banks of valuable genetic individuals already created using old or inappropriate techniques of sperm freezing (even after the death of the donkey) and particularly to those semen samples with poor results after thawing.

In conclusion, it seems clear that SLC of frozen–thawed donkey spermatozoa can be a suitable procedure for improving the post-thaw sperm quality of cryopreserved semen doses, in particular those ejaculates with low sperm freezability.

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CHAPTER 3

"Effect of single-layer centrifugation or washing on frozen-thawed donkey semen quality: Do they have the same effect regardless of the quality of the sample?"

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Effect of single-layer centrifugation or washing on frozen–thawed donkey semen quality: Do they have the same effect regardless of the quality of the sample?

I. Ortiz [a,*], J. Dorado [a], J.M. Morrell [b], F. Crespo [c], J. Gosálvez [d], M.J. Gálvez [a], D. Acha [a], M. Hidalgo [a]

[a] *Animal Reproduction Group, Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, University of Cordoba, Córdoba, Spain*
[b] *Department of Clinical Sciences, Division of Reproduction, Swedish University of Agricultural Sciences, Uppsala, Sweden*
[c] *Department of Reproduction, Centro Militar de Cría Caballar (FESCCR-Ministry of Defense), Ávila, Spain*
[d] *Department of Biology, Universidad Autónoma de Madrid, Madrid, Spain*

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The aims of this study were to determine the sperm quality of frozen–thawed donkey sperm samples after single-layer centrifugation (SLC) using Androcoll-E in comparison to sperm washing or no centrifugation and to determine if the effect on the sperm quality after SLC or sperm washing depends on the quality of the sample. Frozen–thawed sperm samples from Andalusian donkeys were divided into three aliquots, and they were processed using three different techniques after thawing: uncentrifuged diluted control (UDC), sperm washing (SW), and SLC. Afterward, sperm quality index was estimated by integrating all parameters (total and progressive sperm motility, membrane integrity, and DNA fragmentation) in a single value. The relationship between the sperm quality of thawed UDC samples and the effect on sperm parameters in SW and SLC-selected samples was assessed. Sperm quality index was significantly higher ($P < 0.001$) in SLC (0.8 ± 0.0) samples than that in UDC (0.6 ± 0.0) and SW (0.6 ± 0.0) samples, regardless of the sperm quality index after thawing of the sperm sample. In conclusion, SLC of frozen–thawed donkey spermatozoa using Androcoll-E-Small can be a suitable procedure for selecting frozen–thawed donkey sperm with better quality, in particular in those samples where an improvement in motility is needed.

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1. Introduction

Spanish donkey breeds are in danger of extinction; considering the importance of biodiversity and of preservation of domestic species resources, the creation of genetic banks for these donkey breeds is needed [1]. However, freezing and thawing causes major damage to the spermatozoa, particularly to their plasma and organelle membranes [2–4]. The most important factors causing cryoinjury are considered to be the osmotic stress caused by dehydration of the extender during freezing and thawing [5,6] and the toxicity caused by unequal distribution of cryoprotectants [7].

Different strategies have been proposed to improve postthaw sperm quality, including dilution or removing freezing extenders by simple sperm washing (SW) to reduce the concentration of cryoprotectant in the semen sample. It has been hypothesized that glycerol, the cryoprotectant most commonly used for horse semen cryopreservation, may be toxic for donkey jack sperm [8] or exert a negative effect on donkey jenny fertility [9]. These

* Corresponding author. Tel.: +34 957 218363; fax: +34 957 211093.
E-mail address: v52orjai@uco.es (I. Ortiz).

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facts could be responsible for the disappointing results of artificial insemination (AI) with frozen–thawed donkey sperm [1]. However, when several studies investigated this phenomenon, glycerol could not be confirmed as responsible for either toxicity of donkey sperm [10] or female uterus inflammation [9,11]. Thus, that hypothesis currently remains unclear.

Additionally, replacement of freezing extenders with seminal plasma by dilution or simple SW after thawing has recently been proposed for processing donkey sperm straws before AI [1]. However, in that study, better post-thaw sperm motility was shown when frozen–thawed donkey sperm was reextended with semen extender rather than with seminal plasma.

An alternative approach to improve the quality of frozen–thawed sperm doses and to remove the cryoprotectant used would be to select those spermatozoa that are most likely to achieve fertilization from the rest of the semen sample [12]. In this sense, centrifugation of stallion sperm through a species-specific single layer of silica colloid (single-layer centrifugation [SLC]), called Androcoll-E, has been shown to select sperm with better motility, normal morphology, membrane integrity, and intact chromatin from the rest of the ejaculate [13–15], and it also increases pregnancy rates [16]. However, to our knowledge, no studies comparing redilution, SLC, and SW have been performed in donkey frozen–thawed sperm samples.

The aims of this study were to (1) determine if SLC using Androcoll-E on frozen–thawed donkey sperm samples could select high-quality sperm in comparison to SW or no centrifugation, (2) determine if the effect on the sperm quality after SLC or SW depends on the quality of the sample.

2. Materials and methods

2.1. Animals

All animal procedures were performed in accordance with the Spanish laws for animal welfare and experimentation. Six healthy, mature, Andalusian donkeys, aged from 6 to 15 years, were used as semen donors. The jackasses were owned by "Donkey's House Foundation" (Rute, Córdoba, Spain) and were housed in individual paddocks placed at the Veterinary Teaching Hospital of the University of Córdoba (Spain). The feeding consisted of alfalfa hay and water "*ad libitum*."

2.2. Semen collection

Semen was collected using a Missouri artificial vagina with an in-line gel filter (Minitüb, Tiefenbach, Germany) in the presence of a jenny in natural or induced estrus to stimulate copulatory activity. Semen was collected from each animal twice a week. Three ejaculates were collected from each donkey on different sampling occasions obtaining a total number of 18 ejaculates. Total and progressive sperm motility were objectively evaluated from fresh semen by using the Sperm Class Analyzer (SCA 2011 v.5.0.1; Microptic S.L., Barcelona, Spain) system as described later. Gel-free volume (mL) was measured in a graduated collector. Sperm concentration ($\times 10^6$ spermatozoa/mL) was assessed with a sperm photometer (Spermacue; Minitüb).

2.3. Sperm freezing and thawing

Immediately after collection, semen was extended 1:1 (v:v) in EquiPro (Minitube) and centrifuged 7 minutes at $400 \times g$. After that, the supernatant was removed and the sperm pellet was reextended in a commercial freezing medium with glycerol (Gent; Minitube) to a final concentration of 200×10^6 sperm/mL. Semen was slowly cooled to 5 °C within 2 hours, loaded in 0.5-mL plastic straws, and frozen horizontally in racks placed 2.5 cm above the surface of the liquid nitrogen for 5 minutes. The straws were then directly plunged in liquid nitrogen, and after at least 1 week of storage, they were thawed in a water bath at 37 °C for 30 seconds.

2.4. Postthawing sperm processing

2.4.1. Uncentrifuged diluted control

One semen straw was thawed and directly diluted with INRA 96 (IMV Technologies, L'Aigle, France) to a final concentration of 25 million sperm/mL. This treatment was considered as uncentrifuged diluted control (UDC). Post-thaw sperm parameters were analyzed as described in the following.

2.4.2. Sperm washing

Immediately after thawing, one semen straw was extended at the 1:1 ratio and centrifuged at $400 \times g$ for 7 minutes. The supernatant was removed, and the sperm pellet was resuspended to a final concentration of 25 million sperm/mL for sperm analysis.

2.5. Single-layer centrifugation

Sperm selection was carried out on frozen–thawed semen samples using Androcoll-E-Small which is a glycidoxypropyltrimethoxysilane-coated silica colloid. Androcoll-E-Small was allowed to equilibrate to room temperature (22 °C) for 30 minutes before use. Then, 2 mL of frozen–thawed semen (three straws extended until a final concentration of 50–100 $\times 10^6$ sperm/mL) was carefully layered on the top of 4 mL of Androcoll-E-Small located in a 15-mL corning tube. The suspension was centrifuged at $300 \times g$ for 20 minutes. The supernatant was removed, and the sperm pellet was recovered and transferred to a clean tube containing extender. The sperm concentration of the resuspended sperm pellet in 1 mL of extender was measured using the SCA. After that, the sperm concentration of SLC-selected samples was adjusted to a final concentration of 25 million sperm/mL, and then, sperm parameters were analyzed as described in the following. The yield of selected spermatozoa was calculated according to the following formula: yield = (number of spermatozoa* in sperm pellet/number of spermatozoa* in initial load) $\times$ 100; *yield was separately calculated for total, motile, progressively motile, intact membrane, and unfragmented-DNA spermatozoa.

2.6. Postthaw sperm analysis

2.6.1. Computer-assisted sperm analysis of sperm motility

Sperm motility was evaluated by computer-assisted sperm analysis using the SCA system. Settings were as follows: one photograph every 40 ms, cell size from 15 to 75 μm^2; connectivity 12; progressive spermatozoa greater than 75% of the straightness (STR) coefficient. An aliquot of each semen sample was extended to a final concentration of 25×10^6 sperm/mL and then incubated at 38 °C for 10 minutes. After that, 5 µL of each diluted semen sample was placed in a Makler counting chamber (Sefi Medical Instruments Ltd., Haifa, Israel). Three drops, with two randomly chosen microscopic fields per drop, were analyzed in each semen sample. The trajectory of each individual spermatozoon was determined by the SCA software obtaining computer-assisted sperm analysis sperm kinematic parameters: total motility (TM), progressive motility (PM), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, VSL/VCL × 100), STR (VSL/VAP × 100), wobble (VAP/VCL × 100), amplitude of lateral head displacement (µm), and beat cross frequency (BCF, Hz).

2.6.2. Sperm membrane integrity

Sperm membrane integrity was assessed using the sperm Duo-Vital Kit (Halotech DNA SL, Madrid, Spain) on the basis of green and red emission of two fluorescent dyes: acridine orange and propidium iodide, respectively. At least 200 sperm were counted, considering green spermatozoa as intact membrane sperm and red or red–green as damaged membrane sperm. The percentage of sperm with intact membrane was recorded (IMS, %).

2.6.3. Sperm DNA fragmentation analysis

Sperm DNA fragmentation was assessed using the Halomax kit for stallions (Halotech DNA SL) on the basis of the sperm chromatin dispersion test. For each sample, 20 µL of diluted spermatozoa ($10–15 \times 10^6$ spermatozoa/mL) was added to a vial containing low–melting point agarose and mixed. This vial was previously immersed in a water bath at 90 °C to 100 °C for five minutes and subsequently was left to warm at 38 °C for five minutes. A 10-µL aliquot of the agarose–sperm mixture was then spread onto pretreated slides provided in the kit, covered with a glass coverslip, and placed in a refrigerator on a cold metallic plate for 5 minutes. After solidification, the coverslip was carefully removed and the "sperm–gel" slide was placed horizontally for 5 minutes in 10 mL of the lysing solution. The sperm–gel preparation was then washed in distilled water for 5 minutes before dehydration in a sequential series of 70% and 100% ethanol baths for 2 minutes each.

DNA damage was visualized under fluorescence microscopy after staining the smears with a commercial kit for green fluorescence (Halotech DNA SL). The emission of green provided enough discrimination of sperm heads containing either fragmented DNA (large halos of chromatin dispersion, at least the double diameter than the core) or unfragmented DNA (small halos or no halos of chromatin dispersion). At least 300 spermatozoa per sample were counted at × 40 objective magnification. Sperm DNA fragmentation was expressed as the percentage of spermatozoa with unfragmented DNA (sDU, %) or fragmented DNA (sDF, %).

2.6.4. Sperm quality index

The sperm quality index (SQi) was calculated as the score of the sperm quality parameters assessed according to the following formula: $SQi = \Sigma Sp/100n$, where Sp is a sperm parameter (independent variable) and n is the number of parameters. The sperm parameters included in this formula were TM, PM, membrane integrity, and DNA fragmentation. Therefore, the SQi was calculated as follows: $SQi = (TM + PM + IMS + sDU)/(100 \times 4)$. Values for SQi ranged between 0 and 1, where SQi = 1 represents the maximum level of sperm quality.

2.7. Experimental design

2.7.1. Experiment 1: Comparison of sperm quality parameters and yield between no centrifugation, SW, and SLC in frozen–thawed samples

Three aliquots of each frozen–thawed semen sample were taken and processed using three different procedures: (1) UDC, (2) SW, and (3) SLC using Androcoll-E-Small. After that, sperm parameters assessed, including the SQi, were statistically compared between the treatments.

2.7.2. Experiment 2: Comparison between SW and SLC in sperm samples with low, medium, or high quality after thawing

The relationship between the sperm quality of thawed uncentrifuged control samples (UDC) and the effect on sperm parameters in SW and SLC-selected samples was assessed. Uncentrifuged control samples (UDC) were divided according to their SQi into low-, medium-, and high-quality groups using the 25th and 75th percentiles as thresholds. Frozen–thawed samples with SQi values below the 25th percentile were considered as low, values between the 25th and 75th as medium, and values higher than the 75th percentile as high. The effect of SW and SLC was assessed in each group separately.

2.8. Statistical analysis

Data analysis was performed using the Statistical Analysis Systems software (SAS version 9.0; SAS Institute Inc, Cary, NC, USA). Analysis of the data was carried out using a general linear model (PROC MIXED), with animals, treatments, and ejaculates as fixed effects. The Duncan test for *post hoc* analyses was performed to assess differences between the treatments. Values were expressed as the mean ± standard error of the mean. Significant differences were considered when $P < 0.05$.

3. Results

All the sperm parameters assessed in fresh semen samples used in this study were within the physiological values for donkey semen evaluation [17]: sperm concentration greater than 208.7×10^6 sperm/mL, gel-free volume greater than 44 mL, sperm TM greater than 90.3%, and sperm PM greater than 67.3%.

3.1. Experiment 1: Comparison of sperm quality parameters and yield between no centrifugation, SW, and SLC in frozen–thawed samples

Total motility and PM were significantly higher in the SLC-selected samples compared to both SW and uncentrifuged diluted samples. Intact membrane sperm percentage was also higher in SLC than that in the SW samples. There were no significant differences between sperm parameter values obtained in UDC and SW samples except for membrane integrity, which was significantly lower in the SW group (Table 1).

Sperm quality index showed significantly ($P < 0.001$) higher values after SLC. No differences were found between the SW and uncentrifuged control samples.

All the kinematic parameters assessed were significantly improved ($P < 0.001$) in the SLC samples compared to the SW and UDC samples. There were also differences between the SW and UDC samples for some sperm kinematic parameters (VSL, LIN, STR, BCF) obtaining higher values after SW in comparison to the control (Table 1).

Sperm yields considering each parameter were also studied in Table 2. In general, SW showed higher sperm recovery rates than SLC except for yield of motile and yield of progressive where no significant differences were obtained between the treatments.

3.2. Experiment 2: Comparison of treatments (UDC, SW, and SLC) according to the SQi (low, medium, high) after thawing of sperm samples

Mean values of SQi obtained after classification of frozen–thawed donkey sperm were 0.5 ± 0.0 (range, <0.5) for the low SQi group, 0.6 ± 0.2 (range, 0.5–0.7) for the medium SQi group, and 0.8 ± 0.0 (range, >0.7) for the high-SQi group. Significantly higher values of SQi ($P < 0.001$) were observed for SLC in comparison to SW and UDC, regardless of the sperm quality group (Table 3).

Table 3 shows the comparison between the three different processing protocols on low, medium, and high sperm quality groups. Sperm TM and PM were significantly higher after colloid centrifugation in all sperm quality groups. In general, SLC did not select sperm membrane integrity in comparison to control; however, it was better than SW in medium- and high-quality groups. No significant differences in sperm DNA fragmentation were found between the treatments except for high sperm quality group where SW significantly increased this parameter.

