#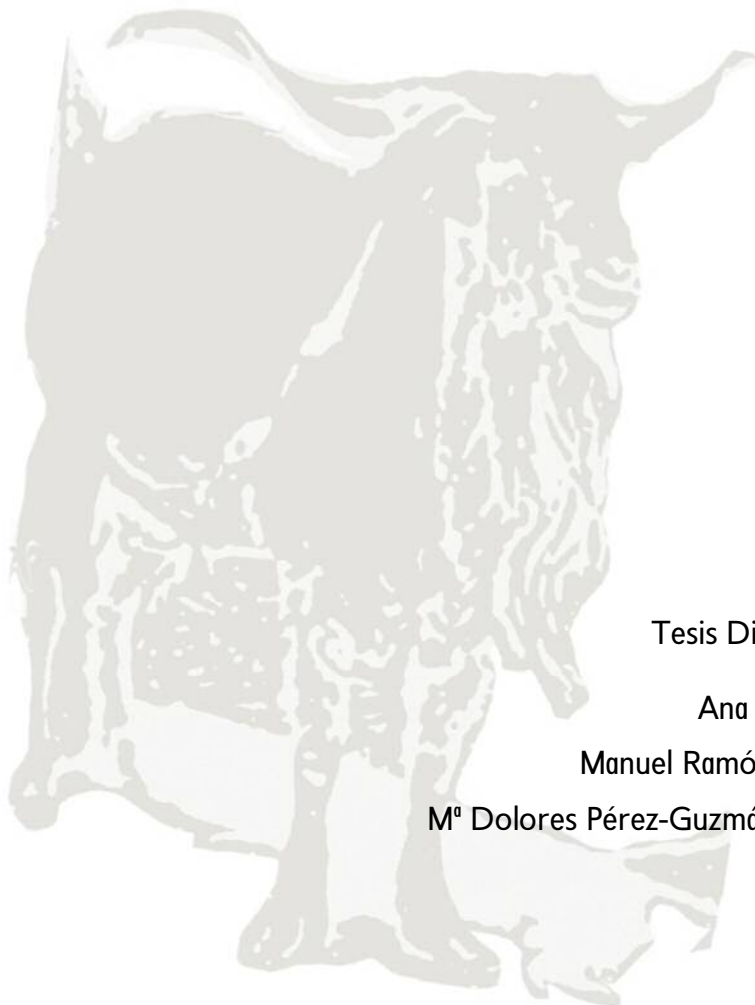 Advances in sperm cryopreservation of samples collected by vagina artificial and electroejaculation from Blanca-Celtibérica goat breed

DOCTORAL THESIS

by

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Advances in sperm cryopreservation of samples collected by vagina artificial and electroejaculation from Blanca-Celtibérica goat breed

por

Mª del Pilar Jiménez Rabadán

TESIS DOCTORAL

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Y por último y muy especialmente a mis padres, Bernardo y Mª Carmen, por haberme enseñado que con sacrificio y esfuerzo todo es posible y por confiar en mí con los ojos cerrados. A Carlos, por animarme a embarcarme en esta experiencia y acompañarme a lo largo de ella, por su comprensión, por su apoyo y por su inmensa paciencia.

A todos ellos, muchas gracias.

A mi familia

A Carlos

SUMMARY

Blanca-Celtibérica goat is an endangered autochthonous breed from Spain. To prevent the disappearance of these autochthonous breeds and in order to maintain the genetic diversity, the Food and Agriculture Organization of the United Nations (FAO) recommends the preservation of these breeds by *in situ* and *ex situ* conservation programs (FAO, 2010). The creation of Genetic Resource Banks (GRB) are a measure of *ex situ* conservation and allow us the storage of semen, oocytes and embryos indefinitely, being an essential tool in order to preserve the genetic diversity of species. The most widespread application in the development of GRB has been the semen collection and its freezing. This requires the knowledge of reproductive physiology as well as the suitable assisted reproductive technologies for each species. In the case of the Blanca-Celtibérica goat breed, the geographical location joined to extensive production systems and lack of buck center lead to semen collection by routine techniques would be non-viable. Thus, when animals can not be trained to semen collection by artificial vagina, there are other alternatives such as the electroejaculation, being possible to obtain semen of males living in the countryside. Besides the semen collection, the conservation of semen samples is also a key aspect. Up to date, although several studies have been carried out on sperm cryopreservation in caprine, most of them have used samples collected by artificial vagina. However, it is known that the collection method influences on the sperm production and composition of seminal plasma (Marco-Jiménez *et al.*, 2005; Marco-Jiménez *et al.*, 2008) as well as the cryoresistance of sperm samples (Álvarez *et al.*, 2012). Another problem during the buck semen cryopreservation is the low seminal quality found after thawing when freezing extenders based on egg yolk or skim milk are used. Some components of egg yolk and skim milk have negative interactions with seminal plasma producing toxic compounds for the spermatozoa. This effect could be more pronounced on sperm samples collected by electroejaculation.

With this background, the general aim of this Doctoral Thesis has been to develop an optimal method in order to collect and cryopreserve buck semen of Blanca-Celtibérica breed which allow us to storage seminal doses to create a Germoplasm Bank.

In the first Chapter of this Doctoral Thesis, it has been evaluated the effect of method of semen collection, artificial vagina and electroejaculation, on sperm quality at thawing in Blanca-Celtibérica buck, comparing the results obtained in other close-related small ruminant species, ovine, which have more available information regarding to cryopreservation (Marco-Jiménez *et al.*, 2005; Marco-Jiménez *et al.*2008; Álvarez *et al.*, 2012). The goal was to establish a starting point to cryopreserve semen of Blanca-Celtibérica goat breed using a freezing standard protocol performed for small

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ruminant, and two collection methods, artificial vagina as usual collection method for small ruminant, and electroejaculation as alternative method. In both species, the sperm samples collected by electroejaculation had higher volume and lower concentration and no differences were observed for sperm motility for fresh semen between both collection methods. However, the collection method and species influenced on the resistance of sperm samples to freezing. Furthermore, after thawing, the sperm motility parameters were lower in bucks than rams, being the lowest those values for samples collected by electroejaculation. Moreover, sperm samples obtained by electroejaculation in bucks showed lower values of viability and active mitochondria and higher DNA fragmentation than ovine samples and those collected by artificial vagina.

In the second Chapter of this Thesis, the effect of method of ejaculates collection (artificial vagina or electroejaculation) and season of collection (breeding season or non-breeding season) on thawed sperm quality were studied. In addition, it has been studied the influence of seminal plasma removal by centrifugation before freezing and the use of different freezing extenders, two commercial, Biladyl® (based on egg yolk) and Andromed® (based on soy lecithin) and a non-commercial extender based on skim milk. Finally, interactions between studied factors were also evaluated. These studies were conducted with sperm samples collected by artificial vagina and electroejaculation. Samples obtained by electroejaculation showed lower thawed sperm quality than those collected by artificial vagina. Moreover, the season of collection influenced on this quality being more suitable the sperm collection during breeding season irrespective of the collection method. The removal or not of seminal plasma before freezing by centrifugation had not any effect on sperm quality at thawing regardless of collection method and extender used. However, it was observed a beneficial effect of seminal plasma removal when the semen collection was performed during non-breeding season. Otherwise, commercial extenders based on egg yolk and soy (Biladyl® and Andromed®, respectively) provided good values of seminal quality at thawing, being even higher for viability when Biladyl® was used. Due to worse sperm quality at thawing for samples collected by electroejaculation in relation to artificial vagina, it was necessary to carry out modifications in the freezing extender and procedure in order to improve the sperm quality after cryopreservation. Therefore, it was proposed the next Chapter of this Doctoral Thesis.

In the third Chapter of this Thesis, the effect of removal of the seminal plasma by sperm selection using single layer centrifugation (SLC), before freezing and after thawing, on sperm quality at thawing was researched. Sperm samples selected by SLC before freezing showed lower sperm quality than samples selected after thawing or not

selected. The highest values were those in samples selected after thawing. Moreover, these sperm samples had lower percentage of DNA fragmentation and free-radical production. However, the sperm recovery after SLC was very low for samples selected at thawing in relation to those selected before freezing.

Although high sperm quality was obtained when SLC was performed after thawing, the total number of spermatozoa was lower and so, this technique is not suitable from a technical standpoint. Therefore, a fourth experiment was proposed, in which were modified some aspects of freezing extender as well as other related to cryopreservation protocol in order to obtain high quality sperm samples. In this way, in the fourth Chapter of this Thesis, the effect of egg yolk concentration (0, 1.5, 10 y 20%) contained in the freezing extender was studied. It was also evaluated the effect of cooling rates (decrease of temperature from 30 °C to 5 °C in 90 minutes (slow rate) or 10 minutes (fast rate)), the effect of temperature of glycerol addition (30 or 5 °C) and equilibration times at 5 °C (0, 1, 2 or 3 h). Extenders containing 10 and 20% of egg yolk provided higher sperm quality than 0 and 1.5 %, showing 20% of egg yolk similar values than control samples, except for motility parameters. Moreover, slow cooling rates (90 min) had higher sperm quality than the fast ones (10 min) and no differences were observed in relation to control samples. Otherwise, no differences were found between temperatures of glycerol addition and longer equilibration periods at 5 °C (3 hours) provided better results at thawing than shorter periods (1 hour) or no equilibration time. Finally, a heterologous *in vitro* fertilization test was performed using samples cryopreserved with the best protocol observed (20% egg yolk, slow cooling rate and equilibration for 3 h) for samples collected by electroejaculation and the results were compared to sperm samples obtained by artificial vagina and frozen by standard protocol (control samples). The fertility of samples collected by electroejaculation and frozen by protocol which better results provided was slightly higher than for those obtained by artificial vagina and frozen by the standard protocol.