Table 1
Sperm quality and kinetic parameters of frozen–thawed sperm samples from six donkeys subjected to three different processing techniques after thawing: uncentrifuged diluted control (UDC), sperm washing (SW), and single-layer centrifugation (SLC).

Postthaw sperm parameters	Treatment		
	UDC	SW	SLC
TM (%)	58.3 ± 4.6^y	57.0 ± 4.6^y	81.2 ± 3.4^x
PM (%)	47.7 ± 4.1^y	47.1 ± 4.6^y	73.4 ± 4.2^x
IMS (%)	57.5 ± 2.7^A	45.5 ± 3.6^B	61.7 ± 3.8^A
sDF (%)	12.7 ± 1.5	13.4 ± 1.2	12.3 ± 1.6
SQi	0.6 ± 0.0^y	0.6 ± 0.0^y	0.8 ± 0.0^x
VCL (μm/s)	150.4 ± 1.1^y	150.4 ± 1.2^y	156.5 ± 0.9^x
VSL (μm/s)	122.7 ± 1.1^z	127.2 ± 1.2^y	133.4 ± 0.9^x
VAP (μm/s)	140.7 ± 1.1^y	141.2 ± 1.2^y	147.0 ± 0.9^x
LIN (%)	73.3 ± 0.4^z	76.3 ± 0.5^y	79.8 ± 0.4^x
STR (%)	80.1 ± 0.4^z	83.2 ± 0.4^y	86.0 ± 0.3^x
WOB (%)	87.4 ± 0.3^y	87.9 ± 0.3^y	90.0 ± 0.2^x
ALH (μm)	2.4 ± 0.0^y	2.4 ± 0.0^y	2.5 ± 0.0^x
BCF (Hz)	7.6 ± 0.1^z	8.2 ± 0.1^y	9.1 ± 0.1^x

Values are expressed as mean ± standard error of the mean.
Different superscript letters indicate significant differences at different P values: $^{A, B, C}P < 0.05$; $^{a, b, c}P < 0.01$; $^{x, y, z}P < 0.001$.
Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; IMS, intact membrane sperm; LIN, linearity; PM, progressive motility; sDF, sperm DNA fragmented; SQi, sperm quality index; STR, straightness; TM, total motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, wobble.

4. Discussion

According to the results obtained in this study, colloid centrifugation using Androcoll-E selected high-quality sperm, showing significantly higher ($P < 0.001$) SQi values than those after SW and no centrifugation (UDC). These results are in agreement with previous studies where sperm parameters were improved after colloid centrifugation in donkey semen using Androcoll-E-Large [18] and Equipure [19]. Sperm quality is considered a parameter of crucial importance in assisted reproduction. It has been suggested previously that several *in vitro* assays of sperm quality could be used in an attempt to predict the fertilizing capacity of semen [20]. Although some tests seem to correlate well with pregnancy rates after AI in some species and for some males, a single test that is universally reliable across species remains elusive [21]. Previous studies have identified objective measures of sperm quality, such as chromatin integrity and sperm motility, which are linked to pregnancy rates [22,23]. Although preservation of sperm membranes and motility are important measures of sperm quality, they may not reveal the changes in DNA. The analysis of DNA fragmentation (sDF) has explained the infertility of individuals with apparently normal seminograms [24,25]. This approach is particularly interesting because DNA fragmentation correlates poorly with classical parameters of semen quality [26] as also occurs in donkey sperm [27]. Owing to this observation, in this study, we assessed sperm TM and PM, membrane integrity, and DNA fragmentation, and then, we used the SQi, which encompasses these four sperm parameters and allows us to visualize better the sperm quality of the semen sample and the effect that each treatment has on it.

However, when analyzing each parameter in detail, some particular differences are observed. In this regard, sperm membrane integrity was decreased after SW, and no differences were obtained between UDC and SLC. Although centrifugation is normally used in the equine practice to remove seminal plasma from stallion and donkey [28] semen, the sperm motility, membrane integrity, and recovery rate can be adversely affected by the centrifugation time and gravitational force (g-force) [29,30]. The g-force used in this study ($400 \times g$) has been used previously with good results to remove seminal plasma from donkey ejaculates before freezing. However, in this study, we centrifuged semen after thawing. Because of the deleterious

Table 2
Comparison of sperm yields using two different centrifugation protocols (sperm washing [SW] and single-layer centrifugation [SLC]) on frozen–thawed sperm samples (n = 18).

Treatment	Yield (%)	Yield of motile (%)	Yield of progressive (%)	Yield of intact (%)	Yield of unfragmented (%)
SW	58.6 ± 4.5^a	57.8 ± 5.3	57.8 ± 5.3	48.9 ± 6.2^A	58.1 ± 5.3^a
SLC	28.5 ± 3.4^b	40.3 ± 4.0	44.8 ± 4.7	31.1 ± 4.2^B	28.6 ± 3.4^b

Values are expressed as mean ± standard error of the mean.
Different superscript indicates significant differences at different P values: $^{A, B, C}P < 0.05$; $^{a, b, c}P < 0.01$; $^{x, y, z}P < 0.001$.
Yield = (number of spermatozoa* in sperm pellet/number of spermatozoa* in initial load) × 100. *Yield was separately calculated for total, motile, progressively motile, intact membrane, and unfragmented-DNA spermatozoa.

effect that freezing–thawing process has in the spermatozoa, plasma membrane of frozen–thawed sperm could be more sensitive to the centrifugation process even when a low g-force is applied. The apparent damage caused in sperm membranes after SW could be due to this fact. It is also interesting to note that the percentage of IMS was not significantly different in the SLC samples than in the control ones. Taking all these data into account, it seems that centrifugation has a harmful effect on sperm membranes, but when semen is cushioned with a colloid (Androcoll-E) rather than with a milk-based extender (INRA 96), this effect is diminished as it has been shown previously by Morrell et al. [31].

When analyzing sperm DNA fragmentation, no differences were found between the treatments for this parameter. Our results agree with Costa et al. [32]; in this study, PM and IMS were increased after SLC, but it did not have an effect on DNA integrity. Other studies state that SLC improves sperm DNA fragmentation [14,33], but these reports assessed DNA integrity after 48 hours of cold storage. Our results are also in agreement with previous reports performed in different animal species, such as rams [34], humans [35], stallions [36], and even donkeys [27] where no differences were found after the freezing–thawing process. These studies support that DNA fragmentation should be assessed as a dynamic parameter to evaluate the stability of DNA molecule after submitting semen samples to different stressors (temperature, osmolality, and so forth). This theory would explain why after 48 hours of cold storage significant differences are found because the colloid selected sperm with a more resistant DNA molecule. To confirm this justification, further studies should be performed to analyze the sperm DNA fragmentation dynamics after different protocols of centrifugation.

Sperm motility parameters were not adversely affected by any centrifugation procedure. On the one hand, SW enhanced some kinematic parameters related to LIN and sperm vigor (VSL, LIN, STR, and BCF) with respect to the control (UDC). Other authors have shown similar findings after centrifuging raw donkey semen [28,30] to remove seminal plasma from the semen sample. On the other hand, all the sperm parameters related to sperm motility from SLC-selected samples showed significantly higher values ($P < 0.001$) than noncentrifuged and washed samples. Sperm TM and PM are traditionally considered as essential indicators to evaluate the quality of a sperm sample [23]. Our results showed better values of these two parameters after SLC, which are in agreement with previous studies where colloid centrifugation was performed in frozen–thawed sperm of stallion [37] and donkeys [19]. The enhancement of all the sperm velocities after SLC was particularly interesting. Sperm velocities, mainly VCL and VSL, have been related to potential fertility [38]. Curvilinear velocity is of key importance for the formation of the sperm reservoir at the uterotubal junction in mice [39], whereas higher values of VSL have been associated with higher fertility in humans [40]. Average path velocity and STR (VSL/VAP) are used to calculate the percentage of PM of a semen sample. Amplitude of lateral head displacement together with BCF indicates the vigor of flagellar beating which is important for *in vitro* and *in vivo* fertilization to progress through the cervical mucus and perioocyte envelopes [41].

Table 3
Comparison of the three different processing protocols (uncentrifuged diluted control, UDC; sperm washing, SW; and single-layer centrifugation, SLC) after thawing on low, medium, and high sperm quality groups of frozen–thawed sperm samples.

Sperm quality	Treatment	Sperm parameters				
		TM (%)	PM (%)	IMS (%)	sDF (%)	SQi
Low	UDC	34.4 ± 3.9^b	27.8 ± 4.1^b	46.0 ± 2.7	14.3 ± 4.7	0.5 ± 0.0^B
	SW	32.6 ± 6.7^b	24.4 ± 5.8^b	30.5 ± 6.5	13.7 ± 3.1	0.4 ± 0.1^B
	SLC	69.5 ± 10.7^a	63.0 ± 11.7^a	49.4 ± 8.7	14.8 ± 4.7	0.7 ± 0.1^A
Medium	UDC	59.1 ± 4.4^y	47.1 ± 3.8^y	62.1 ± 3.6^a	14.3 ± 1.7	0.6 ± 0.0^y
	SW	59.7 ± 4.7^y	48.2 ± 5.1^y	46.9 ± 4.8^b	14.2 ± 1.6	0.6 ± 0.1^y
	SLC	80.6 ± 3.0^x	70.7 ± 4.8^x	63.4 ± 5.2^a	13.6 ± 1.9	0.8 ± 0.0^x
High	UDC	80.2 ± 4.3^B	69.0 ± 3.9^b	59.2 ± 4.6^{AB}	7.2 ± 1.5^B	0.8 ± 0.0^y
	SW	75.0 ± 3.1^B	67.1 ± 3.7^b	57.0 ± 2.3^B	10.9 ± 2.2^A	0.7 ± 0.0^y
	SLC	94.6 ± 1.0^A	90.5 ± 1.4^a	69.7 ± 4.1^A	6.8 ± 1.6^B	0.9 ± 0.0^x

SQi = ΣSp/100n (Sp = sperm parameter; n = number of parameters).
Values are expressed as mean ± standard error of the mean.
Different superscript indicates significant differences at different P values: $^{A, B, C}P < 0.05$; $^{a, b, c}P < 0.01$; $^{x, y, z}P < 0.001$.
Abbreviations: IMS, intact membrane sperm; PM, progressive motility; TM, total motility; sDF, sperm DNA fragmented; SQi, sperm quality index.

Sperm recovery rates (or yield) after both centrifugation treatments (SW and SLC) were low. Some studies have been carried out to analyze which was the best combination of force and time to remove seminal plasma from raw stallion ejaculates. Alvarenga et al. [42] reported that the lowest sperm loss and least sperm damage were achieved when stallion semen was centrifuged at $600 \times g$ for 10 minutes. In that study, the recovery rate after centrifugation was over 90%, which is much higher than that in this study ($58.6 \pm 4.5\%$). However, to the authors' knowledge, there are no previous references of SW after thawing donkey sperm. Moreover, the aim of this study was to compare SW and SLC, for this reason, the centrifugation protocol described by Serres-Dalmau [43] was chosen instead. This centrifugation protocol was designed and tested for freezing donkey semen, and also, it was a short-time, low-force protocol ($400 \times g$ for 7 minutes). With these characteristics, differences between SLC and SW regardless of time and force could be tested. Further studies are needed to achieve higher recovery rates, which would be more desirable for a practical use. Our results showed higher values of yield after SW than SLC except for yield of motile and yield of progressively motile where no significant differences were found. Although it has been described previously that SLC with Androcoll-E has a beneficial effect on fresh and fresh-cooled stallion sperm motility, membrane integrity, and chromatin integrity [14,33], it is also widely known that sperm motility and DNA fragmentation correlate poorly [44]. Our results suggest that the colloid seems to have more efficiency to select motile donkey sperm than DNA-unfragmented sperm because the yield of motile was higher than the yield of unfragmented. To comprehend this phenomenon, we should understand first the physical relationship between the cell and the colloid. An immature or chromatin-fragmented spermatozoon has a lower density [33]; for this reason, going through the colloid is more difficult than for an intact or a mature one. However, if this DNA-fragmented spermatozoon is rapid and progressive, this lower density would be compensated and the cell would cross the colloid and it would become an SLC-selected spermatozoon. Regarding membrane integrity, although it may appear incomprehensible, it has been reported before that motility and membrane integrity are not correlated [45]. As it has been explained previously, it seems that washing has a prejudicial effect in sperm membranes, but colloid centrifugation attenuates this deleterious effect [13]. Nevertheless, according to our results, SLC could not significantly select sperm with IMS; for this reason, SLC and SW should not be recommended to improve the plasma membrane integrity of the sperm sample in frozen–thawed donkey sperm.

In experiment 2, sperm samples were divided into low, medium, or high quality after thawing. The effect of the different treatments (UDC, SW, and SLC) was compared separately, to determine if the changes on the sperm quality after SLC or SW depend on the quality of the original semen sample. This experiment allowed us to discern whether it is worth performing a centrifugation procedure after thawing. Centrifugation did not affect sperm TM and PM, although SLC significantly selected sperm motility regardless of the initial sperm quality. This finding suggests that in any frozen–thawed donkey sperm sample, spermatozoa with better motility would be selected after carrying out SLC, even when the sperm quality of the sample is very good. In general, SLC did not select sperm membrane integrity in comparison to the control; however, it was better than SW in medium- and high-quality groups. The higher the SQi of the semen sample the more damaged after SW and the less injured after SLC. However, sperm membrane integrity was never significantly higher after centrifugation (SW and SLC) than that in the control (UDC); for this reason, if we want to increase the membrane integrity of the semen sample, we should choose another selection technique without a centrifugation step, such as swim-up. No significant differences in sperm DNA fragmentation were found between the treatments except for high sperm quality group where SW significantly increased this parameter. Single-layer centrifugation seems to show a tendency to select better sperm with intact DNA as SQi is increased. This could be due to the group of high SQi which has a greater percentage of motile and progressively motile sperm; then, the competition between the cells to pass through the colloid is greater, and thus, the density of the nucleus of the sperm is more relevant. If this hypothesis was proven in further studies with a dynamic DNA test, SLC could be used in semen samples with low fertility which show good values in classical parameters of semen quality because some of these problematic cases have been related before with higher levels of DNA fragmentation index [46].

Briefly, if SW is performed, 41.38% of the initial sperm load is lost and there is no beneficial effect on the semen sample. In contrast, although the recovery rate is lower after SLC, this treatment selects high-SQi sperm. Furthermore, this treatment is worth doing in any semen sample regardless of its SQi after thawing, especially if a better TM or PM motility is required.

In conclusion, SLC of frozen–thawed donkey spermatozoa using Androcoll-E-Small can be a suitable procedure for selecting high-quality sperm after thawing cryopreserved donkey sperm doses; in particular, in those samples where an improvement in motility is needed.