From the results obtained in the essays presented in this Doctoral Thesis we can conclude that the thawed sperm quality in Blanca-Celtibérica bucks is influenced by several factors such as the collection method, the season of the year, the freezing extenders, their composition and their addition way, the cooling rate and the time which sperm samples are maintained at subphysiological temperatures. Hence, it is recommended to carry out the semen collection during the breeding season, to remove the seminal plasma if collection is performed during non-breeding season, and to freeze in extenders based on egg yolk such as Biladyl®, irrespective of the collection method. Moreover, higher thawed sperm quality for samples collected by electroejaculation were obtained when extenders with 20% egg yolk, slow

refrigeration rates (from 30 °C to 5 °C in 90 minutes) and equilibration period for 3 hours were used. The temperature of glycerol addition can be performed at 5 °C or 30 °C. Finally, the sperm quality at thawing is improved when sperm samples collected by electroejaculation are selected by SLC after thawing, but not when samples are selected before freezing.

Mª del Pilar Jiménez Rabadán

RESUMEN

La cabra Blanca-Celtibérica es una raza autóctona española que se encuentra catalogada en peligro de extinción. Para evitar la desaparición de razas autóctonas y mantener la diversidad genética, la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO) recomienda la preservación de dichas razas mediante programas de conservación *in situ* y *ex situ* (FAO, 2010). Entre las medidas de conservación *ex situ* está contemplada la creación de Bancos de Recursos Genéticos (BRG) que permiten el almacenamiento de semen, óvulos y embriones de forma indefinida, convirtiéndose así, en una herramienta fundamental para asegurar la máxima preservación de la diversidad genética de las especies. La técnica reproductiva que más ha contribuido al desarrollo y difusión de los BRG ha sido la obtención y congelación de semen. Para asegurar el éxito en la aplicación de estas técnicas, es necesario conocer la fisiología reproductiva así como las técnicas de reproducción asistida más adecuadas a cada especie. En el caso concreto de la raza caprina Blanca-Celtibérica, la localización geográfica junto con los sistemas de producción extensivos y la ausencia de un centro de sementales, hace que la recogida de semen con las técnicas rutinarias como la vagina artificial no resulte viable. Así, en los casos en los que no sea posible el entrenamiento de los animales para la obtención de eyaculados con la vagina artificial se dispone de otros métodos alternativos como, por ejemplo, la electroeyaculación, siendo posible la recogida de semen de machos localizados en el campo. Además del método de recogida seminal, otro aspecto fundamental a tener en cuenta en el desarrollo de los BRG es la conservación de las muestras seminales. Aunque hasta la fecha se han realizado numerosos trabajos de congelación espermática en caprinos, la mayoría de ellos han utilizado muestras obtenidas mediante vagina artificial. Está descrito cómo el método de recogida influye sobre la producción y la composición del plasma seminal (Marco-Jiménez *et al.*, 2005; Marco-Jiménez *et al.*, 2008), así como sobre la resistencia a la congelación de las muestras espermáticas (Álvarez *et al.*, 2012). Otro problema añadido en la criopreservación de semen caprino es la baja calidad seminal encontrada tras la descongelación cuando se usan diluyentes a base de yema de huevo y leche desnatada. Determinados componentes de la yema de huevo y de la leche interaccionan con el plasma seminal dando lugar a compuestos tóxicos para los espermatozoides. Así, este efecto podría ser más marcado en las muestras obtenidas mediante electroeyaculación.

Por todo lo descrito anteriormente, el objetivo general de esta Tesis Doctoral ha sido el desarrollo de una metodología óptima de recogida y congelación de semen de macho cabrío de la raza Blanca-Celtibérica que permita almacenar con garantías dosis seminales para la creación de un Banco de Germoplasma.

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En el primer Capítulo de esta Tesis se ha evaluado el efecto del método de recogida de semen, vagina artificial y electroeyaculación, sobre la calidad seminal a la descongelación en la raza caprina Blanca-Celtibérica, comparando los resultados obtenidos con los de otra especie de pequeño rumiante filogenéticamente cercana, el ovino de raza Manchega, y de la que se dispone de más información al respecto (Marco-Jiménez *et al.*, 2005, Marco-Jiménez *et al.*, 2008; Álvarez *et al.*, 2012). El objetivo fue establecer una situación de partida para la congelabilidad del semen en la raza caprina Blanca-Celtibérica empleando un protocolo de congelación estándar utilizado en pequeños rumiantes, y dos métodos de recogida, la vagina artificial como método de recogida habitual empleado en pequeños rumiantes, y la electroeyaculación como método alternativo. En ambas especies las muestras seminales obtenidas por electroeyaculación se caracterizaron por un mayor volumen y una menor concentración espermática, no observándose diferencias para la motilidad espermática en semen fresco entre ambos métodos de recogida dentro de cada especie. Sin embargo, el método de recogida y la especie fueron factores que influyeron en la resistencia a la congelación de las muestras espermáticas. Así, tras la descongelación, los parámetros de motilidad fueron menores en los machos cabríos en relación al ovino, registrándose los valores más bajos cuando el semen fue obtenido por electroeyaculación. Además, las muestras espermáticas recogidas mediante elecroeyaculación en machos cabríos mostraron menores porcentajes de viabilidad y de espematozoides con mitocondrias activas y mayores porcentajes de fragmentación de ADN en relación al ovino y a la recogida mediante vagina artificial.

En el segundo Capítulo de esta Tesis se evaluaron los efectos que el diluyente de criopreservación y diversas modificaciones del protocolo de congelación estándar utilizado en pequeños rumiantes tienen sobre la calidad seminal a la descongelación en muestras de machos de la raza caprina Blanca-Celtibérica. Así, se estudió el efecto de eliminar o no el plasma seminal mediante centrifugación antes de la congelación y la utilización de diferentes diluyentes de congelación, dos comerciales, Biladyl® (a base de yema de huevo) y Andromed® (a base de lecitina de soja), y uno no comercial a base de leche desnatada. Además, se examinaron los efectos que la época de recogida (estación reproductiva o no reproductiva) podrían tener sobre la calidad seminal a la descongelación. Finalmente se evaluaron las interacciones entre todos los factores estudiados. Estos trabajos se realizaron tanto para muestras espermáticas recogidas mediante vagina artificial como por electroeyaculación. Las muestras obtenidas mediante electroeyaculación presentaron peor calidad seminal a la descongelación que las muestras recogidas con vagina artificial, siendo más adecuada la recogida espermática durante la época reproductiva, independientemente del método de

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recogida. En relación al efecto de eliminar o no el plasma seminal mediante centrifugación, observamos que la eliminación del mismo no tuvo ningún efecto sobre los valores de calidad seminal a la descongelación, independientemente del método de recogida y el diluyente utilizado. Sin embargo, se observó un efecto beneficioso de la eliminación del plasma seminal cuando la recogida de los eyaculados se llevó a cabo durante la época no reproductiva. Por otra parte, los diluyentes comerciales a base de yema de huevo y lecitina de soja (Biladyl® y Andromed®, respectivamente) fueron los que mejores valores de calidad seminal a la descongelación mostraron, obteniéndose incluso valores más altos de viabilidad para el diluyente Biladyl®. Debido a la peor calidad seminal a la descongelación de las muestras obtenidas mediante electroeyaculación en relación a la vagina artificial, se plantearon nuevas modificaciones en el protocolo de congelación para mejorar la calidad espermática tras la criopreservación, planteándose para ello el siguiente Capítulo de esta Tesis Doctoral.

En el Capítulo 3 de esta Tesis se ha estudiado el efecto que tuvo sobre la calidad espermática a la descongelación, la eliminación del plasma seminal mediante centrifugación en una sola capa de coloide (SLC) aplicada antes o después de la criopreservación. Las muestras espermáticas que fueron seleccionadas mediante SLC antes de la congelación mostraron menores valores de calidad espermática que las muestras seleccionadas después de la congelación o no seleccionadas, registrándose los valores de calidad más altos en aquellas muestras espermáticas seleccionadas después de la congelación. Las muestras seleccionadas después de la descongelación mostraron además, menores niveles de fragmentación del ADN y de producción de radicales libres de oxígeno (ROS). Sin embargo, el porcentaje de espermatozoides recuperados tras seleccionar con SLC después de la descongelación fue muy bajo en relación al obtenido de muestras seleccionadas antes de la congelación.

A pesar de la mejor calidad espermática obtenida con el uso de métodos de selección sobre muestras descongeladas, el bajo número total de espermatozoides recuperados hace que esta técnica sea poco rentable desde un punto de vista técnico. Por esta razón, fue planteado un cuarto experimento en el que se llevaron a cabo modificaciones en la composición del diluyente de criopreservación así como en el protocolo de congelación con el objetivo de obtener muestras espermáticas de alta calidad seminal. De este modo, en el Capítulo 4 de esta Tesis se estudió el efecto de la concentración de yema de huevo (0, 1,5, 10 ó 20%) contenida en el diluyente de congelación sobre la calidad seminal a la descongelación. Además, se evaluó el efecto de la velocidad de refrigeración (disminución de la temperatura de 30 °C a 5 °C en 90 minutos (velocidad lenta) o 10 minutos (velocidad rápida)), el efecto de la temperatura de adición del glicerol (30 °C ó 5 °C) y el efecto del tiempo de equilibración a 5 °C (0,

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1, 2 ó 3 horas) sobre la calidad espermática a la descongelación. Las concentraciones de 10% y 20% de yema de huevo en el diluyente de congelación proporcionaron valores de calidad espermática superiores a las concentraciones de 0% y 1,5%, presentando la concentración de 20% de yema de huevo valores muy similares a las muestras control, excepto para los parámetros de motilidad. Además, se observó que para las muestras refrigeradas lentamente los valores de calidad espermática a la descongelación fueron más altos en relación a las refrigeradas rápidamente, no existiendo diferencias con respecto a las muestras control. Por otra parte no se encontraron diferencias en la calidad seminal entre muestras a las que se les añadió el glicerol a 30 °C en relación a aquellas en las que se añadió a 5 °C. Por otro lado, largos periodos de equilibración (3 horas) a 5 °C proporcionaron los mejores resultados a la descongelación en relación a periodos más cortos (1 hora) o a muestras congeladas sin periodo de equilibración (0 horas). Finalmente, utilizando el protocolo de congelación con el que se obtuvieron los mejores resultados de calidad seminal (20% yema de huevo, velocidad de enfriamiento lenta y equilibrado durante 3 h) para las muestras recogidas por electroeyaculación, se realizó un test de fecundación *in vitro* heteróloga y se compararon los resultados de fertilidad con aquellos obtenidos de muestras seminales recogidas mediante vagina artificial y congeladas siguiendo el protocolo de congelación estándar (muestras control). Se observó que la fertilidad fue ligeramente superior para las muestras espermáticas obtenidas mediante electroeyaculación y congeladas usando el protocolo de criopreservación que proporcionó mejores resultados respecto a la fertilidad obtenida de muestras espermáticas recogidas mediante vagina artificial y congeladas siguiendo el protocolo estándar.

Con los resultados obtenidos en esta Tesis Doctoral podemos concluir que la calidad seminal a la descongelación de machos cabríos de raza Blanca-Celtibérica se ve afectada por diversos factores como son el método de recogida, la época del año, los diluyentes de congelación, sus componentes y su forma de adición, la velocidad de enfriamiento y el tiempo que se mantienen a temperaturas subfisiológicas. De este modo, se recomienda tanto para muestras espermáticas obtenidas por vagina artificial como por electroeyaculación realizar la recogida durante la época reproductiva, eliminando el plasma seminal mediante lavado si se hace fuera de época reproductiva y congelarlas con un diluyente a base de yema de huevo como el Biladyl®. Asímismo, sobre muestras recogidas mediante electroeyaculación los valores más altos de calidad seminal a la descongelación se obtuvieron con diluyentes con un 20% de yema de huevo, refrigerando de forma lenta (de 30 °C a 5 °C en 90 minutos) y con un periodo de equilibración de 3 horas, siendo independiente añadir el glicerol a 5 °C o 30 °C. Finalmente, la calidad seminal a la descongelación mejora cuando se exponen las

muestras espermáticas descongeladas recogidas mediante electroeyaculación a un proceso de selección utilizando SLC, pero no cuando el plasma seminal es eliminado mediante este proceso antes de la congelación.

GENERAL INTRODUCTION

Blanca-Celtibérica goat breed

The Blanca-Celtibérica goat breed was originated from *Capra prisca* and nowadays still maintain many features from this primitive species.

The production is orientated toward meat production by means of extensive systems in mountain areas with extreme temperatures.

Currently, this breed is considered as Spanish autochtonous endangered breed (RD 2129/2008). Its census is around 8500 animals located mainly in regions of Castilla-La Mancha, Andalucía and Murcia (http://www.magrama.gob.es/es/). Each region has an association of farmers whose principal aim is to preserve and improve the breed in the natural environment.

Genetic Resource Banks

Food and Agriculture Organization of the United Nations (FAO) has developed a global plan of action for animal genetic resources (2007) in order to fight against the loss of animal genetic diversity, being a priority the *in situ* and *ex situ* preservation of zoogenetic resources. *In situ* conservation means animal preservation under the environment where they have grown, while *ex situ* conservation implies the maintenance of populations out of their natural environment. The creation of Genetic Resource Banks (GRB) is contemplated among the measures of *ex situ* conservation. The GRB allow us the storage of semen, oocytes, embryos and other tissues indefinitely, being a key tool in order to ensure the genetic diversity of species (Holt *et al.*, 1996a; Roldán *et al.*, 2006 Wildt *et al.*, 1997). The most widespread use of GRB has been the collection and freezing of semen (Roldán and Garde, 2004; Watson and Holt, 2001). However, it is necessary to know deeply the reproductive physiology of species or breed which we want to preserve, as well as the reproductive advances on biotechnology in order to use those assisted reproduction techniques more suitable to each species (Yoshida, 2000).

Sperm cryopreservation

Sperm cryopreservation together with different assisted reproduction techniques such as the artificial insemination allow us the use of semen from valuable animals, the preservation of endangered species, to solve problems of male infertility in humans and the exchange of semen between subpopulations that may become geographically or biologically isolated (Andrabi and Maxwell, 2007; Pope and Loskutoff, 1999; Watson 2000; Watson and Holt, 2001; Wildt, 1992).

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The first buck semen cryopreservation was carried out by Smith and Polge in 1950. After that, Barker (1957) stated that fertility of frozen-thawed goat semen was too low to be of practical value, being the starting point in the study of buck sperm freezing for many researchers. For these studies, freezing extenders and protocols used were those which proved successful in bull semen cryopreservation (Leboeuf *et al.*, 2000).

Semen cryopreservation involves common stages to any protocol such as collection and extension of semen, addition of cryoprotectant, cooling above 0 °C, cooling below 0 °C, storage and thawing (Curry, 2007). However, there are noticeable differences between spermatozoa from different species which will have an effect on sensitivity of spermatozoa to the freezing-thawing procedure (Curry, 2007; Medeiros *et al.*, 2002). This fact leads to specific modifications in each stage of the cryopreservation process (Watson, 1995; Wildt and Wemmer, 1999).

Semen cryopreservation causes ultrastructural, biochemical and functional damage to spermatozoa (Leboeuf *et al.*, 2000) due to dramatic temperature changes, submission to osmotic and toxic stresses derived from exposure to cryoprotectants and formation and dissolution of ice in the intracellular and extracellular environment (Medeiros *et al.*, 2002). As a consequence, it would be produced sperm cell damage, cytoplasm fracture or even effects on the cytoskeleton or genome related structures (Isachenko, 2003). These effects would result finally in a reduction on motility, viability, impaired transport and fertility (Leboeuf *et al.*, 2000).

There are many factors affecting the sperm cryopreservation and they need to be examined in detail before a criopreservation procedure can be initiated due to these factors will influence the outcome of the process (Purdy, 2006; Roldán *et al.*, 2006). Among these factors can be highlighted: species and individuals, sperm collection method, collection season, extender compostition, cooling rate, equilibration time, freezing rate and thawing rate.

Species and individuals

Knowledge of sperm biology for the species is essential to maximize post-thaw recovery of spermatozoa and consequently fertility (Purdy, 2006). Sperm cryopreservation protocols vary between species due to differences on cell shape and volume as well as organelles size and composition (Curry *et al.*, 1996; Medeiros et al., 2002). In addition to this, it has been observed that differences in other factors such as sperm membrane lipid composition influence on the susceptibility of spermatozoa to damage produced during the cryopreservation (Holt, 2000). In this line, boar is a classical example of a species that is cold shock-sensitive while spermatozoa from bull

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and ram are more resistant. On the other side, humans, rabbits and rooster are the most resistant to cold temperatures (Wales and White, 1959; Holt, 2000). Therefore, it is important to recognize these species differences in spermatozoal sensitivity to cold shock and apply appropriate handling protocols to avoid cellular damage during cryopreservation.

In small ruminants, it has been observed specific differences in sperm susceptibility to freezing methods, particularly obvious in goat males (Barbas and Mascarenhas, 2009). Moreover, there is some evidence for physiological differences between spermatozoa from individuals of the same species (Curry, 2000). Therefore, inter-male or even inter-ejaculates variability in sperm freezability has been recognized as a source of variation during the cryopreservation procedure (Holt, 2000; Ramón *et al.*, 2013; Soler *et al.*, 2003; Watson, 1995). For goats, several studies have showed individual differences in sperm freezability and fertility (Furtoss *et al.*, 2010; Medrano *et al.*, 2010). Besides, a particularity of this species is the large variability not only at individual level but also at level of ejaculates within the same buck (Furtoss *et al.*, 2010).