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CHAPTER 4

"Comparison of EquiPure and Androcoll-E for donkey sperm selection after thawing: effect on sperm motility, membrane integrity and motile sperm subpopulations"

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1 **Comparison of EquiPure and Androcoll-E for donkey sperm selection after**
2 **thawing: effect on sperm motility, membrane integrity and motile sperm**
3 **subpopulations**

4 I. Ortiz[1*], J. Dorado[1], J.M. Morrell[2], M.J. Gálvez[1], D Acha[1], M. Hidalgo[1]
5
6 *[1]Veterinary Reproduction Group, Department of Medicine and Animal Surgery,*
7 *Faculty of Veterinary Medicine, University of Cordoba, 14071 Cordoba, Spain.*
8 *[2]Department of Clinical Sciences, Division of Reproduction, Swedish University*
9 *of Agricultural Sciences, Box 7054, SE-75007 Uppsala, Sweden*
10
11 Corresponding author: Isabel Ortiz. Email: v52orjai@uco.es
12
13 Short title: Comparison of two colloids for donkey sperm
14
15 **Abstract**

16 EquiPure and Androcoll are colloids developed for stallion semen, both of which
17 have been used separately to improve sperm parameters in donkey semen after
18 thawing. The aim of this study was to compare two colloids for stallion semen
19 suitable for donkey sperm in order to assess if there is any difference in their
20 ability to select sperm with good motility, membrane integrity and a better
21 distribution of the motile subpopulations. Semen samples were divided between
22 the following treatments: one aliquot prior to freezing (Fresh), and four after
23 thawing: uncentrifuged diluted control, sperm washing, colloid centrifugation
24 using EquiPure Bottom Layer (SLC-EquiPure) and Androcoll-E (SLC-Androcoll).
25 Total (TM), progressive (PM) and sperm motility kinematics (assessed by

computer-assisted sperm analysis); membrane integrity (IMS, evaluated under fluorescence microscopy); and distribution of motile sperm subpopulations (sP) were compared between treatments. SLC-EquiPure and SLC-Androcoll showed sperm with higher TM and PM than the other treatments. The highest mean values in most kinematics were obtained for SLC-Androcoll, but IMS was improved after SLC-EquiPure. Four sP were found; SLC-Androcoll selected more sperm in sP4, i.e those with more progressive and faster sperm. In conclusion, colloid centrifugation using Androcoll or Equipure has a different effect on sperm features. EquiPure selects sperm with intact membranes and progressive but relatively slow sperm whereas Androcoll selects the fastest and most progressively motile sperm.

Keywords: Donkey, Cryopreservation, Sperm; Single layer centrifugation, Sperm selection.

Implications:

According to FAO, Andalusian donkey breed is endangered and accounted for 793 individuals, of which only 100 are considered as breeding males. One of the conservation strategies for such situation was the creation of cryopreserved semen banks from jackasses with desirable genetic features. However, freezing and thawing exert a deleterious effect on spermatozoa. Two colloids designed for stallion semen (EquiPure and Androcoll) have been shown to select good-quality donkey sperm after thawing. Nevertheless, there is not any study which compares if one colloid is more suitable than the other to select motile and viable sperm after thawing.

51

52 **Introduction**

53 Centrifugation through a single layer of silica colloid (SLC) has been
54 shown useful to improve sperm quality and the distribution of sperm
55 subpopulations in different animal species (Martinez-Alborcia *et al.*, 2013; Stoll *et*
56 *al.*, 2013; Urbano *et al.*, 2013; Anel-López *et al.*, 2015). EquiPure^{TM} and
57 AndroColl-Equine are commercial colloids developed for stallion sperm
58 separation. Although both colloids have been used to select stallion sperm (Stoll
59 *et al.*, 2013; Heutelbeck *et al.*, 2015), there is no specific colloid designed for
60 donkey semen processing. Recently, EquiPure Bottom Layer (Ortiz *et al.*, 2015a)
61 and Androcoll-E (Ortiz *et al.*, 2014) have been also used to select spermatozoa
62 with better parameters in this species. However, the effect of colloid
63 centrifugation on the structure of the motile subpopulations in donkey sperm has
64 not been assessed yet.

65 It is widely known that freezing and thawing cause major damage to
66 stallion and donkey sperm, mainly due to osmotic stress and penetrant
67 cryoprotectants (Peña *et al.*, 2011). SLC has been proposed as a strategy to
68 improve post-thaw sperm quality (Ortiz *et al.*, 2015a; Ortiz *et al.*, 2015b) and
69 fertility in donkeys (Serres *et al.*, 2014). Moreover, although comparisons of
70 different products and protocols for sperm selection have been carried out in
71 some animal species (Makkar *et al.*, 1999; Samardzija *et al.*, 2006; Phillips *et al.*,
72 2012; Sabatini *et al.*, 2014), such a comparison has not been carried out with
73 donkey semen.

74 Hence, the aim of this study was to compare two commercial stallion
75 colloids suitable for donkey sperm in order to assess if one of them is better at

76 selecting sperm with good motility, membrane integrity and distribution of the
77 motile subpopulations after thawing using dilution and sperm washing as
78 controls.
79

80 **Materials and methods**

81

82 *Semen collection and evaluation*

83 All animal procedures were performed in accordance with the Spanish
84 laws for animal welfare and experimentation, under the supervision of the
85 Bioethical and Biosafety Committee of the University of Cordoba (UCO). Semen
86 was collected from four clinically healthy Andalusian donkeys (aged 6-15 years)
87 using an artificial vagina in the presence of a jenny in estrus. Semen was
88 collected from each animal twice per week on different sampling occasions
89 obtaining twelve ejaculates in total (three ejaculates per animal). All the semen
90 samples had at least a gel-free volume > 44.0 mL, sperm concentration > 208.7
91 x 10^6 sperm/mL, total sperm motility > 90.3%, progressive sperm motility > 67.3%
92 and normal forms > 72.7% evaluated as described by Ortiz *et al.* (2014).

93 An aliquot from each ejaculate was diluted with a skimmed milk extender
94 (EquiPro, Minitüb, Tiefenbach, Germany) to reach a sperm concentration of
95 approximately 25x10^6 spermatozoa per mL, incubated at 37ºC for 10 minutes and
96 then assessed for sperm motility and membrane integrity as described later.

97

98 *Semen freezing and thawing*

99 Semen samples were frozen following a protocol previously used for donkeys
100 (Ortiz *et al.*, 2015b). Briefly, fresh semen was diluted in a ratio 1:1 (v:v) with

101 EquiPro and then centrifuged 7 min at 400 *g*. The sperm pellet was re-extended
102 in semen freezing medium with glycerol (Gent, Minitüb, Tiefenbach, Germany) to
103 a final concentration of 200 x 10^6 sperm/mL. Sperm were slowly cooled to 5ºC
104 for 2 hours into an Equitainer and then loaded in 0.5 mL plastic straws. The straws
105 were frozen horizontally in racks placed 2.5 cm above the surface of liquid
106 nitrogen (LN_2) for 5 minutes and placed into LN_2 tanks. Straws were thawed by
107 immersion in a 37ºC water bath for 30s.
108

109 *Computer-Assisted Sperm Motility Analysis (CASA)*

110 Sperm motility was evaluated in fresh and frozen-thawed semen samples
111 by CASA (Sperm Class Analyzer, SCA v5.01, Microptic S.L., Barcelona, Spain).
112 The features of this system have been described previously (Ortiz *et al.*, 2014).
113 Three drops, with two randomly chosen microscopic fields per drop, were
114 evaluated in a minimum of 200 spermatozoa per sample, obtaining the following
115 kinematic parameters: total (TM, %) and progressive motility (PM, %), curvilinear
116 (VCL, µm/s), straight line (VSL, µm/s) and average path velocities (VAP, µm/s),
117 linearity (LIN, VSL/VCLx100), straightness (STR, VSL/VAPx100), wobble (WOB,
118 VAP/VCLx100), amplitude of lateral head displacement (ALH, µm) and beat cross
119 frequency (BCF, Hz).
120

121 *Sperm membrane integrity*

122 The integrity of plasma membrane was assessed in fresh and frozen-
123 thawed sperm using Duo-Vital (Halotech SL, Madrid, Spain) as described by
124 Cortes-Gutierrez *et al.* (2008). At least 200 sperm were counted per slide using
125 fluorescence microscopy (Olympus BX40, Tokyo, Japan) and a U-ND25-2 filter

126 (a 460 to 490 nm excitation filter). The percentage of sperm with intact membrane
127 was recorded (IMS, %).
128
129 *Sperm Yield*
130 The yield of recovered (SW) or selected spermatozoa (SLC-EquiPure and
131 SLC-Androcoll) was calculated according to the following formula:
132 *Yield = (number of spermatozoa* in sperm pellet/number of spermatozoa* in*
133 *initial load) x 100*
134 **Yield was separately calculated for: total sperm, motile, progressively motile and*
135 *intact membrane sperm*
136
137 *Experimental design*
138 Four aliquots of each frozen-thawed semen sample were processed
139 according to the procedures described below. Sperm parameters in fresh semen
140 and post-thaw sperm samples were compared statistically (Figure 1).
141
142 *Uncentrifuged diluted control (UDC).* One semen straw was thawed and directly
143 diluted with INRA 96 (IMV Technologies, L'Aigle, France) to a final concentration
144 of $25x10^6$ sperm/mL. Post-thaw sperm parameters were analysed as described
145 above.
146
147 *Sperm washing (SW).* Immediately after thawing, one semen straw was extended
148 1:1 in INRA 96 and then centrifuged at 400 *g* for 7 minutes. The supernatant was
149 removed and the sperm pellet was resuspended as described for UDC.
150

151 *Single layer centrifugation using EquiPure (SLC-EquiPure).* Single layer
152 centrifugation (SLC) was performed using the highest density layer of EquiPure
153 (EquiPure^{TM} bottom layer, Nidacon International AB, Gothenburg, Sweden) as
154 described previously by Ortiz *et al.* (2015a). Briefly, 1.5 mL of frozen-thawed
155 semen were carefully layered on top of 4 mL EquiPure located in a 15 mL corning
156 tube at room temperature (≈22ºC). The suspension was centrifuged at 300 *g* for
157 20 min. The supernatant was removed and the sperm pellet resuspended as
158 described above.
159
160 *Single layer centrifugation using Androcoll-E (SLC-Androcoll).* AndroColl-Equine
161 (Minitüb, Tiefenbach, Germany) consists of a glycidoxypropyltrimethoxysilane-
162 coated silica commercial colloid for large volumes of equine semen. In this
163 experiment, we used the original version (non-commercialised) for small volumes
164 (Androcoll-E-Small, Swedish University of Agricultural Sciences, Uppsala,
165 Sweden) following the methodology described by Ortiz *et al.* (2015b). Briefly, 2
166 mL of frozen-thawed semen were carefully layered on top of 4 mL of Androcoll-
167 E-Small in a 15-mL corning tube. The suspension was centrifuged at 300 *g* for
168 20 min. The supernatant was removed and the sperm pellet diluted as described
169 for the other treatments.
170
171 *Statistical analysis*
172 Results were expressed as mean ± standard deviation. Differences
173 between mean values of sperm motility, membrane integrity and recovery rate
174 were analyzed using a general linear model (PROC GLM) with animals,
175 treatments and ejaculates as fixed effect. The Duncan test was used for *post hoc*

176 analyses. Secondly, sperm subpopulations analysis was performed over a data
177 matrix of 32,993 individual motile spermatozoa. A principal component analysis
178 (PRINCOMP) followed by the FASTCLUS clustering procedure was used to
179 classify the spermatozoa of the data set into a reduced number of subpopulations
180 according to their patterns of movement as previously described (Martinez-Pastor
181 *et al.*, 2005a). The summary statistics of the relative frequencies of spermatozoa
182 belonging to each subpopulation were calculated and compared by ANOVA and
183 chi-square test (FREQ procedure). All analyses were performed with SAS
184 statistic package v9.0 (SAS Institute Inc., Cary, NC, USA). The level of
185 significance was set at $P < 0.05$.

186

187 **Results**

188 In general, TM, PM and IMS were higher in fresh semen in comparison to
189 frozen-thawed semen samples. Additionally, colloid single layer centrifugation
190 resulted in higher sperm parameters in comparison to SW and UDC. In particular,
191 IMS was higher in SLC-EquiPure in comparison to SLC-Androcoll but lower than
192 fresh semen; however, SLC-Androcoll obtained similar values for PM in
193 comparison to fresh semen (Table 1). On the other hand, most of the sperm
194 kinetics parameters were significantly higher in SLC-Androcoll in comparison to
195 the other post-thaw procedures obtaining values similar (VCL) or even higher
196 (VSL, VAP, LIN, STR and WOB) than fresh semen (Table 1).

197 In general, yield of sperm were similar between SLC-EquiPure and
198 Androcoll but lower than SW. Only the yield of IMS obtained with SLC-EquiPure
199 was similar to SW but also to the value obtained for SLC-Androcoll (Table 2).
200 Summary statistics for the motility characteristics of the four subpopulations

201 identified are shown in Table 3. Subpopulation 1 (sP1) included spermatozoa with
202 relatively low velocity (medium VCL, VSL and VAP) but with high
203 progressiveness (high LIN, STR, WOB, BCF and low ALH), yielding 32.2% of the
204 total motile population. Subpopulation 2 (sP2) comprises 11.1% of sperm
205 population, including highly active but non-progressive spermatozoa (high VCL
206 and ALH, low LIN and STR and moderate BCF). Subpopulation 3 (sP3) contained
207 the lowest number of spermatozoa (9.5%) and included spermatozoa whose
208 movements were less vigorous (low VCL, VAP, ALH and BCF) and less
209 progressive (low VSL, LIN and STR). Subpopulation 4 (sP4) contained the largest
210 number of spermatozoa (51.5%), which were the most vigorous spermatozoa
211 (highest VCL and BCF and high ALH) and progressive (highest VSL and VAP).
212 There were significant differences in the distribution of the four sperm
213 subpopulations between (P=0.034, letters a-e) and within (P=0.010, letters A-D)
214 treatments (Figure 2). Sp4 was the most representative subpopulation in all
215 treatments except for EquiPure where sP1 was the largest.
216
217 **Discussion**
218 In the present study, two commercial colloids developed for stallion semen
219 were compared to select donkey sperm with better motility, membrane integrity
220 and belonging to the best motile subpopulation after thawing.
221 According to our results, SLC using both colloids increased TM and PM
222 compared to controls (SW and UDC), which is in agreement with other studies
223 performed in donkey semen (Ortiz *et al.*, 2015a; Ortiz *et al.*, 2015b).
224 Nevertheless, not only is it important that the spermatozoa are motile but also the
225 features (called kinematics) of this movement. Both methods (SLC-EquiPure and

226 SLC-Androcoll) significantly increased ALH and BCF, related to sperm vigor
227 (Cancel *et al.*, 2000). Moreover, VCL, VSL and, VAP, which indicate rapidness,
228 are correlated with fertility rates in several mammalian species (Olds-Clarke,
229 1996; Robayo *et al.*, 2008; Macías García *et al.*, 2009). Surprisingly, the
230 significantly highest values of these velocities were shown after colloid
231 centrifugation using Androcoll, being even greater than those obtained in fresh
232 semen. SLC-EquiPure did not achieve such values and they were lower than SW.
233 In brief, the procedure which selects the most rapid, progressive and vigorous
234 spermatozoa was SLC-Androcoll.

235 On the other hand, EquiPure was the only procedure able to select sperm
236 with intact membrane after thawing. It could be happening that Androcoll and
237 EquiPure exert different effects on the sperm membrane such as different
238 glycoprotein removal that could justify the results obtained. At this point we are
239 facing a dilemma: SLC-Androcoll selects better velocity and progressivity,
240 nevertheless, SLC-EquiPure shows better membrane integrity values. According
241 to Foster *et al.* (2011), sperm motility and viability address different aspects of
242 equine sperm quality. Since they are independent measures, motility and
243 membrane integrity cannot be used on their own to ascertain that one colloid is
244 preferred over another. Even though the exact composition of both colloids
245 remains unknown, it would make sense if Androcoll had higher density, because
246 faster sperm would get to the pellet more easily and it would be saturated before,
247 but the plasma membrane damage would be also greater (Morrell *et al.*, 2009).
248 Nonetheless, increasing density might have some drawback apart from the
249 damage of the plasma membrane, for instance a lower recovery rate. In this

250 regard, there were no significant differences between SLC-EquiPure and SLC-
251 Androcoll in the yields obtained.

252 It is important to highlight the fact that until now, we have been working
253 using mean values of sperm motility. This fact oversimplifies the motility analysis
254 because the sample is considered as homogeneous and its internal variability is
255 not taken into account. This means that the further relationship between motility
256 and fertility is impaired (Martinez-Pastor *et al.*, 2005b). In this sense, the
257 ejaculates of several mammalian species (Abaigar *et al.*, 1999; Quintero-Moreno
258 *et al.*, 2003; Martinez-Pastor *et al.*, 2005a; Muiño *et al.*, 2009; Dorado *et al.*,
259 2011a), including donkeys (Flores *et al.*, 2008; Dorado *et al.*, 2013c), are known
260 to contain motile sperm subpopulations, which have been related to
261 cryoresistance (Flores *et al.*, 2008; Dorado *et al.*, 2011a) and fertility (Quintero-
262 Moreno *et al.*, 2003; Dorado *et al.*, 2013c). Our study revealed four sperm
263 subpopulations as previously described in fresh, cooled and frozen-thawed
264 donkey semen (Flores *et al.*, 2008; Miró *et al.*, 2009; Dorado *et al.*, 2013b). In
265 agreement with these studies, cryopreservation had a significant effect on the
266 distribution of these subpopulations. According to the results obtained in the
267 subpopulation distribution of post-thaw control samples (UDC), it seems clear
268 that sperm subpopulations with non-progressive movement (sP2 and sP3) are
269 not able to survive to freezing and thawing and become immotile (Flores *et al.*,
270 2008; Dorado *et al.*, 2011b). When thawed sperm was submitted to sperm
271 washing, the proportions of sP1 (low velocity and high progressiveness), sP2
272 (high activity but non-progressiveness) and sP3 (less vigor and less
273 progressiveness) were decreased and sP4 (highest vigor and progressivity) was

274 increased. It seems that fast and progressive sperm can withstand the stress
275 caused by centrifugation and they pass more easily into the pellet.

276 When it comes to SLC-EquiPure, the subpopulation with a higher number
277 of spermatozoa was sP1. This could be due to two possibilities: on the one hand,
278 the colloid EquiPure could mainly select this kind of spermatozoa or, this colloid
279 selects motile and progressive sperm, regardless of its velocity. Therefore, it is
280 possible that progressive and fast spermatozoa (sP4) lose velocity after
281 centrifugation through this colloid, and appear in sP1. The same effect would
282 occur to those sperm which were originally in sP1, becoming sP2 and sP3. This
283 possible damage would not affect membrane integrity. After colloid centrifugation
284 with Androcoll, the highest number of sperm belonged to sP4. This colloid could
285 either select fast and progressively motile sperm (sP4) or have a less deleterious
286 effect on sperm motility than EquiPure. In stallions, Androcoll selected
287 progressive spermatozoa from frozen-thawed semen with relatively low velocity
288 (similar to sP1 in this study) (Macias Garcia *et al.*, 2009). In dogs, samples
289 centrifuged with Androcoll-C and Puresperm increased the high speed and
290 progressive subpopulation (similar to sP4 in this study) (Dorado *et al.*, 2011b;
291 Dorado *et al.*, 2013a). Our results cannot be compared with other studies in
292 donkey semen since, to the best of the author's knowledge, this is the first study
293 which assesses the effect of colloid centrifugation in donkey sperm
294 subpopulations and it is also the first one which studies the effect of EquiPure in
295 motile sperm subpopulations.

296 When AI (artificial insemination) is performed with frozen-thawed donkey
297 semen, pregnancy rates obtained in jennies are very low (0-36%), (Trimeche *et*
298 *al.*, 1998; Oliveira *et al.*, 2006; Vidament *et al.*, 2009) (particularly when compared

299 to mares (36-53%) (Vidament *et al.*, 2009; Canisso *et al.*, 2011) Different
300 strategies have been developed to improve fertility rates using cryopreserved
301 donkey semen including addition of different cryoprotectants and seminal plasma
302 to frozen-thawed semen (Rota *et al.*, 2012), study of the jennies endometrial
303 response after AI (Vilés *et al.*, 2013a and 2013b) or the influence of different
304 insemination protocols (Oliveira *et al.*, 2016). Recently, Serres *et al.* (2014) found
305 that SLC-selected donkey semen obtained better pregnancy rates in comparison
306 to unselected samples. Moreover, the subpopulation of rapid and highly
307 progressive spermatozoa, which correspond to sP4 in this study, has been found
308 in a higher proportion in fresh ejaculates of donkeys with better fertility rates
309 (Dorado *et al.*, 2013c). According to Dorado *et al.* (2013c) and Serres *et al.*
310 (2014), it could be expected higher fertility values in SLC-Androcoll selected
311 samples with a higher proportion of sP4. Unfortunately, it is not possible to
312 assume that a higher proportion of sP4 in SLC-Androcoll samples is responsible
313 for higher pregnancy rates without fertility trials.

314 In conclusion, EquiPure selects membrane intact, progressive and
315 relatively slow sperm whereas Androcoll selects the fastest and more progressive
316 sperm. Further studies are needed to clarify which aspects of sperm quality are
317 more related with pregnancy rates after AI.

318

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331

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485

Table 1. *Comparison of sperm parameters assessed before freezing (fresh semen) and after different procedures performed after thawing.*

Sperm parameters	Fresh semen	Post-thaw sperm processing				RMSE
		UDC	SW	SLC-Equipure	SLC-Androcoll	
TM (%)	96.3[a]	64.8[c]	60.8[c]	83.5[b]	83.9[b]	9.5
PM (%)	83.0[a]	51.6[c]	50.9[c]	70.8[b]	77.2[ab]	11.7
IMS (%)	83.6[a]	59.8[c]	46.2[d]	73.6[b]	61.0[c]	8.8
VCL (μm/s)	169.7[a]	137.9[c]	156.0[ab]	140.2[bc]	165.1[a]	19.4
VSL (μm/s)	107.1[b]	111.0[b]	132.4[a]	107.8[b]	140.6[a]	13.4
VAP (μm/s)	140.7[bc]	127.6[c]	146.3[ab]	126.8[c]	155.7[a]	17.0
LIN (%)	59.9[d]	70.3[c]	76.6[b]	72.3[c]	80.7[a]	3.9
STR (%)	72.1[d]	78.5[c]	83.7[b]	80.7[bc]	87.1[a]	3.9
WOB (%)	80.1[d]	84.9[c]	87.7[b]	86.5[bc]	90.4[a]	2.1
ALH (μm)	4.2[a]	2.4[b]	2.4[b]	2.7[b]	2.7[b]	0.4
BCF (Hz)	7.7[b]	8.2[b]	8.1[b]	9.6[a]	9.3[a]	0.8

RMSE= Root mean square error; UDC= Uncentrifuged diluted control; SW= Sperm washing; SLC=Single layer centrifugation; TM = Total motility; PM = Progressive motility; IMS = Intact membrane sperm; VCL= Curvilinear velocity; VSL = Straight-line velocity; VAP = Average path velocity; LIN = Linearity; STR = Straightness; WOB = Wobble; ALH = Amplitude of lateral head displacement; BCF = Beat-cross frequency.

Values within a row with different superscripts differ significantly at $P<0.001$.

Values are expressed as mean values.

Table 2. *Sperm recovery rate (yield) obtained after different post-thaw sperm procedure.*

	Yield			
Treatments	Total sperm	TM	PM	IMS
Sperm Washing	60.1^{a}	61.2^{a}	63.2^{a}	51.3^{a}
SLC-Equipure	30.3^{b}	41.0^{b}	42.6^{b}	38.8^{ab}
SLC- Androcoll	28.2^{b}	36.7^{b}	44.7^{b}	29.4^{b}
RMSE	12.5	14.2	17.7	16.1
P-value	P<0.001	P<0.001	P=0.017	P=0.011

SLC=Single layer centrifugation; TM=Total Motility, PM=Progressive Motility; IMS=Intact Membrane Sperm.

Values within a column with different superscripts differ significantly at *P*<0.05.

Table 3. *Motility parameters for the four sperm subpopulations (sP1, sP2, sP3, and sP4) defined after analysis of the entire set of semen samples.*

		Sperm motility patterns							
Subpopulation	n (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
sP1	10632 (32.2)	128.1^{c}	102.9^{b}	117.1^{c}	81.2^{b}	88.6^{b}	91.3^{b}	2.5^{d}	8.7^{b}
sP2	3658 (11.1)	201.8^{b}	80.7^{c}	158.9^{b}	39.3^{c}	51.4^{d}	78.4^{c}	5.3^{a}	7.8^{c}
sP3	3150 (9.5)	77.7^{d}	30.9^{d}	57.8^{d}	39.1^{c}	56.4^{c}	70.8^{d}	2.8^{c}	5.5^{d}
sP4	15553 (47.1)	208.34^{a}	177.3^{a}	194.0^{a}	85.3^{a}	91.3^{a}	93.1^{a}	3.3^{b}	10.2^{a}
RMSE		32.2	31.2	31.2	13.3	13.4	7.4	1.1	3.4

n= number of spermatozoa; VCL= curvilinear velocity; VSL = straight-line velocity; VAP = average path velocity; LIN = linearity; STR = straightness; WOB = wobble; ALH = amplitude of lateral head displacement; BCF = beat-cross frequency.

Different superscript letters (a-d) in the same column indicate significant differences (P<0.001) between subpopulations.

1

2 **Figure captions**

3 **Figure 1.** Experimental design.

4 Fresh= fresh semen; UDC= Uncentrifuged diluted control; SW= Sperm washing;

5 SLC= Single layer centrifugation.

6

7 **Figure 2**. Relative frequency distribution of motile sperm subpopulations (sP1-

8 sP4) between and within treatments.

9 Different letters (A-D) inside columns indicate significant differences within

10 treatments (P=0.01). Different letters (a-e) in the table below indicate significant

11 differences between treatments.

12

13

Figure 1

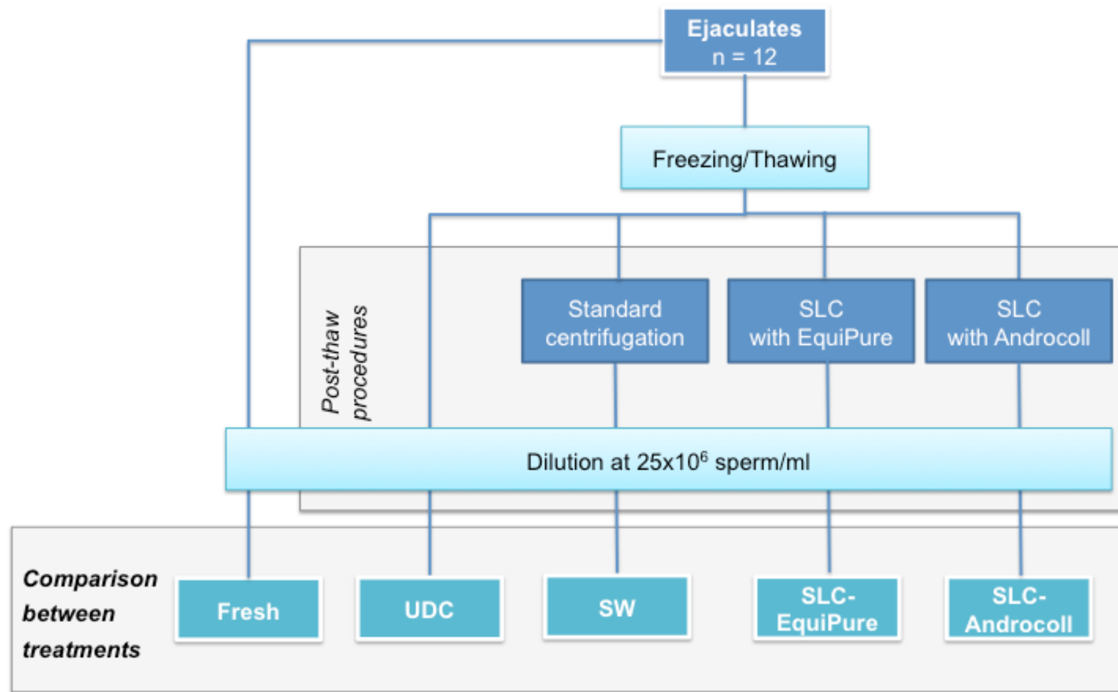
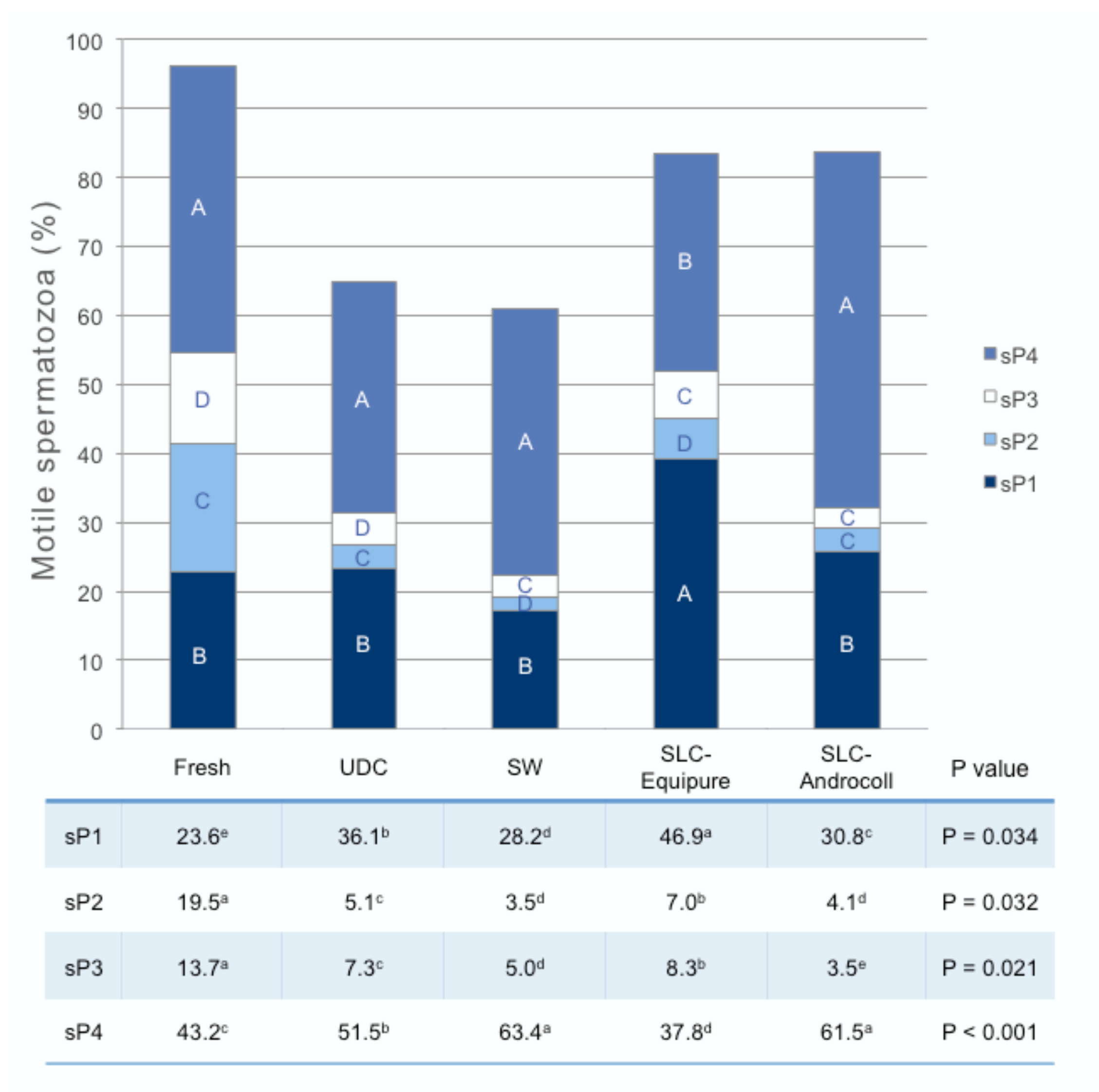


Figure 2



	Fresh	UDC	SW	SLC-Equipure	SLC-Androcoll	P value
sP1	23.6^{e}	36.1^{b}	28.2^{d}	46.9^{a}	30.8^{c}	P = 0.034
sP2	19.5^{a}	5.1^{c}	3.5^{d}	7.0^{b}	4.1^{d}	P = 0.032
sP3	13.7^{a}	7.3^{c}	5.0^{d}	8.3^{b}	3.5^{e}	P = 0.021
sP4	43.2^{c}	51.5^{b}	63.4^{a}	37.8^{d}	61.5^{a}	P < 0.001

CHAPTER 5

"New approach to assess sperm DNA fragmentation dynamics: Fine-tuning mathematical models"

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New approach to assess sperm DNA fragmentation dynamics: Fine-tuning mathematical models.