Collection of sperm samples

Another factor which should be taken into account during the cryopreservation procedure is the collection method used to obtain the sperm samples. In small ruminants, semen from live animals is obtained mainly by artificial vagina (AV) or electroejaculation (EE). Semen collection by AV is a widespread method used due to sperm samples obtained by this method are similar than ejaculates collected by natural service (Wulster-Radcliffe *et al.*, 2001). Moreover, this technique is fast, no appalling and allows the collection of various ejaculates from the same male (Herrera *et al.*, 1994; Maxwell and Evans, 1990). However, the major disadvantage is that this technique requires a previous training period of males (Wulster-Radcliffe *et al.*, 2001). The EE is an alternative when males cannot be trained to AV or for wild species, and may be a viable method of repeatedly collection of ejaculates from individual specimens without causing death (Santiago-Moreno *et al.*, 2009). This collection method has been successfully used in a variety of wild ruminants (Asher *et al.*, 1990; Cassinello *et al.*, 1998; Garde *et al.*, 2003; Santiago-Moreno *et al.*, 2009; Watson, 1976). Nevertheless, the sperm characteristics are usually different between collection methods. Thus, several authors have observed that ejaculates collected by EE showed higher volume and lower concentration than those obtained by AV in bucks (Austin *et al.*, 1968; Memon *et al.*, 1986), rams (Marco-Jiménez *et al.*, 2005; Marco-Jiménez *et al.*, 2008; Mattner and Voglmayr, 1962; Terrill, 1940) and bulls (Dziuk *et al.*, 1954). In addition to

this, studies in domestic caprids have reported that sperm quality after thawing of semen collected by EE is lower than that obtained by AV (Greyling and Grobelaar, 1983), suggesting that seminal plasma has negative effects on spermatozoa survival during the cryopreservation procedure (Kawano *et al.*, 2004).

Seminal plasma

Seminal plasma roles on spermatozoa have been widespreadly studied and a variety of results have been found including: i) motility activation, ii) setting of osmotic pressure and source of nutrients, iii) prevention against premature activation during female reproductive tract transport and plasmmalema stabilization by capacitation inhibitors (Desnoyer and Manjunath, 1992; Villemure *et al.*, 2003) and iv) influence on fertility (Rozeboom *et al.*, 2000). A beneficial effect of seminal plasma has been observed on thawed sperm samples. Thus, addition of seminal plasma or some of their components to thawed sperm samples increases the sperm motility (White *et al.*, 1987), helps to recover some surface proteins (Domínguez *et al.*, 2008), reverts the damage on sperm membrane (Rebolledo *et al.*, 2007) and increases the sperm quality parameters (Maxwell *et al.*, 2007). However, other authors have not observed any effect of seminal plasma or even a harmful effect has been found on thawed sperm motility and viability (García and Graham, 1987; Graham, 1994; Moore *et al.*, 2005).

Seminal plasma contains proteins which are secretions from epididymis and accessory sex glands (Chandonnet *et al.*, 1990). The addition and removal of some proteins during sperm maturation through epididymis and when semen is ejaculated have an important role on the maintenance of plasmalemma stability (Desnoyers and Manjunath, 1992) and motility (Henricks *et al.*, 1998; Sánchez-Luengo *et al.*, 2004). Contrary, other proteins inhibit the movement of epididymal spermatozoa in a physiological way, inhibiting also the motility of ejaculated spermatozoa (Das *et al.*, 2010). Some seminal plasma proteins prevent spermatozoa from damage caused by cold shock (Pérez-Pe *et al.*, 2002). Types and amount of seminal plasma proteins vary between individuals. In addition to this, these proteins could be affected by some environmental factors such as sperm collection season, stress, feeding, temperature (Pérez-Pe *et al.*, 2001a) or even semen collection method (Marco-Jiménez *et al.*, 2008).

Other compounds in the seminal plasma are enzymes. An enzyme which is believed that play a role during acrosome reaction and spermatozoa-oocyte fusion is the phospholipase A2 (Yuan *et al.*, 2003). In caprine, coagulating egg yolk enzyme (EYCE) and bulbourethral III secretion (SBUIII) are secreted by bulbourethral glands and have phospholipase activity. EYCE hydrolyzes egg yolk lecithin contained in freezing extenders into fatty acids and lysolecithin, which is toxic to spermatozoa

(Iritani and Nishikawa, 1961; Iritani and Nishikawa, 1963; Sias *et al.*, 2005). This hydrolysis induces acrosome reaction (Upreti *et al.*, 1999) and chromatin decondensation (Sawyer and Brown, 1995). Similarly, SBUIII hydrolyzes residual triglycerides in the skim milk from freezing extenders that result in fatty acids which are toxic to spermatozoa (Pellicer-Rubio *et al.*, 1997; Pellicer and Combarnous, 1998). As a result, it is caused a decrease in the percentage of motile spermatozoa, deterioration in the quality of movement, breakage of acrosomes and finally, cellular death. However, the toxicity of these enzymes differs with pH, temperature, seminal plasma concentration and season of semen production (Leboeuf *et al.*, 2000).

In order to avoid the harmful effect of these enzymes on egg yolk and skim milk, some authors have suggested that the removal of seminal plasma from ejaculates by means of centrifugation is an useful method to increase the sperm motility, membrane integrity and fertility after freezing-thawing procedure (Kozdrowski *et al.*, 2007; Machado and Simplicio, 1995). Nevertheless, other studies have showed no effect of seminal plasma removal on sperm quality after thawing (Cabrera *et al.*, 2005; Daskin and Tekin, 1996). Sperm selection techniques are an alternative to remove seminal plasma from buck ejaculates and also, to enrich the amount of cells with normal morphology and higher motility (Hollinshead *et al.*, 2004; Mortom *et al.*, 2006). Recently, a sperm selection technique using single layer centrifugation (SLC) has been reported, with important advantages in relation to conventional density gradient centrifugation (DGC) (Morrell and Rodríguez-Martínez, 2009). This technique (SLC) use a species-specific formulation (Androcoll®), resulting in the selection of motile, morphologically normal spermatozoa with intact membranes and good chromatin integrity (Morrell *et al.*, 2009a; Morrell *et al.*, 2009b). SLC could improve sperm quality at thawing of buck semen samples as it has been observed in other species, where this technique selected a sperm subpopulation with high quality and with improved functionality (Martínez-Alborcia *et al.*, 2012).

Season of ejaculate collection

The reproductive activity of goats living at high or mid-levels is influenced by the season, with photoperiod being the main determining factor (Gordon, 1997; Ritar, 1993; Roca *et al.*, 1992). Through autumn and winter it is observed the best sperm quality, being this period considered as the breeding season in this species (Coloma *et al.*, 2011; Karagiannidis *et al.*, 2000; Pérez and Mateos, 1996; Roca *et al.*, 1992). Several effects associated to season have been reported such as changes in testicular weight and size, chemical composition and volume of ejaculates, mating activity and fertility (Coloma *et al,.* 2011; Gordon, 1997; La Falci *et al.*, 2002; Maxwell *et al.*, 2007).

Moreover, it has been observed an effect of season on the response of sperm cells to the freezing-thawing process in several mammals, including goats (Chemineau *et al.*, 1999; Coloma *et al.*, 2011; D´Alessandro and Martemucci, 2003; Fiser and Fairful, 1983; Janett *et al.*, 2003; Koonjaenak *et al.*, 2007; López-Fernández *et al.*, 2011; Medrano *et al.*, 2010). In rams, improvements on fertility of sperm samples collected and frozen during breeding season have been related with changes on seminal plasma proteins (Smith *et al.*, 1999). Thus, several authors have reported that the sperm samples supplementation with seminal plasma from breeding season provided a beneficial effect on sperm quality at thawing (Domínguez *et al.*, 2008; Leahy *et al.*, 2010). This way, higher sperm quality was observed in sperm samples collected and frozen during breeding season (D´Alessandro and Martemucci, 2003). In goats, a study on Spanish ibex showed higher resistance to freezing during winter (Coloma *et al.*, 2011).

Regarding the interaction between breeding season and seminal plasma composition, some studies carried out in goats showed that the addition of seminal plasma proteins collected during non-breeding season led to decrease in sperm quality (La Falci *et al.*, 2002). Other studies have reported an effect of seminal plasma removal on resistance to freezing of sperm samples collected during different season. Thus, Cabrera *et al.* (2005) observed an improvement on thawed seminal quality when seminal plasma was removed before cryopreservation procedure during autumn and spring, while sperm parameters were higher when seminal plasma was no removed during winter. Coloma *et al.* (2010) did not find a significant interaction between seminal plasma removal and season, although they observed lower percentage of intact acrosomes when seminal plasma was no removed during decreasing daylenght and no effect of seminal plasma removal during increasing photoperiod.