I. Ortiz[a], J. Dorado[a], J. Morrell[b], J. Gosálvez[c], F. Crespo[d], J.M. Jiménez[e], M. Hidalgo[a]

[a]*Veterinary Reproduction Group, Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Cordoba, 14071 Cordoba, Spain.*[b]*Department of Clinical Sciences, Division of Reproduction, Swedish University of Agricultural Sciences, Box 7054, SE-75007 Uppsala, Sweden.* [c]*Department of Biology, Universidad Autónoma de Madrid, 28049 Madrid, Spain.* [d]*Department of Reproduction, Centro Militar de Cría Caballar (FESCCR-Ministry of Defense), 05005 Ávila, Spain.* [e]Department of Chemistry and Physics of Materials, University of Salzburg, Hellbrunner Straße 34/III, A-5020 Salzburg, Austria.

Abstract

Background: Sperm DNA fragmentation (sDF) has been proved to be an important parameter in order to predict *in vitro* the potential fertility of a semen sample. Colloid centrifugation could be a suitable technique to select those donkey sperm more resistant to DNA fragmentation after thawing. Previous studies have shown that to elucidate the latent damage of the DNA molecule, sDF should be assessed dynamically, where the rate of fragmentation between treatments indicate how resistant is DNA to iatrogenic damage. The rate of fragmentation is calculated using the slope of a linear regression equation. However, it has not been studied if sDF dynamics fits this model.

The objectives of this study were to evaluate the effect of different after-thawing centrifugation protocols on sperm DNA fragmentation and elucidate the most accurate mathematical model (linear regression, exponential or polynomial) for DNA fragmentation over time in frozen-thawed donkey semen

Results: After submitting post-thaw semen samples to no centrifugation (UDC), sperm washing (SW) and single layer centrifugation (SLC) protocols, sDF values were significantly lower after 6 h of incubation in SLC samples than in SW and UDC. Coefficient of determination (R2) values were significantly higher for a second order polynomial model than for linear or exponential. The highest values for acceleration of fragmentation (aSDF) were obtained for SW, followed by SLC and UDC.

Conclusion: SLC after thawing seems to preserve longer DNA longevity in comparison to UDC and SW. Moreover, the fine-tuning of models has shown that sDF dynamics in frozen-thawed donkey semen fits a second order polynomial model, which implies that fragmentation rate is not constant and fragmentation acceleration must be taken into account to elucidate hidden damage in the DNA molecule.

Keywords: Sperm DNA fragmentation; dynamics; fine-tuning; colloid centrifugation; mathematical models.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

104 **Background**

105 The importance of the assessment of sperm chromatin to predict the
106 potential fertility is well proved in humans and animals [0]. This crucial role
107 that sperm DNA fragmentation (sDF) plays in sperm analysis is due to its
108 relationship with infertility problems after obtaining apparently normal values
109 for routine sperm parameters such as motility, morphology or integrity of
110 sperm membranes [1]. The assessment of this parameter is even more critical
111 when sperm quality is limited or compromised, as it happens in some
112 subfertile males, cool-shipped or frozen-thawed semen samples [2].
113 Therefore, it becomes of the utmost importance to select spermatozoa with
114 intact DNA in order to achieve a higher success in pregnancy rates [3].

115 Previous studies have evaluated the effect of different centrifugation
116 techniques to select frozen-thawed donkey sperm [4,5] concluding that,
117 although sperm quality was improved when colloid centrifugation was
118 performed, this procedure did not select intact DNA spermatozoa performing
119 a static analysis of sDF (baseline value). Nevertheless, it has been shown in
120 several studies [6,7] that a dynamic assessment of sDF is more accurate to
121 simulate *ex vivo* sperm maintenance and to evaluate latent chromatin damage
122 than only considering baseline values. These studies submitted semen
123 samples to thermal stress and recorded sDF values at different times. Then, a
124 linear regression equation was calculated and the rates of fragmentation (the
125 slope of the linear regression equation, sDF%/time) of the treatments were
126 compared.

127 This approach solved the issue of the encrypted DNA damage,
128 however, another question arose: Do DNA dynamics fit to a linear regression

model? Although linear regression is the simplest model, it entails that DNA damage is then a simple process with constant speed. Before accepting this statement as an actual fact, a fine-tuning of mathematical models for DNA fragmentation over time should be carried out.

In light of the aforementioned, the objectives of this study were to evaluate the effect of different after-thawing centrifugation protocols on sperm DNA fragmentation and elucidate the most accurate mathematical model for DNA fragmentation over time in frozen-thawed donkey semen.

Methods

Animals and semen collection

Six healthy Andalusian donkeys (aged from 6 to 15 years) were used for this study. Semen collection was performed using a Missouri artificial vagina with an in-line gel filter (Minitüb, Tiefenbach, Germany) in the presence of a jenny in natural or induced estrus. Three ejaculates per animal were collected, obtaining a total number of 18 ejaculates. All animal procedures were performed in accordance with the Spanish laws for animal welfare and experimentation.

Sperm freezing and thawing

Sperm was frozen and thawed following the methodology described by Ortiz et al. [5]. Briefly, seminal plasma was removed by centrifugation (400 x *g* for 7 minutes) and the sperm pellet was resuspended with a commercial freezing medium containing egg-yolk and glycerol (Gent; Minitüb, Tiefenbach,

Germany). Then, semen was slowly cooled for 2 hours, loaded into 0.5ml straws, placed 2.5cm above the surface of the liquid nitrogen (LN_2) for 5 minutes and plunged in LN_2. Thawing was performed in a water bath at 37ºC for 30 seconds.

Sperm processing after thawing

After thawing, each semen sample was divided into three aliquots and submitted to different centrifugation protocols.

Uncentrifuged diluted control (UDC):

After thawing, sperm was extended (INRA96; IMV Technologies, L'Aigle, France) to a final concentration of $25x10^6$ sperm/ml. Sperm parameters were analyzed as described below.

Sperm washing (SW):

Sperm was thawed, diluted at the 1:1 ratio and centrifuged (400 x g for 7 minutes). The sperm pellet was resuspended to $25x10^6$ sperm/ml for sperm analysis.

Single Layer Centrifugation (SLC):

Sperm selection was carried out using the colloid Androcoll-E-Small (Swedish University of Agricultural Sciences, Uppsala, Sweden) as described by Ortiz et al. [5]. In short, 2ml of thawed sperm were carefully placed on 4ml of colloid. The suspension was centrifuged (300 x g for 20 minutes) and the

pellet was resuspended to a final concentration of 25x10^6 sperm/ml. Then, sperm parameters were assessed as described in the following.

Sperm analysis after thawing

Sperm motility and membrane integrity

Total (TM, %) and progressive (PM, %) sperm motility were evaluated by computer-assisted sperm analysis (CASA) using the Sperm Class Analyzer (SCA 2011 v.5.0.1; Microptic S.L., Barcelona, Spain) with the settings described by Ortiz et al. [8]. Membrane integrity was assessed using Vital Test kit (Halotech DNA, Madrid, Spain) following manufacturer's instructions[5]. Red-stained sperm were considered as membrane-damaged and green sperm were considered as intact membrane sperm (IMS, %).

Sperm DNA fragmentation (sDF) analysis

The degree of DNA damage in each sample was quantified using the sperm DNA fragmentation index (sDF%). This parameter was assessed using the Halomax kit (Halotech DNA SL, Madrid, Spain) as previously described by Ortiz et al. [5]. This test is based on the dispersion of the chromatin (halo) after an exposure to a lysing solution. In order to evidence the halos of chromatin, samples were stained with a commercial kit for green fluorescence (Halotech DNA SL). Those sperm with large halos (at least double diameter than the core) were considered to have fragmented DNA. At least 300 spermatozoa per sample were counted and the percentage of fragmented DNA cells was recorded (sDF, %).

203 **Experimental design:**

204 *Experiment 1: Effect of UDC, SW and SLC on DNA fragmentation dynamics.*

205 A dynamic assessment of DNA fragmentation was carried out by
206 incubating an aliquot (from UDC, SW and SLC samples) for 24 h at 37ºC. sDF
207 was evaluated at T0 (baseline), T3, T6 and T24h and compared between and
208 within treatments.

209

210 *Experiment 2: Comparison between the coefficient of determinations (R^2) of*
211 *linear, exponential and polynomial regression in sDF dynamics.*

212 In order to fit the regression model for sDF, coefficient of determination
213 (R^2) of sDF dynamics (T0, T3, T6, T24) was calculated for different regression
214 models (linear, exponential and polynomial) and compared within treatments
215 (UDC, SW, and SLC).

216 The accuracy of three different regression models (linear, exponential,
217 and polynomial) was evaluated by comparing the coefficient of determination
218 (R^2). Then, sDF dynamics were compared between treatments using the most
219 accurate regression model.

220

221 **Statistical analysis:**

222 In Experiment 1 statistical analysis was performed using the Statistical
223 Analysis Systems software (SAS v.9.0; SAS Institute Inc., Cary, NC, USA). A
224 general linear model (PROC MIXED) with animals, ejaculates, treatments and
225 time as fixed effects was performed. Then, differences between treatments
226 (UDC vs. SW vs. SLC) and times (T0 vs. T3 vs. T6 vs. T24) were assessed.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

227 In Experiment 2, sDF (%) values (*y* coefficient) at 0, 3, 6 and 24 hours
228 (*x* coefficient) were adjusted to linear, exponential and second order
229 polynomial models. R^2 was calculated for each replica and model using
230 Microsoft Excel for Mac v.14 (Microsoft Corporation, Redmond, WA, USA). R^2
231 was compared, separately for each treatment (UDC, SW and SLC), among
232 models (linear, exponential and polynomial) with PROC MIXED (SAS) using
233 animals and ejaculates as fixed effects.

234 Since second order polynomial functions are parabolic lines, the
235 derivative function $\frac{d\,sDF}{dt}\left(\frac{\%}{h}\right)$ was calculated for each treatment (UDC, SW and
236 SLC). Afterwards, a graphic was represented using the rate of change of sDF
237 (%/h, DNA fragmentation velocity) of the polynomial function $\frac{d\,sDF}{dt}\left(\frac{\%}{h}\right)$ as *y*
238 coefficient and time (0, 3, 6 and 24 h) as *x* coefficient. The slopes of these
239 straight lines (DNA fragmentation acceleration $\%/h^2$) were compared between
240 treatments (UDC vs. SW vs. SLC) using GraphPad Prism v.6 for Mac OS v.6
241 (GraphPad Software, San Diego, CA, USA).

242 All values were expressed as the mean ± standard error of the mean
243 (SEM). Significant differences were considered when $P<0.05$. Duncan *post*
244 *hoc* test was carried out to assess differences between treatments.

245

246

247 Results

248

249 Sperm parameters after thawing

Mean values of sperm parameters obtained immediately after thawing were as follows: TM = 58.31 ± 4.57, PM = 47.66 ± 4.07, IMS =57.53 ± 2.71, and sDF = 12.98 ± 1.52.

Sperm DNA Fragmentation (sDF) Dynamics

Comparison between UDC, SW, and SLC after thawing up to 24 h of incubation at 37ºC is shown in Figure 1. Significantly lower values of sDF (P<0.001) were found for SLC after 24h of incubation.

Table 1 illustrates the sDF dynamics within treatments. Significantly higher values (P<0.001) of sDF were obtained after 6 h of incubation for UDC and SW. However, in SLC-selected aliquots there were not significant differences until 24 h of incubation at 37ºC.

Regression models fit to the data

R^2 for SDF dynamics was significantly higher (P=0.001) in polynomial regression models in comparison to linear and exponential models for UDC (0.9699 ± 0.0087 vs. 0.8694 ± 0.0335 vs. 0.8014 ± 0.343), SW (0.9667 ± 0.0120 vs. 0.9324 ± 0.0190 vs. 0.8828 ± 0.0251), and SLC (0.9706 ± 0.0097 vs. 0.8326 ± 0.0605 vs. 0.0826 ± 0.0581) (Figure 2).

sDF dynamics in polynomial regression

Figure 3 shows the graphical representation of the polynomial regression models for UDC, SW and SLC.

Since second order polynomial functions are graphically represented with parabolic lines, they cannot be compared with each other as a whole.

275 The derivative of the polynomial function $\frac{d\,sDF}{dt}\left(\frac{\%}{h}\right)$ is the rate of change of the
276 function (fragmentation rate, %/h). Figure 4 represents the velocity of
277 fragmentation with respect to time for UDC, SW and SLC. The slopes of these
278 lines are the acceleration of fragmentation (aSDF, fragmentation rate/time,
279 $\%/h^2$). Significant differences between the slopes of UDC (-0.0683 ± 0.0265),
280 SW (0.0106 ± 0.0130) and SLC (-0.0073 ± 0.0141) were obtained (P=0.0141).
281

282 Discussion

283 The results of this study indicate that DNA in frozen-thawed donkey
284 sperm selected by SLC is more resistant to a stressor (incubation at 37°C up
285 to 24h) than control or SW. In order to compare sDF values obtained after
286 each centrifugation procedure (UDC, SW and SLC), semen samples were
287 submitted to incubation at 37°C for 24 h. Afterwards, a static and a dynamic
288 assessment of the DNA fragmentation dynamics were carried out. On the one
289 hand, the static analysis of the sDF dynamics, which consisted of a
290 comparison of treatments (UDC vs. SW vs. SLC) right after finishing the
291 centrifugation protocols (T0) and after 3h (T3), 6h (T6) and 24h (T24) of
292 incubation at 37°C. There were not differences in sDF values between
293 treatments until 24 hours. On the other hand, it was also evaluated how stable
294 was the DNA molecule in each centrifugation protocol (T0 vs. T3 vs. T6 vs.
295 T24). In this regard, sDF values did not increase until 6 h of incubation for
296 UDC and SW. However, sDF remain stable for up to 24 h of incubation in SLC
297 samples. Previous studies performed in stallion semen and donkey semen
298 have not found differences after colloid centrifugation in DNA fragmentation

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

299 baseline values (T0) [5,9]. However, according to other studies, sDF must be
300 studied dynamically by submitting the semen sample to a stressor in order to
301 find a possible encrypted damage in the DNA [6,7,10]. When a dynamic
302 assessment was performed, other studies did show that SLC was able to
303 select those stallion sperm more resistant to DNA fragmentation [11,12].
304 Thus, the joint interpretation of the static and dynamic assessment of sDF
305 dynamics seem to agree that SLC slow down the DNA fragmentation process
306 in comparison to UDC and SW.