Freezing extender

The purpose of a cryopreservation extender is to supply the sperm cells with sources of energy, protect the cells from temperature-related damage, and maintain a suitable environment for the spermatozoa to survive temporarily (Purdy, 2006). In general, a goat sperm cryopreservation medium includes a non-penetrating cryoprotectant, a penetrating cryoprotectant, a buffer, one or more sugars, salts and antibiotics (Evans and Maxwell, 1987).

The most common non-penetrating cryoprotectant used in buck semen cryopreservation is egg yolk (Ritar and Salamon, 1982; Tuli and Holt, 1994) due to its effect protecting acrosomal and plasmatic membranes (Salamon and Maxwell, 2000). However, as it was stated above, the interaction between this cryoprotectant and a

seminal plasma enzyme (EYCE) leads to consider the egg yolk concentration as an important factor. Thus, a variety of results has been observed when egg yolk is included in a range of 2-20% (Aboagla and Terada, 2003; Aboagla and Terada, 2004; Ritar and Salamon, 1982; Ritar and Salamon, 1991; Tuli and Holtz, 1994). Another non-penetrating cryoprotentant widely used is skim milk but an interaction between SBUIII enzyme from seminal plasma and compounds from skim milk has been demonstrated in a similar way than egg yolk interactions (Leboeuf *et al.*, 2000). Due to negative interactions between egg yolk and skim milk with some seminal plasma compounds, other extenders based on soybeans have been suggested as an alternative. These extenders have been used in different species, obtaining similar results than egg yolk (Aires *et al.*, 2003; Forouzanfar *et al.*, 2010; Sariözkan *et al.*, 2010).

Several penetrating cryoprotentants have been tested for buck semen cryopreservation (Kundu *et al.*, 2000; Leboeuf *et al.*, 2000; Ritar *et al.*, 1990a, Ritar *et al.*, 1990b; Singh *et al.*, 1995; Tuli and Holtz, 1994), but the most frequently used is glycerol. Its protective effect is attributed to its colligative or water-binding property (Salamon and Maxwell, 1995). Glycerol concentration usually used varies from 3 to 9 % (Leboeuf *et al.*, 2000). However, glycerol has also a toxic effect on spermatozoa (Hammerstedt *et al.*, 1990; Holt, 2000) which depends on extender composition, method of glycerol addition and cooling and freezing rates (Salamon and Maxwell, 1995; Salamon and Maxwell, 2000). Thus, sperm quality at thawing differs depending on temperature of glicerol addition. Glycerol can be added at different temperatures in 1, 2 or 3 steps (Corteel, 1974; Salamon and Ritar, 1982; Tuli and Holtz, 1994). Some authors have obtained good results when glicerol was added at 4 °C (Corteel, 1974; Corteel, 1975; Corteel, 1990), while other studies reported better results after glicerol addition at 30 or 37 °C (Farshad *et al.*, 2009; Salamon and Ritar, 1982; Tuli and Holtz, 1994). Otherwise, Coloma *et al.* (2010) did not find differences on buck thawed sperm quality when glycerol was added at room temperature or 5 °C.

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Cooling and equilibration

When buck semen is diluted in the freezing extender, it is cooled to 4-5 °C. This temperature decrease produces a reduction in metabolic activity of cells and an increase in the spermatozoa lifespan. However, changes in sperm membrane which alter its functionality are produced during this stage (Amann, 1999). This problem can be solved by modifying cooling rates before freezing (White, 1993). Hence, different rates (slow and fast) has been studied in several species with variable results (Fernández-Santos *et al.*, 2006; Januskauskas *et al.*, 1999; Memon *et al.*, 2013; Mocé and Vicente, 2002; Salazar *et al.*, 2011).

Equilibration period is the interval in which the sperm cell remains in contact with glycerol before freezing, to allow cryoprotectant to penetrate the cells, enabling equilibrium between intra and extracellular medium (Evans and Maxwell, 1987). The equilibration time varies between species. Effective cryoprotection after short contact with glycerol has been demonstrated for bull, boar and ram (Berndston and Foote, 1969; Berndston and Foote, 1972; De Matos *et al.*, 1992; Wilmut *et al.*, 1973). However, although some authors have frozen buck semen successfully without equilibration period (Ritar and Salamon, 1983; Ritar *et al.*, 1990b), most of researchers include, for this species, an equilibration time at 5 °C from 1 to 3 hours (Choe *et al.*, 2006; Corteel, 1974; Deka and Rao, 1986).

Sperm freezing

After dilution, cooling and equilibration, the freezing of sperm samples is done in straws or pellet form. Freezing of sperm in pellets is rapid and inexpensive, but inventory management is problematic because the semen samples can not be labeled. Semen aliquots of 0.1-0.3 mL are dispensed into indentations on a block of dry ice (solid carbon dioxide at -79 °C) and frozen for 2-4 min. After that, the pellets are plunged into liquid nitrogen for storage (Chemineau *et al.*, 1991; Evans and Maxwell, 1987). On the other hand, freezing can be carried out in straws. This method is more expensive and laborious than the pellet technique, but each sample can be labeled for accurate inventory management. Ritar *et al.* (1990a; 1990b) demonstrated that sperm samples frozen in pellets have higher motility than samples frozen in straws. Nevertheless, similar motility was observed for sperm samples frozen in 0.25 mL straws in relation to 0.5 mL.

Sperm thawing

Thawing sperm samples is determined by the method used to freeze the semen. Thus, sperm pellets should be thawed in dry tubes at 37 °C without a thawing solution, while the thawing of straws may be performed using various methods (Evans and Maxwell, 1987). Tradicionally, straws are thawed by placing them in a 37 °C water bath for 12-30 seconds (Deka and Rao, 1987). Other authors observed good sperm quality after thawing at 70-75 °C for 7-10 seconds (Andersen, 1969; Tuli *et al.*, 1991). However, thawing at 37 °C is more suitable under practical conditions and moreover, the risk of overheating, which can result in tremendous sperm mortalities, is excluded.

OBJECTIVES

The main goal of this Doctoral Thesis has been the development of an optimal methodology to collect and cryopreserve buck semen of Blanca-Celtibérica breed which allows us to store seminal doses successfully in a Germoplasm Bank. Therefore, the following specific objectives have been suggested:

1. Study the effect of semen collection method (artificial vagina and electroejaculation) on sperm quality after thawing in Blanca-Celtibérica goat breed and compare the results to those obtained from a phylogentically close related small ruminant species which is more studied (Manchego ram breed).

2. Evaluate the effects of different factors on sperm quality at thawing in sperm samples obtained by artificial vagina and electroejaculation:

   a) Season of semen collection.

   b) Removal of seminal plasma by centrifugation/washing.

   c) Freezing extender: two commercial extenders, Biladyl® (egg yolk-based extender) and Andromed® (soy lecithin-based extender), and a non-commercial extender based on skim milk.

3. Evaluate the effect of sperm selection technique by single layer centrifugation (SLC) before and after freezing on sperm quality at thawing in samples collected by electroejaculation.

4. Study the effect of different modifications in freezing extender and cryopreservation protocol on sperm quality after thawing in samples collected by electroejaculation:

   a) Egg yolk concentration (0, 1.5, 10 and 20%) contained in the freezing extender.

   b) Cooling rate from 30 °C to 5 °C (fast: 10 minutes or slow: 90 minutes) and temperature of glycerol addition (30 °C or 5 °C).

   c) Equilibration time at 5 °C (0, 1, 2 and 3 hours).

CHAPTER 1

Does the semen collection method affect the same way on sperm cryoresistance in goat and sheep?

1.1 Abstract

The semen collection in domestic small ruminants is mainly carried out by artificial vagina (AV), but electroejaculation (EE) is an alternative when males are not trained to AV. In this sense, some studies have been conducted in sheep and few research works have been done in goat. In this work, the effect of semen collection method using the same procedure in two phylogenetically close species of small ruminant, sheep and goat, was studied. Semen from seven Blanca-Celtibérica bucks and five Manchega rams was collected by AV and EE at same time. For both species, sperm samples were diluted with the same extender (Biladyl® with 20% egg yolk) and frozen over nitrogen vapors. The assessment of motility by CASA, and flow cytometry analyses, including an analysis of chromatin integrity, were performed at thawing. The collection method influenced on the sperm quality at thawing in bucks, whereas no differences between both methods were observed in rams. Higher values were found for sperm motility and progressive motility in ram samples collected by both methods, followed by buck samples collected by AV and EE, respectively. Flow cytometry analysis showed lower percentage of intact spermatozoa, percentage of viable spermatozoa with active mitochondria and higher chromatin damage for buck samples collected by EE in relation to samples collected by AV and those from rams obtained by both methods. In conclusion, the semen collection by EE provided lower sperm quality after thawing in Blanca-Celtibérica buck, while the sperm cryoresistance for samples collected by VA and EE was unaffected in sheep.

Keywords: artificial vagina, electroejaculation, buck, ram, sperm cryopreservation

1.2. Introduction

Many local breeds, some of which are endangered, have features like resistance to climatic stress, diseases and parasites, which make them well adapted to local conditions. Loss of local breeds leads to many problems such as cultural erosion, reduction of rural economies and negative environmental factors. Appropriate conservation measures should ensure that farmers and researchers have access to a diverse gene pool for further breeding and research. The storage of genetic material for breeding purposes is common for some commercial breeds, but not in all species. Thereby, for local breeds, the collection and storage of animal genetic material has not been adequate. In such cases, it is important to support planned and targeted collection of animal genetic resources and to expand *ex situ* conservation activities (FAO, 2010; FAO 2012).

Potential means of genetic diversity preservation include the storage of semen, embryos, oocytes and somatic cells, being the semen cryopreservation the most widespread methodology because of it is cheap and easy to use. The first step in the semen freezing is the recovery of sperm samples. For domestic small ruminants, the sperm collection by artificial vagina (AV) is the preferred method (Leboeuf *et al.*, 2000), but this technique requires a previous training period (Wulster-Radcliffe *et al.*, 2001). Another method of collection such as the electroejaculation (EE) is an alternative when males are not trained to AV and may be a viable method of repeatedly collecting ejaculates from individual specimens without causing death (Santiago-Moreno *et al.*, 2009). However, differences on sperm characteristics between ejaculates collected by AV and EE have been found, showing the ejaculates obtained by EE higher volume and lower concentration (Memon *et al.*, 1986; Marco-Jiménez *et al.*, 2005; Marco-Jiménez *et al.*, 2008). In addition, the EE procedure changes the secretory function of one or more accessory glands modifying the composition of the seminal plasma (Marco-Jiménez *et al.*, 2008). Moreover, it has been described that seminal plasma proteins have an important function for preventing the cold-shock sperm membrane damage (Barrios *et al.*, 2005), and their composition may vary with the EE procedure influencing on cryoresistance of sperm samples. For breeds bred and reared in extensive systems, such as the Blanca-Celtibérica endangered goat breed, the EE method is an option to collect semen (Jiménez-Rabadán *et al.,* 2012a, Jiménez-rabadán *et al.,* 2012b). This method could also be used in sheep with the same management system or problems with training to AV. Nevertheless, results for the latter small ruminant species are confusing and little research has been conducted in goat. Thus, Quinn *et al.* (1968) and Álvarez *et al.* (2012) in sheep reported that spermatozoa collected by AV were more resistant to cold shock than those collected by EE,

although other authors observed greater sperm quality for samples from ram obtained by EE compared to samples collected by AV (Marco-Jiménez *et al.*, 2005). Controversy results could be due to differences between studies related to collection schemes, freezing extenders used, season of semen collection, samples handling or even to different males were used for each collection method.

With this background, this study was aimed to evaluate the effect of collection method (artificial vagina or electroejaculation) in two species phylogenetically close of small ruminants, Blanca-Celtibérica goat and Manchego sheep, using for both species the same methodology of semen collection and freezing protocol to know if the differences in sperm quality are properly due to sperm collection.

1.3 Material and methods

1.3.1 Animals and reagents

Animal handling was performed in accordance with Spanish Animal Protection Regulation, RD 1201/2005, which conforms to European Union Regulation 2010/63. Seven males of Blanca-Celtibérica goat breed (age > 1.5 years) and five males of Manchega sheep breed (age > 1.5 years) were used. All males were maintained and managed at the Regional Center of Animal Selection and Reproduction (CERSYRA) located in Valdepeñas (Spain). The thawing procedure was conducted at laboratories from Group of Biology of Reproduction in Albacete (Spain).

Chemicals were of reagent grade and purchased from Sigma (Madrid, Spain). Biladyl® was purchased from Minitüb (Tiefenbach, Alemania). Fluorescence probes were purchased from Invitrogen (Barcelona, Spain), except for propidium iodide (PI) which was purchased from Sigma. Chromatographically purified acridine orange (AO) was purchased from Polysciences Inc. (Warrington, PA, USA).

1.3.2 Semen collection

For each species and male, the collection of ejaculates was performed using AV and EE, both on the same day. Males were trained to conduct collections by AV and ejaculates were routinely collected once per week. The procedure of EE was carried out using the protocol described by Garde *et al.* (2003). Males were anesthetized with xylacine (0.2mg/kg Rompun® 2% i.m.; Bayer S.A., Barcelona, Spain), the rectum was cleaned of faeces and the prepucial area was shaved and washed with physiologic saline serum. A three electrode probe connected to a power source that allowed voltage and amperage control was used (P.T. Electronics, Boring, OR, USA). Probe diameter, probe length and electrode length were 3.2, 35.0 and 6.6 cm, respectively. The EE

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regime consisted of consecutive series of 5-sec pulses of similar voltage, each separated by 5-sec break. Each series consisted of a total of four pulses. The initial voltage was 1 V which was increased in each series until a maximum of 5 V. Urine contamination was tested and ejaculates with urine were rejected. Ejaculates were collected through of year and two ejaculates per ram and buck and collection method were obtained for the present study.

1.3.3 Evaluation of ejaculates

Immediately after the collection of ejaculates, the volume of ejaculate (measured in a conical graduated tube), sperm concentration (by spectrophotometer) and wave motion (scored on a scale of 0 to 5) were evaluated. Spermatozoa total number (STN) was calculated with the volume and concentration (Volume × Concentration). The proportion of motile spermatozoa (SM) was evaluated subjectively in aliquots of semen diluted (1:200) in a phosphate buffer saline (PBS) and incubated for 5 min at 37 °C, using a phase-contrast microscope (× 100).

1.3.4. Semen cryopreservation

Semen from each male and specie was diluted in a commercial extender, Biladyl® (20% clarified egg yolk) by the two-step dilution method. First, non-glycerolated fraction was added at 30 °C and the diluted semen was cooled to 5 °C for 2 h. Then, it was further diluted (v:v) with fraction containing glycerol. The diluted sperm samples were then held at 5 °C for 2 h more before freezing. At the end of this time, the diluted semen was loaded into 0.25 mL plastic straws and frozen. Straws were frozen over nitrogen vapours for 10 min, by placing the straws 4 cm above the surface of nitrogen liquid. The straws were subsequently plunged into liquid nitrogen and stored. Sperm samples were frozen to a final concentration of 140 to 200 × 10^6 spermatozoa/mL.

1.3.5. Thawed sperm evaluation

Thawing was performed by placing the straws in a water bath at 37 °C for 30 sec, emptying the contents into dry tubes and incubating for 5 min at the same temperature. One straw from each male, method of collection and replicate was thawed and analyzed for both species.

Frozen-thawed spermatozoa were evaluated for motility by Computer Assisted Semen Analysis (CASA) using the Sperm Class Analyzer software (SCA® 2002, Microptic, Barcelona, Spain). Sperm samples were diluted (1:1) with TALP-HEPES medium (87 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 40 mM HEPES, 21.6 mM sodium lactate, 1 mM sodium pyruvate, 50 µg/mL kanamycin, 10

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µg/mL phenol red, and 6 mg/mL bovine serum albumin (BSA) (pH 7.5)) and 5 µL were put on a Makler chamber recording the characteristics of sperm motility. The following motility parameters were assessed: total motile spermatozoa (TM, %), progressive motile spermatozoa (PM, %), velocity according to the straight path (VSL, µm/s), linearity index (LIN, %) and amplitude of lateral head displacement (ALH).

Also, aliquots of thawed semen were used to conduct flow cytometry analysis. We assessed the membrane stability with YO-PRO-1, the viability with PI and the mitochondrial activity with Mitotracker Deep Red (Martínez-Pastor *et al.*, 2008). A staining solution using TALP-HEPES was prepared by adding 50 nM YO-PRO-1 (stock: 100 µM in DMSO) and 15 µM propidium iodide (stock: 7.5 mM in milli-Q water). Twenty µL of sample were diluted in 0.5 mL of staining solution in polypropylene tubes for flow cytometry and were kept in dark for 15 min. Mitochondrial membrane potential of the samples was evaluated with 0.1 µM YO-PRO-1 and 100 nM Mitotracker Deep Red solution (stock: 1 mM in DMSO) in TALP-HEPES. Sperm samples were diluted to 1 x 10^6 spermatozoa/mL, and 300 µL were transferred to a polypropylene tube and left in dark for 30 min. After incubation times, sperm samples were analyzed using a Cytomics FC500 flow cytometer (Beckman coulter, Inc. USA). YO-PRO-1-/PI- spermatozoa were considered as intact spermatozoa (indicating live spermatozoa with intact plasmalemma) and Mitotracker+/YO-PRO-1- as viable spermatozoa with active mitochondria.

Chromatin stability was assessed by using the SCSA® (Sperm Chromatin Structure Assay) technique (SCSA® Diagnostics, Inc., Brookings, SD, USA) (Evenson and Jost, 2000). This technique is based on the susceptibility of the sperm DNA to acid-induced denaturation *in situ* and the metachromatic staining Acridine Orange. This stain fluoresces green when combined with double-stranded DNA, and red when combined with single-stranded DNA (denaturated). Thawed spermatozoa were diluted with TNE buffer to 2 × 10^6 cells/mL. Samples were flash frozen in LN_2 and stored at -80 °C until analysis. We calculated the DNA fragmentation Index (DFI) for each spermatozoon as the ratio of red fluorescence respect to total fluorescence (red + green). High values of DFI indicates chromatin abnormalities. The %DFI was calculated as the percentage of spermatozoa with DFI > 25.