307 Dynamical processes (such as sperm DNA fragmentation) are very
308 common in biology as they provide insight into how a force (e. g. incubation at
309 37°C over time) acts to change a cell, an organism, a population, or an
310 assemblage of species [13]. Since we want to know how sDF changes over
311 time *(t)*, that is, *sDF(t)*. There are two main types of dynamical models,
312 "discrete time" and "continuous time", depending on whether time is
313 represented in discrete steps or along continuous axis. In practice, it is not
314 possible to evaluate sDF continually, we must divide the sample into aliquots
315 at intervals of time instead. Thus, it is crucial to choose a suitable time scale
316 for our study. Previous reports in donkeys [14] and horses [7] have shown that
317 significant changes in sDF between treatments and individuals occurred from
318 6 to 24 h of incubation at 37°C when using a chromatin dispersion test or 4 h
319 of incubation for sperm chromatin structure assay (SCSA) by flow cytometry
320 [15]. However, since one of the objectives of this study was to fine-tune a
321 model, in order to adjust the model to reality, we need to have as many points
322 as possible, then we set the points at 0, 3, 6 and 24 h of incubation.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

Once the variable (sDF) and the units of time (0, 3, 6 and 24 h) are stablished, it is needed to evaluate how they are related. The most common single-variable functions or mathematical models which regularly arise in all areas of biology are linear, exponential and polynomial. It is common to think of a relationship between two variables as a straight line, in fact, it has been taken for granted that sDF follows a linear regression equation

$$y = f(x) = ax +$$

where *y*=sDF(%), *x*=time(h); *a*=fragmentation rate(%/h); *b*=intercept(%). This model has been applied to calculate the sDF rate (rSDF) using the slope of the linear regression line. Nonetheless, a linear regression model, as its name indicates, is a straight line. This would mean that rSDF is constant over time which lead us to face some limitations. In this sense, a phenomenon called “Plateau effect” has been previously described when using linear regression to assess sDF dynamics in stallion sperm [7]. This so-called Plateau effect is nothing but a change in the slope of the regression line, due to a change in the velocity of fragmentation. This singularity leads to confounding results if the equation is not divided in two different lines: 0-6h and 6-24h of incubation. Nonetheless, that provisional adjustment shows that a sDF dynamics does not fit a linear regression equation.

Non-linear equations are those which can be represented as curves. In this sense, exponential and polynomial functions are very popular for modelling biological functions. Exponential function is probably one of the most important function in dynamic models of biology, it is represented as

$$y = f(x) = b \cdot e^{ax};$$

its main application is describing fast growth [16]. The exponential model has previously been applied to explain the behavior of sDF dynamics in human ejaculates [17], nevertheless, this explanation was merely descriptive since there was not a statistic comparison between other mathematical models. Last but not least, second order polynomial function (or quadratic),

$$y = f(x) = ax^2 + bx + ;$$

has a wide variety of important uses in biology. It describes the rate of growth when resources are limited [18]. Although previous studies have shown sDF curves which might fit a polynomial model [19,20], to date, no study has tried to explained sDF dynamics using this model. Actually, to the best of the authors' knowledge, a fine-tuning of models has never been performed for sDF dynamics. Furthermore, this quadratic model explains by itself the behavior of sDF from 0 to 24 h of incubation, without the need to split the 0-6h and 6-24h as it occurs with the traditional assessment by linear regression [7].

On the whole, mathematical models are mainly use to make predictions about the behavior of a variable at any time. Logically, the closer to reality the model is the more accurate the prediction will be. In statistics, R^2 provides a measure of how well observed outcomes are replicated by the model, based on the proportion of total variation of outcomes explained by the model [21]. Surprisingly, second order polynomial achieved the significantly highest R^2 in all the treatments studied (UDC, SW and SLC), becoming a more accurate model than linear regression or exponential to predict sDF over time. The more than acceptable R^2 mean values obtained for UDC, SW and

SLC (0.9699, 0.9667 and 0.9706, respectively) make that the conclusions obtained from this model are very close to reality.

The fact that our model is a parabola implies that sDF rate is not constant, which also means that there is sDF acceleration, fact that has never been described before. In quadratic functions, we can track the rate of change of the function using a difference equation. A difference equation specifies how much a variable changes from one time unit to the next. In quadratic functions, the rate of change over time is expressed as follows

$$y' = f'(x) = \frac{dy}{dx} = 2ax + b;$$

in our function, the rate of change of the function is the rate of change of rSDF. Therefore, sDF rate (rSDF) in quadratic functions is expressed as

$$rSDF = \frac{dsDF}{dt}\left(\frac{\%}{h}\right),$$

and the fragmentation acceleration (aSDF) is the rate of change over time, or the slope of the derivative of the quadratic function,

$$aSDF = \frac{dsDF/dt}{t}\left(\frac{\%}{h^2}\right).$$

Surprisingly, when representing the rates of change for UDC, SW and SLC, the three lines obtained were very different. Although UDC showed faster rSDF for about 10 h, it was also the treatment with significantly lower acceleration ($aSDF_{UDC}$= -0.0683 ± 0.0265), this marked deceleration explains the Plateau effect described in stallions. In SW samples, rSDF values were lower than UDC until 10 h of incubation, but a higher acceleration ($aSDF_{SW}$= 0.0106 ± 0.0130) increased rSDF from that point on. SLC also showed a negative acceleration ($aSDF_{SLC}$= -0.0073 ± 0.0141), not as marked as in UDC samples. On the one hand, centrifuged samples after thawing (SW and

396 especially SLC) showed lower values than control (UDC) for 10 and 15 h,
397 respectively. On the other hand, centrifugation increased acceleration of
398 fragmentation, in particular in SW samples. It could be possible that post-
399 thawing centrifugation, mainly SW, damaged the fixing mechanism of the
400 DNA molecule [22,23]. However, sDF values are lower during the time studied
401 for SW and SLC. Further studies involving fertility are needed to study this
402 hypothetical damage and evaluate if it is more relevant than sDF values prior
403 to fertilization time, which are significantly lower for SLC in this study.

404 Nonetheless, we need to keep in mind that rSDF values have been
405 obtained from a model, then, they are expected not observed values. In order
406 to fit the model more, further studies with more frequent assessments
407 (between 6 and 24h and after 24h) are needed to obtain predictions even
408 more accurate. It is of the utmost importance to fit data to an accurate model
409 in order to know exactly how this molecule behaves. In this sense, DNA
410 cannot have been correlated to any other sperm parameter [14,24] using
411 linear regression to work with sDF dynamics. Hopefully, this study provides
412 new tips so that correlation between DNA fragmentation dynamics and sperm
413 quality is focused from a new perspective.

414

415 Conclusions

416 SLC after thawing seems to preserve longer DNA longevity in
417 comparison to UDC and SW. Moreover, the fine-tuning of models has shown
418 that sDF dynamics in frozen-thawed donkey semen fits a second order
419 polynomial model, which implies that fragmentation rate is not constant and

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fragmentation acceleration must be taken into account to elucidate hidden damage in the DNA molecule.

List of abbreviations

aSDF= Sperm DNA Fragmentation Acceleration; **CASA=** Computer-Assisted Sperm Analysis; **Expon.=** Exponential; **h=** hours of incubation at 37ºC; **IMS=** Intact Membrane Sperm; **LN_2=** Liquid Nitrogen; **PM=** Progressive Sperm Motility; **Poly.=** Second order polynomial; **R^2=** Coefficient of Determination; **rSDF=** sperm DNA Fragmentation Rate; **SAS=** Statistical Analysis Systems Software; **sDF=** Sperm DNA Fragmentation; **SEM=** Standard Error of the Mean; **SLC=** Single Layer Centrifugation; **SW=** Sperm Washing; **T0-T3-T6-T24=** 0-3-6-24 hours of incubation at 37ºC; **TM=** Total Sperm Motility; **UDC=** Uncentrifuged Diluted Control.

Declarations

Ethics approval

All animal procedures were performed in accordance with the Spanish laws for animal welfare and experimentation, under the supervision of the Bioethical and Biosafety Committee of the University of Cordoba (UCO).

Consent for publication

Not applicable

Availability of data and material

The dataset will be shared as soon as the article is accepted for publication.

445

446 **Competing interests**

447 The authors declare that they have no competing interests.

448

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457

458 **Authors' contribution**

459 I Ortiz contributed to all sections. J Dorado and M Hidalgo contributed to the
460 study design, data analysis and interpretation, preparation of the manuscript
461 and final approval of the manuscript. J M Morrell, J Gosálvez and F Crespo
462 collaborated in the experimental design. J M. Jiménez revised modelling and
463 calculation.

464

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558 Figures

559 **Figure 1.** Effect of centrifugation (UDC, SW, and SLC) on the percentage of
560 sperm DNA fragmentation (sDF, %) of frozen-thawed donkey sperm for 24h of
561 incubation at 37ºC.
562

563 Values are expressed as means (bars) ± SEM (error bars).

564 Different superscripts letters indicate significant differences (P<0.05).

565 UDC=Uncentrifuged diluted control; SW= Sperm washing; SLC=Single Layer

566 Centrifugation; T0, T3, T6, T24= Incubation for 0, 3, 6, 24 h at 37°C.

567

568 **Figure 2**. Coefficient of determination (R^2) for linear, exponential and

569 polynomial regression models within treatments (UDC, SW, SLC).

570 sDF= Sperm DNA fragmentation; UDC= Uncentrifuged diluted control; SW=

571 Sperm washing; SLC= Single layer centrifugation; Expon.= Exponential;

572 Poly.= Polynomial.

573

574 **Figure 3**. Polynomial regression lines for UDC, SW and SLC.

575 sDF= Sperm DNA fragmentation; UDC= Uncentrifuged diluted control; SW=

576 Sperm washing; SLC= Single layer centrifugation; Poly.= Polynomial.

577

578 **Figure 4.** Sperm DNA fragmentation rate (%/h) in relation to time of

579 incubation at 37C for UDC, SW and SLC.

580 sDF= Sperm DNA fragmentation; UDC= Uncentrifuged diluted control; SW=

581 Sperm washing; SLC= Single layer centrifugation.

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Table

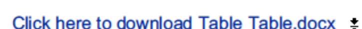


Table 1. Effect of time of incubation of sperm at 37°C on sperm DNA fragmentation (sDF, %) within each centrifugation procedure (UDC, SW and SLC).

	T0	T3	T6	T24	P (a-d)
UDC	12.98±1.52[c]	16.47±1.40[c]	20.90±1.34[b]	35.86±3.21[a]	P<0.001
SW	13.35±1.17[c]	15.01±1.27[bc]	18.28±2.21[b]	34.84±2.60[a]	P<0.001
SLC	11.48±1.67[b]	14.02±2.16[b]	15.70±2.26[b]	24.91±2.19[a]	P<0.001

Values are expressed as mean ± standard error of the mean (SEM).

Different letters indicate significant differences (P<0.001).

T0, T3, T6, T24= Incubation for 0, 3, 6, 24 h at 37°C; UDC= Uncentrifuged diluted control; SW= Sperm washing; SLC= Single Layer Centrifugation.

Figure 1

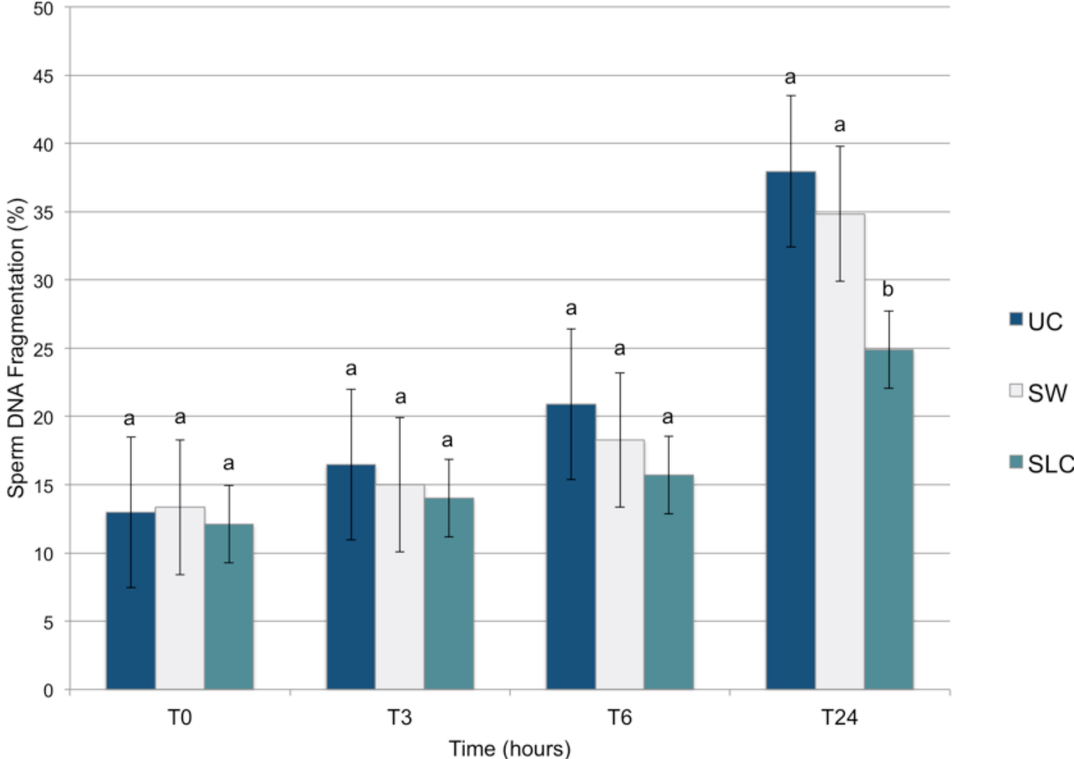


Figure 2

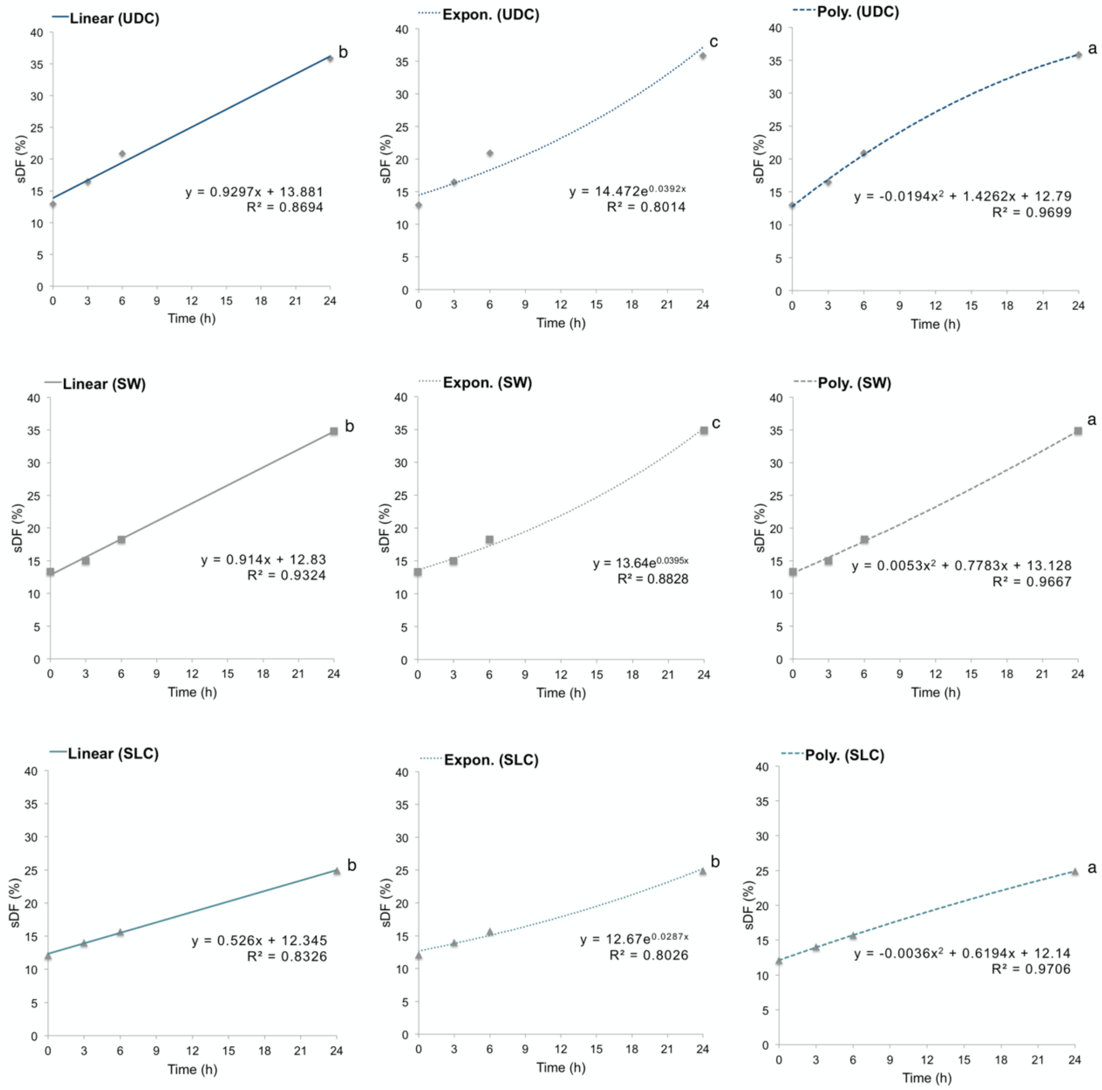


Figure 3

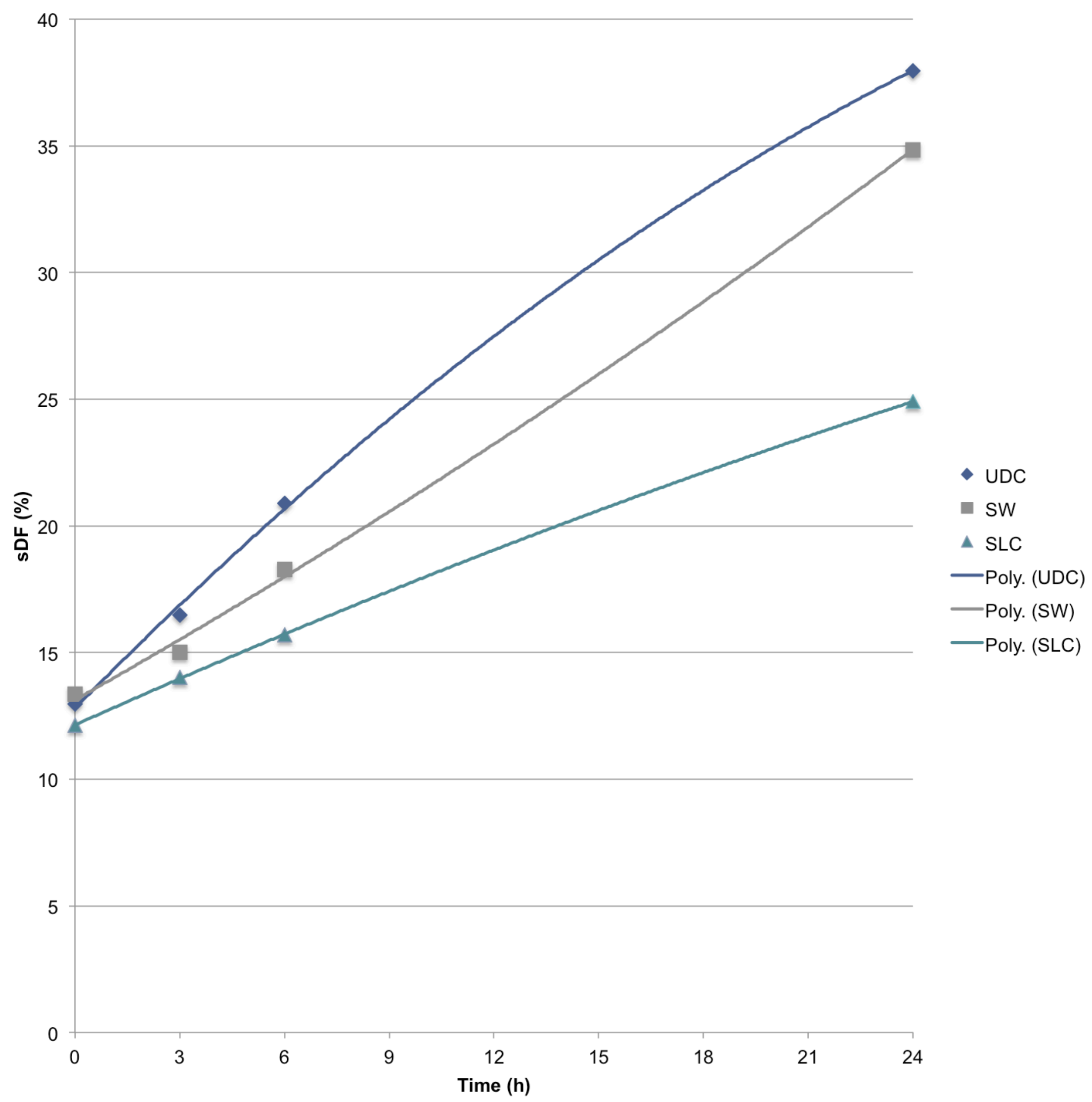
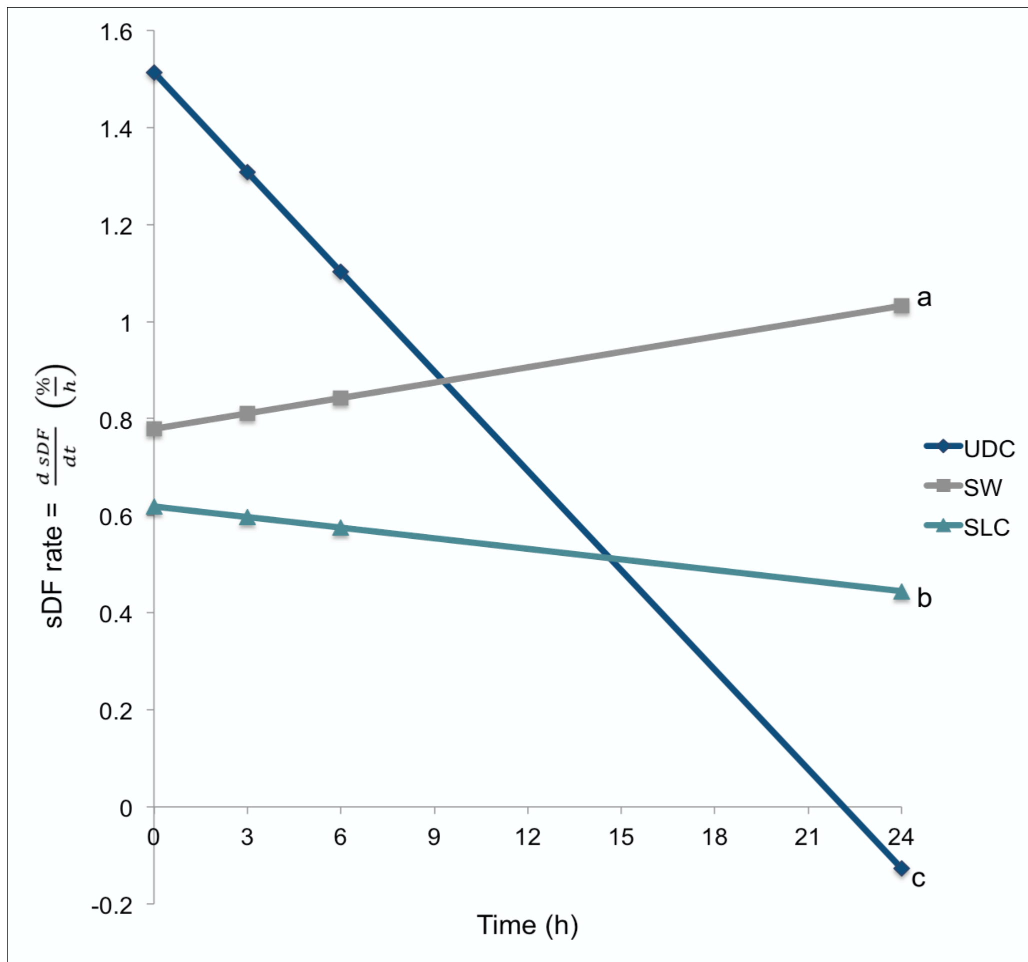
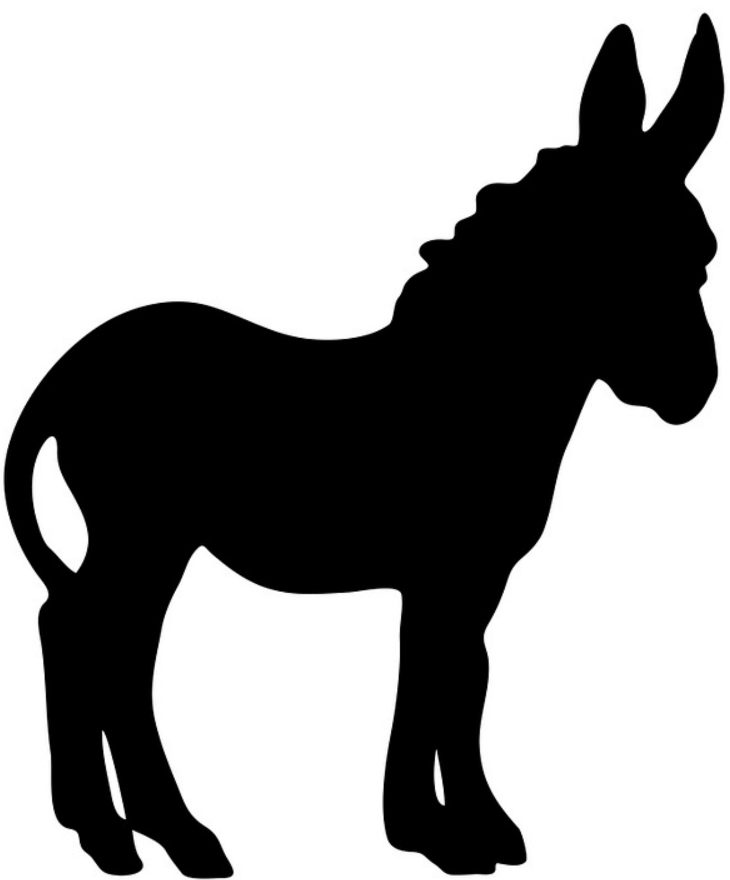


Figure 4



CONCLUSIONES



CONCLUSIONES

De acuerdo a los resultados obtenidos en la presente Tesis Doctoral, se pueden extraer las conclusiones que se enumeran a continuación:

Conclusión 1. (Primera publicación: "Effect of single layer centrifugation using Androcoll-E-Large on the sperm quality parameters of cooled-stored donkey semen doses". *I. Ortiz, J. Dorado, L. Ramírez, J. M. Morrell, D. Acha, M. Urbano, M. J. Gálvez, J. J. Carrasco, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo.* Animal (2014), 8:2, 308-315):

La centrifugación coloidal de una sola capa usando Andrcoll-E-Large mejora el movimiento total y progresivo, vitalidad, morfología, así como la mayoría de los parámetros cinéticos valorados en la totalidad de las muestras de semen de asno procesadas tras 24 horas de refrigeración.

Conclusión 2. (Segunda publicación: "Colloid single-layer centrifugation improves post-thaw donkey (Equus asinus) sperm quality and is related to ejaculate freezability". *I. Ortiz, J. Dorado, D. Acha, M.J. Gálvez, M. Urbano, M. Hidalgo.* Reproduction, Fertily and Development (2015), 27, 332-340):

La centrifugación coloidal de una sola capa del semen congelado-descongelado de asno puede ser un procedimiento adecuado para mejorar la calidad de las muestras post-descongelación de dosis criopreservadas de esperma, esta mejora es especialmente patente en los eyaculados con baja resistencia a la congelación.

Conclusión 3. (Tercera publicación: "Effect of single-layer centrifugation or washing on frozen-thawed donkey semen quality: Do they have the same effect regardless of the quality of the sample?" *I. Ortiz, J. Dorado, J. M. Morrell, F. Crespo, J. Gosálvez, M.J. Gálvez, D. Acha, M. Hidalgo.* Theriogenology (2015), 84, 294-300):

La centrifugación coloidal del semen de asno congelado-descongelado usando Androcoll-E puede ser un procedimiento útil para seleccionar espermatozoides de gran calidad en dosis de esperma de asno criopreservadas, este procedimiento es particularmente útil en aquellas muestras de semen en las que se requiera una mejora del movimiento espermático.

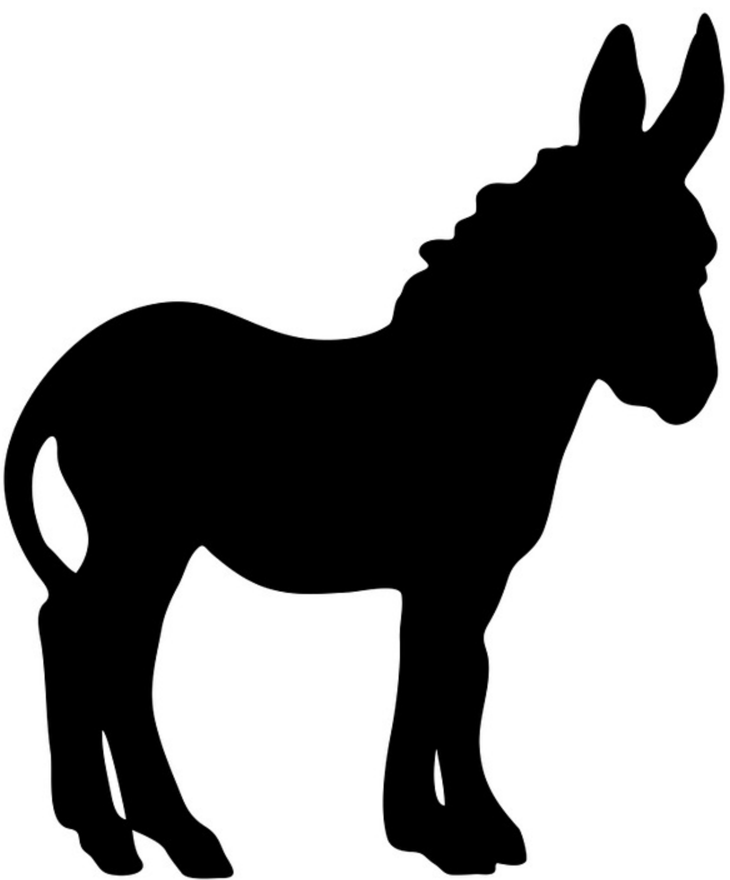
Conclusión 4. (**Cuarta publicación:** "Comparison of EquiPure and Androcoll-E for donkey sperm selection after thawing: effect of sperm motility, membrane integrity and motile sperm subpopulations". *I. Ortiz, J. Dorado, J. M. Morrell, M. J. Gálvez, D. Acha, M. Hidalgo.* Enviado a Animal Journal):

El coloide EquiPure seleccione espermatozoides con la membrana intacta, progresivos y relativamente lentos, mientras que el coloide Androcoll selecciona los espermatozoides más rápidos y progresivos. Se requieren futuros estudios para esclarecer qué aspectos de la calidad espermática están más relacionados con mayores tasas de gestación tras inseminación artificial.