1.3.6. Statistical analysis

The statistical analysis was performed using SPSS for windows v. 19 (SPSS Inc, Chicago, Ill). Data were considered statistically significant when $P \leq 0.05$.

A mixed effect model with male as random effect was conducted. The effects of species and collection method on characteristics of ejaculates and sperm quality at thawing were studied. Multiple comparison analyses were carried out using the Bonferroni adjustment.

1.4 Results

1.4.1 Effect of semen collection method on characteristics of ejaculates.

For both species, ejaculates collected by EE had greater volume than those obtained by AV, although only significant differences ($P \leq 0.05$) were found between collection methods for bucks (Figure 1.1). Higher sperm concentration ($P \leq 0.05$) was observed for samples collected by AV for both species. However, goat samples had lower ($P \leq 0.05$) concentration than sheep ones for each collection method (Figure 1.1). Samples collected by EE from bucks had the lowest ($P \leq 0.05$) STN (Figure 1.1) and no differences ($P > 0.05$) were found for SM between collection method and species (Figure 1.1).
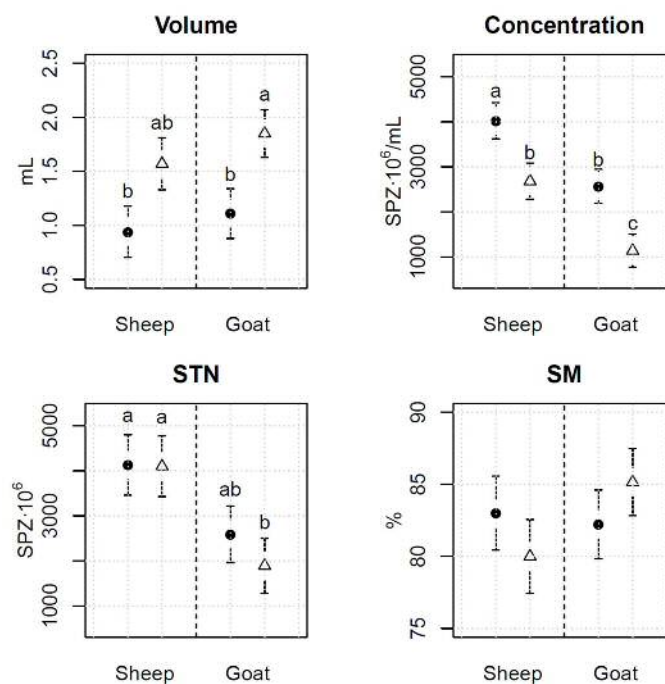
Figure 1.1 Effect of semen collection method on characteristics of ejaculates in Manchega sheep and Blanca-Celtibérica goat. Data are mean ± standard error of mean (SEM). Different letters show significant differences ($P \leq 0.05$). ●: artificial vagina; △: electroejaculation; STN: spermatozoa total number; SM: sperm motility.

1.4.2 Effect of semen collection method on sperm quality after thawing

The semen collection method influenced ($P \leq 0.05$) on sperm motility at thawing in Blanca-Celtibérica bucks, whereas no differences were found between both collection methods in Manchego rams (Figure 1.2). Results showed higher ($P \leq 0.05$) total motile and progressive motile for ram samples collected by both methods, followed by buck samples obtained by AV and EE, respectively. There were no differences ($P \leq 0.05$) for velocity parameters between species and collection methods. Flow cytometry analysis showed lower ($P \leq 0.05$) proportion of intact spermatozoa, viable spermatozoa with active mitochondria and higher %DFI for buck sperm samples obtained by EE and there were no differences ($P > 0.05$) for theses parameters in ram semen collected by both methods and buck semen obtain by AV (Figure 1.3).

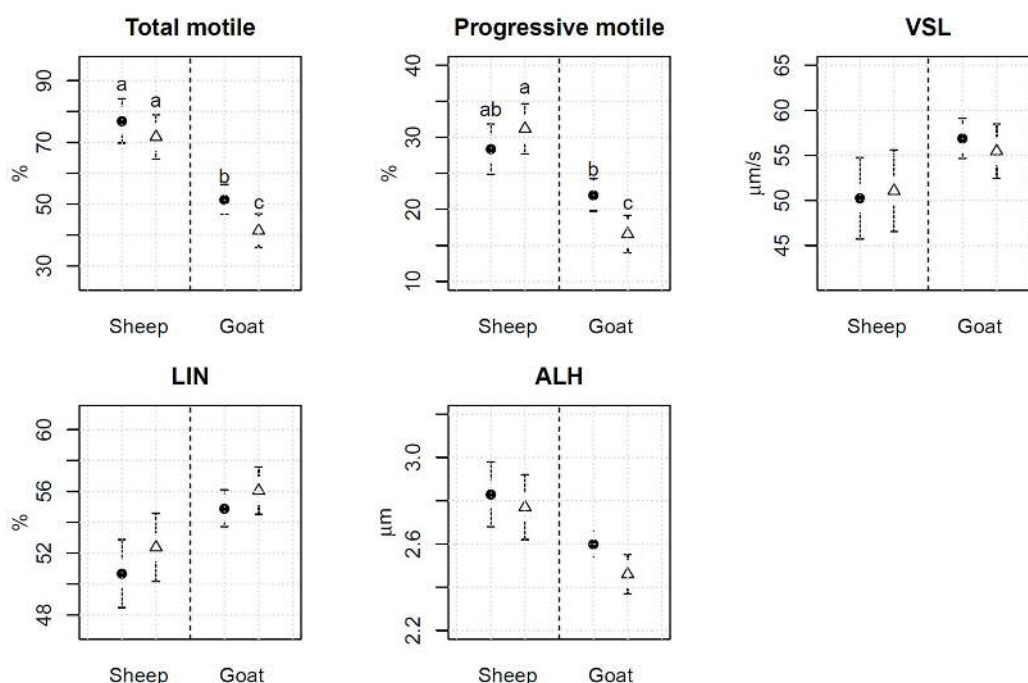**Figure 1.2** Effects of sperm collection method on motility parameters after thawing in Manchega sheep and Blanca-Celtibérica goat. Data are mean ± standard error of mean (SEM). Different letters show significant differences ($P \leq 0.05$). ●: artificial vagina; △: electroejaculation; VSL: velocity according to the straight path; LIN: linearity index; ALH: amplitude of lateral head displacement.
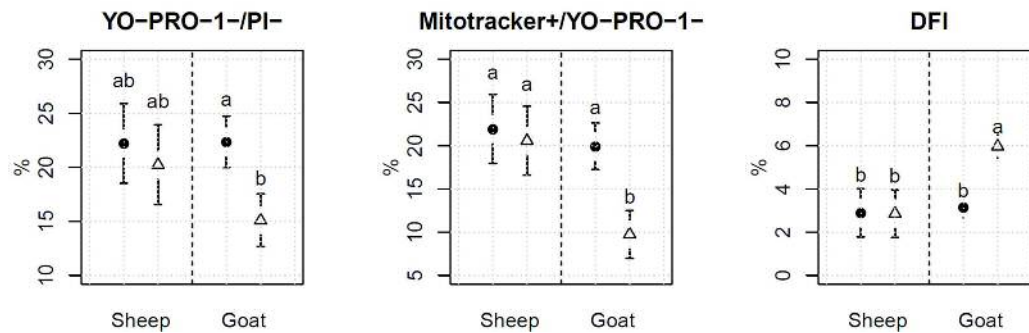
Figure 1.3 Effects of sperm collection method on parameters assessed by flow cytometry after thawing in Manchega sheep and Blanca-Celtibérica goat. Data are mean ± standard error of mean (SEM). Different letters show significant differences ($P \leq 0.05$). ●: artificial vagina; Δ: electroejaculation; YO-PRO-1-/PI-: intact spermatozoa; Mitotracker+/YO-PRO-1-: viable spermatozoa with active mitochondria; DFI: DNA fragmentantion index.

1.5 Discussion

The development of genetic resource banks (GRB) by gametes cryopreservation allows the storage of genetic resources indefinitely (Watson and Holt 2001) and it is a key tool to conserve local breeds, some of which are endangered. The first step for sperm cryobank creation is the collection of ejaculates which will be obtained by different methods depending on species and production systems. Thus, the EE is an alternative to collect semen from untrained males or wild males whose training to AV is not possible.