Conclusión 5. (**Quinta publicación:** "New approach to assess sperm DNA fragmentation dynamics: Fine-tuning mathematical models". *I. Ortiz, J. Dorado, J. M. Morrell, J. Gosálvez, F. Crespo, J. M. Jiménez, M. Hidalgo.* Enviado a Journal of Animal Science and Biotechnology):

La centrifugación coloidal post-descongelación parece preservar durante más tiempo la longevidad del ADN en comparación con la dilución sin centrifugación y la centrifugación simple. Además, el ajuste de modelos ha mostrado que las dinámicas de fragmentación del ADN en semen congelado-descongelado de asno se ajustan a un modelo polinómico de segundo grado, lo que implica que la velocidad de fragmentación no es constante y que la aceleración de fragmentación debe ser tenida en cuenta para evidenciar un daño oculto en la molécula de ADN.

CONCLUSIONS



CONCLUSIONS

According to the results obtained in the present Doctoral Thesis, we can hereby conclude:

Conclusion 1. (First publication: "Effect of single layer centrifugation using Androcoll-E-Large on the sperm quality parameters of cooled-stored donkey semen doses". *I. Ortiz, J. Dorado, L. Ramírez, J. M. Morrell, D. Acha, M. Urbano, M. J. Gálvez, J. J. Carrasco, V. Gómez-Arrones, R. Calero-Carretero, M. Hidalgo.* Animal (2014), 8:2, 308-315):

> Single Layer Centrifugation with Androcoll-E-Large improved total and progressive motility, viability, morphology as well as most of sperm kinematics parameters assessed over the entire set of donkey semen doses processed after 24 hours of cool storage.

Conclusion 2. (Second publication: "Colloid single-layer centrifugation improves post-thaw donkey (Equus asinus) sperm quality and is related to ejaculate freezability". *I. Ortiz, J. Dorado, D. Acha, M.J. Gálvez, M. Urbano, M. Hidalgo.* Reproduction, Fertily and Development (2015), 27, 332-340):

> Single layer centrifugation of frozen-thawed donkey spermatozoa can be a suitable procedure for improving the post-thaw sperm quality of cryopreserved semen doses, specially evidenced in those ejaculates with low sperm freezability.

Conclusión 3. (Third publication: "Effect of single-layer centrifugation or washing on frozen-thawed donkey semen quality: Do they have the same effect regardless of the quality of the sample?" *I. Ortiz, J. Dorado, J. M. Morrell, F. Crespo, J. Gosálvez, M.J. Gálvez, D. Acha, M. Hidalgo.* Theriogenology (2015), 84, 294-300):

> Single layer centrifugation of frozen-thawed donkey spermatozoa using Androcoll-E-Small can be a suitable procedure for selecting high-quality sperm after thawing cryopreserved donkey sperm doses, it is particularly recommended for those samples where an improvement in motility is needed.

Conclusión 4. (Fourth publication: "Comparison of EquiPure and Androcoll-E for donkey sperm selection after thawing: effect of sperm motility, membrane integrity

and motile sperm subpopulations". *I. Ortiz, J. Dorado, J. M. Morrell, M. J. Gálvez, D. Acha, M. Hidalgo.* Submitted to Animal Journal):

> EquiPure selects membrane intact, progressive and relatively slow sperm whereas Androcoll selects the fastest and more progressive sperm. Further studies are needed to clarify which aspects of sperm quality are more related with pregnancies after artificial insemination.

Conclusión 5. (Fifth publication: "New approach to assess sperm DNA fragmentation dynamics: Fine-tuning mathematical models". *I. Ortiz, J. Dorado, J. M. Morrell, J. Gosálvez, F. Crespo, J. M. Jiménez, M. Hidalgo.* Submitted to Journal of Animal Science and Biotechnology):

> Single layer centrifugation after thawing seems to preserve longer DNA longevity in comparison to uncentrifuged diluted control and sperm washing. Moreover, the fine-tuning of models has shown that sperm DNA fragmentation dynamics in frozen-thawed donkey semen fits a second order polynomial model, which implies that fragmentation rate is not constant and fragmentation acceleration must be taken into account to elucidate hidden damage in the DNA molecule.

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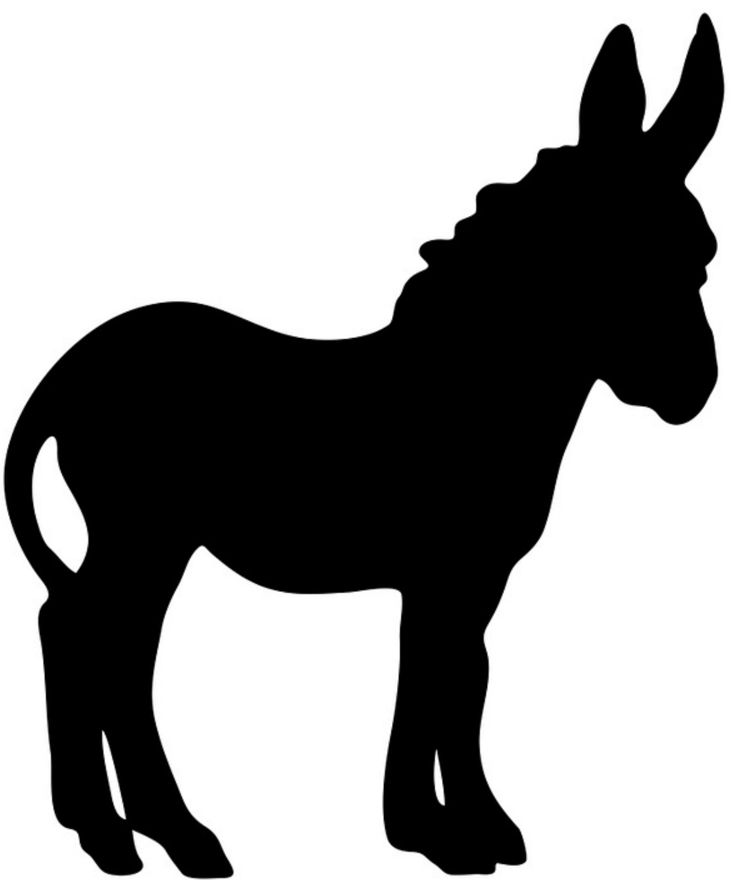

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ÍNDICES DE CALIDAD

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Primera publicación:

- Título: *Effect of single layer centrifugation using Androcoll-E-Large on the sperm quality parameters of cooled-stored donkey semen doses.*
- Autores (p.o. de firma*): I Ortiz, J Dorado, L Ramírez, J.M. Morrell, D Acha, M Urbano, MJ Gálvez, JJ Carrasco, V Gómez-Arrones, R Calero-Carretero, M Hidalgo*
- Revista (año,vol.,pág.): *Animal 2014, 8, 308-315*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports (JCR). 2014.*
- Área temática en la Base de Datos de referencia: *Agriculture, Dairy and Animal Science*
- Índice de impacto de la revista en el año de publicación del Artículo: *1,841*
- Lugar que ocupa/Nº de revistas del Área temática: *7/57 (Q1)*

Segunda publicación:

- Título*: Colloid single layer centrifugation improves post-thaw donkey (Equus asinus) sperm quality and it is related to ejaculate freezability.*
- Autores (p.o. de firma*): I. Ortiz, J. Dorado, D Acha, M.J. Gálvez, M Urbano, M. Hidalgo*
- Revista (año,vol.,pág.): *Reproduction, Fertility and Development, 2015, 27, 332-340*
- Base de Datos Internacional o Nacional en las que está indexada: *JCR. (2015)*
- Área temática en la Base de Datos de referencia: *Zoology*
- Índice de impacto de la revista en el año de publicación del Artículo: *2,135*
- Lugar que ocupa/Nº de revistas del Área temática: *22/161 (Q1)*

Tercera publicación:

- Título: *Effect of single-layer centrifugation or washing on frozen-thawed donkey semen quality: Do they have the same effect regardless of the quality of the sample?*
- Autores (p.o. de firma): *I. Ortiz, J. Dorado, JM Morrell; F. Crespo, J. Gosalvez, D Acha, M.J. Gálvez, M. Hidalgo*
- Revista (año,vol.,pág.): *Theriogenology, 2015, 84, 294-300*
- Base de Datos Internacional o Nacional en las que está indexada: *JCR (2015)*
- Área temática en la Base de Datos de referencia: *Veterinary Sciences*
- Índice de impacto de la revista en el año de publicación del Artículo: *1,838*
- Lugar que ocupa/Nº de revistas del Área temática: *18/137 (Q1)*

Cuarta publicación:

- Título: *Comparison of EquiPure and Androcoll-E for donkey sperm selection after thawing: effect on sperm motility, membrane integrity and motile sperm subpopulations*
- Autores (p.o. de firma): *I. Ortiz, J. Dorado, J. M. Morrell, M. J. Gálvez, D. Acha, M Hidalgo.*
- Revista (año,vol.,pág.): *Animal. Submitted on September 9th, 2016*
- Base de Datos Internacional o Nacional en las que está indexada: *JCR (2015)*
- Área temática en la Base de Datos de referencia: *Agriculture, Dairy and Animal Science*
- Índice de impacto de la revista en el año de publicación del Artículo: *2,056*
- Lugar que ocupa/Nº de revistas del Área temática: *4/58 (Q1)*

Quinta publicación:

- Título: *New approach to assess sperm DNA fragmentation dynamics: Fine-tuning mathematical models*
- Autores (p.o. de firma): *I. Ortiz, J. Dorado, J. M. Morrell, J. Gosálvez, F. Crespo, J. M. Jiménez, M. Hidalgo.*
- Revista (año,vol.,pág.): *Journal of Animal Science and Biotechnology (Submitted on August 31st, 2016)*
- Base de Datos Internacional o Nacional en las que está indexada: *JCR (2015)*
- Área temática en la Base de Datos de referencia: *Agriculture, Dairy and Animal Science*
- Índice de impacto de la revista en el año de publicación del Artículo: *2,037*
- Lugar que ocupa/Nº de revistas del Área temática: *4/58 (Q1)*

PRODUCCIÓN CIENTÍFICA

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Otras aportaciones científicas derivadas directamente de la Tesis Doctoral:

Otras Publicaciones en Revistas Indexadas en el JCR:

- **I Ortiz,** J Dorado, JM Morrell, D Acha, L Ramírez, M Urbano, JJ Carrasco, V Gómez-Arrones, R Calero, M Hidalgo. (2012). Sperm motility differences between donkey cooled sperm processed by colloid centrifugation. Journal of Equine Veterinary Science, 32 (n8): 504-505.

- **I. Ortiz**, J. Dorado, J.M. Morrell, D. Acha, L. Ramírez, M. Urbano, J.J. Carrasco, V. Gómez-Arrones, R. Calero, M. Hidalgo. (2012). Androcoll-E Large improves kinematic parameters on donkey semen after 24 hours of cool storage. Reproduction in Domestic Animals, 47(s3): 90-123.

- **I Ortiz**, S Demyda, J Dorado, D Acha, L Ramírez, M Urbano, MJ Gálvez, L Alcaraz, JM Portero, F Quesada, C González, M Hidalgo. (2012). Differences on CASA sperm kinematics between donkey frozen-thawed sperm processed or not with Equipure. Reproduction, Fertility and Development: 24 (1): 183.

- **I Ortiz**, J Dorado, J Morrell, J Gosálvez, F Crespo, L Ramírez, D Acha, M Urbano, M Gálvez, S Demyda-Peyrás, M Hidalgo. (2013). Comparison of DNA fragmentation dynamics in frozen sperm of Equus asinus between uncentrifuged and SLC selected samples. Reproduction in Domestic Animals, 48(s1): 85.

- **I Ortiz**, J Dorado, JM Morrell, D Acha, MJ Gálvez, MT Urbano, M Hidalgo. (2015). Effect of two commercial colloids on donkey sperm selection after thawing: preliminary results. Reproduction in Domestic Animals, 50 (3): 70.

Contribuciones a Congresos:

- "Mejora de la calidad seminal mediante selección de espermatozoides". **I. Ortiz**, J. Dorado, D. Acha, M.J. Gálvez, M Urbano, L Alcaraz, N Duarte, JM Portero, M. Hidalgo. Congreso de Investigación en Veterinaria y Cyta. Córdoba (España), 7-11 noviembre, 2011.

- Androcoll-E Large improves kinematic parameters on donkey semen after 24 hours of cool storage. **I. Ortiz**, J. Dorado, J.M. Morrell, D. Acha, L. Ramírez, M. Urbano, J.J. Carrasco, V. Gómez-Arrones, R. Calero, M. Hidalgo. 11º Congreso Internacional de la Asociación Española de Reproducción Animal. Córdoba (España), 13-16 junio, 2012.

- Sperm motility differences between donkey cooled sperm processed by colloid centrifugation. **I. Ortiz**, J Dorado, JM Morrell, D Acha, L Ramírez, M Urbano, JJ Carrasco, V Gómez-Arrones, R. Calero, M Hidalgo. 6th International Symposium on Stallion Reproduction. Viena (Austria), 5-7 septiembre, 2012.

- - Differences on CASA sperm kinematics between donkey frozen-thawed sperm processed or not with Equipure. **I Ortiz**, S Demyda, J Dorado, D Acha, L Ramírez, M Urbano, MJ Gálvez, L Alcaraz, ,JM Portero, F Quesada, C González, M Hidalgo. Reproduction, Fertility and Development. 39th Annual Conference of the IETS. Hannover (Alemania), 19-22 enero, 2013.

- Selección de espermatozoides de asno. **I. Ortiz**, J. Dorado, D. Acha, M.J. Gálvez, M. Hidalgo. Congreso investigadores ceiA3. Córdoba (España), 9-10 abril 2013.

- Comparison of DNA fragmentation dynamics in frozen sperm of Equus asinus between uncentrifuged and SLC selected samples. **I Ortiz**, J Dorado, J Morrell, J Gosálvez, F Crespo, L. Ramírez, D Acha, M Urbano, M Gálvez, S Demyda-Peyrás, M Hidalgo. 17th European Society of Domestic Animals Reproduction Conference (ESDAR). Bolonia (Italia), 12-14 septiembre, 2014.

- Effect of two commercial colloids on donkey sperm selection after thawing: preliminary results. **I Ortiz**, J Dorado, JM Morrell, D Acha, MJ Gálvez, MT Urbano, M Hidalgo. 19th European Society of Domestic Animals Reproduction Conference (ESDAR). Helsinki (Finlandia), 10-13 septiembre, 2015.

- Fine-tuning mathematical models for sperm DNA fragmentation. **I. Ortiz**, J. Dorado, F. Crespo, J. Gosálvez, D. Acha, M. J. Gálvez, J. M. Jiménez, M. Hidalgo. 18th International Congress on Animal Reproduction (ICAR). Tours (Francia), 26-30 junio, 2016.