This work was aimed to study the effect of semen collection method (AV *vs.* EE) on sperm quality after cryopreservation in Blanca-Celtibérica bucks and in other small ruminant species more studied, sheep, which has showed conflicting results. The same methodology of semen collection and cryopreservation was used in both species. In our study, the collection method had a similar influence on sperm characteristics assessed in ejaculates in both species. Ejaculates obtained by EE had higher volume and lower concentration than those collected by AV. These results agree with other authors who compared these collection methods in bucks (Austin *et al.*, 1968; Memon *et al.*, 1986; Jiménez-Rabadán *et al.*, 2012a) and rams (Mattner and Volgmayr, 1962; Marco-Jiménez *et al.*, 2005). A higher volume for samples collected by EE could be due to contribution of accessory sex glands because of electrical stimuli as was suggested by Mattner and Volgmayr (1962). Nevertheless, no differences on fresh sperm motility were observed between collection methods for both species as was observed by other authors (Marco-Jiménez *et al.*, 2005; Jiménez-Rabadán *et al.*,2012a; Álvarez *et al.*, 2012).

After thawing, the collection method influenced sperm quality in goat but no in sheep. Thus, sperm quality was higher for samples collected by AV compared to EE in

Blanca-Celtibérica bucks. Moreover, buck sperm samples had lower motility than ram samples and, those collected by EE showed the lowest values. In addition, other sperm parameters such as percentage of intact spermatozoa, percentage of viable spermatozoa with active mitochondrias and DNA fragmentation index had the worst values for buck samples collected by EE and no differences were found between buck samples collected by AV and ram samples obtained by both methods. Differences in the protein profile and ions concentration of seminal plasma between samples collected by AV and EE have been observed in sheep (Marco-Jiménez *et al.*, 2008). Seminal plasma contains proteins and other substances which are useful to spermatozoa in order to withstand cold shock (Barrios *et al.*, 2005) and to prevent against oxidative stress (Wat-sum *et al.*, 2006). The EE could change proteins and enzymes in the seminal plasma affecting the sperm cryoresistance. It is possible that the modifications of proteins and enzymes due to sperm collection method influence deeper on the survival to freezing of spermatozoa in bucks in relation with other species of small ruminants like sheep. It is known the harmful effect of some proteins in the seminal plasma in bucks in some conditions. Thus, it has been reported a specific problem during sperm cryopreservation in this specie when extenders containing egg yolk are used (Sariözkan *et al.*, 2010). This is due to seminal plasma contains an enzyme secreted by the bulbourethral gland, named egg yolk coagulating enzyme (EYCE), which hydrolyzes egg yolk lecithin into fatty acids and lysolecithin being the latter highly toxic to buck spermatozoa (Iritani and Nishikawa, 1963). This enzyme do no exists in other species such as rams (Leboeuf *et al.*, 2000; Purdy, 2006). In addition, the epidydimal fluid from bucks contains a motility inhibiting factor which decreases sperm motility when it is released to ejaculate (Das *et al.*, 2010).

In conclusion, semen from Blanca-Celtibérica bucks collected by EE showed lower sperm quality at thawing than that obtained by AV, whereas the collection method did not influence on thawed sperm quality in Manchega rams. However, due to the importance of EE as sperm collection method in Blanca-Celtibérica goats, further studies should be carried out to improve the sperm quality after thawing for this kind of samples.

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CHAPTER 2

Effect of semen collection method (artificial vagina *vs.* electroejaculation), extender and centrifugation on post-thaw sperm quality of Blanca-Celtibérica buck ejaculates

2.1 Abstract

The aim of this study was to evaluate the effect of semen collection method (artificial vagina compared to electroejaculation), season in which the semen was collected (breeding season compared to non-breeding season), freezing extender (Biladyl®, Andromed® and skim milk based extender) and pre-treatment procedure (washing compared to non-washing) on post-thaw semen quality in buck. Ejaculates from seven bucks of the Blanca Celtibérica breed were collected by artificial vagina and electroejaculation during the breeding (July to December) and non-breeding season (January to June). Samples were split in two aliquots and one of them was washed. Three freezing extenders were evaluated on washing and non-washing sperm samples. Ejaculates collected by artificial vagina had a greater sperm quality after thawing, with greater values ($P \leq 0.05$) for SM (sperm motility), NAR (acrosome intact), YO-PRO-1-/PI- (intact spermatozoa), and and Mitotracker+/YO-PRO-1- (spermatozoa with active mitochondria) and lower % DFI (DNA fragmentation index). Thawed sperm samples which were collected during the breeding season had greater values ($P \leq 0.05$) for NAR, intact spermatozoa and spermatozoa with active mitochondria, than those semen samples obtained during the non-breeding season. Semen freezing with Biladyl® and Andromed® resulted in a greater sperm quality ($P \leq 0.05$) after thawing in relation to milk based extender. Washing procedure had no effect on sperm parameters assessed at thawing. Results from the present study suggest that the success of semen cryopreservation in Blanca-Celtibérica goat depends on semen collection method and season, as well as on the extender used. Thus, the post-thaw sperm quality will be greater ($P \leq 0.05$) when samples are collected by artificial vagina during the breeding season and when Biladyl® or Andromed® are used as freezing extenders.

Keywords: Blanca-Celtibérica buck; Artificial vagina; Electroejaculation; Sperm Cryopreservation; Freezing extender; Breeding season

2.2 Introduction

The Blanca-Celtibérica goat is an autochthonous breed from Spain considered as an endangered breed. Endangered breeds must be preserved by conservation methods. One of the conservation procedures consists of the development of genetic resource banks, cryopreserving gametes and embryos, thus allowing the storage of genetic resources indefinitely (Watson and Holt, 2001).

The first step for the creation of a sperm cryobank is the use of an effective method for the collection of the ejaculates. For domestic males, the artificial vagina (AV) procedure is the preferred method (Leboeuf *et al.*, 2000), but this technique requires a previous training period (Wulster-Radcliffe *et al.*, 2001). Another method of collection such as the electroejaculation (EE) involves an alternative when males are not trained to AV or for wild species, and may be a viable method of repeatedly collecting ejaculates from individual specimens without causing death (Santiago-Moreno *et al.*, 2009). The latter method could be an adequate alternative to collect ejaculates in Blanca-Celtibérica bucks because of there are few herds and they live under an extensive production system, being difficult to train them for AV. However, differences on sperm characteristics between ejaculates collected by AV and EE have been found, obtaining more desirable results when AV is used (Greyling and Gobbelar, 1983). Besides, substantial differences in seminal plasma composition may exist between ejaculates obtained by AV and EE (Marco-Jiménez *et al.*, 2008).

Sperm cryopreservation causes ultraestructural, biochemical and functional impairment to the spermatozoa (Aitken *et al.*, 1998; Purdy, 2006; Watson, 2000). Egg yolk is a common component of sperm cryopreservation extender as a protector of the plasmatic and acrosomal membrane against temperature-related damaged (Purdy, 2006). The Tris-egg yolk-glucose and non-fat dried skimmed milk extenders are most commonly used for cryopreserving buck spermatozoa (Purdy, 2006). A specific problem in the preservation of goat semen has been its limited tolerance to the inclusion of egg yolk in the freezing medium. Seminal plasma has a detrimental effect on the viability of buck spermatozoa during cryopreservation when extender containing egg yolk is used (Sariözkan *et al.*, 2010). This fact is due to seminal plasma contains egg yolk coagulating enzyme (EYCE), which hydrolyzes egg yolk lecithin into fatty acids and lysolecithin (Iritani and Nishikawa, 1961; Iritani and Nishikawa, 1963) which is highly detrimental to buck spermatozoa. Similarly, it has been identified the protein SBUIII from the goat bulbourethral gland which decreased survival of cooled or frozen sperm diluted in milk-based media. This protein could hydrolyze residual triglycerides in skim milk and produce fatty acids exhibiting toxic effects toward spermatozoa (Pellicer-

Rubio *et al.*, 1997). Currently, other extenders based on soybean have been used as an alternative to egg yolk in different species, obtaining for both extenders similar results (Aires *et al.*, 2003; Forouzanfar *et al.*, 2010).

In addition, many studies have reported that the washing procedure of goat semen for removing the seminal plasma from ejaculate is necessary to increase motility, membrane integrity, and fertility after freeze-thaw procedure (Kozdrowski *et al.*, 2007; Machado and Simplicio, 1995). However, other studies have demonstrated that there was no effect of seminal plasma removing on post-thaw sperm quality (Cabrera *et al.*, 2005; Daskin and Tekin, 1996).

Finally, significant seasonal variation in the semen quality of small ruminants living at high or mid-levels altitudes have been demonstrated (Ritar, 1993; Roca *et al.*, 1992) and the chemical composition and volume of the ejaculate may be different depending of season (Maxwell *et al.*, 2007).

With this background, we have evaluated in Blanca-Celtibérica bucks the effects of the collection method (AV compared to EE), season in which the semen was collected (breeding season compared to non-breeding season), freezing extender (Biladyl®, Andromed® and a skim milk-based extender) and pre-treatment procedure (washing compared to non-washing), on sperm quality after cryopreservation.

2.3 Materials and Methods

2.3.1 Animals and reagents

All animal procedures were performed in accordance with Spanish Animal Protection Regulation, RD 1201/2005, which conforms to European Union Regulation 2010/63. Seven males of Blanca-Celtibérica goat breed (age > 1.5 years) were used. Males were maintained and managed at the Regional Center of Animal Selection and Reproduction (CERSYRA) located in Valdepeñas (Spain). The thawing procedure was conducted at laboratories from Group of Biology of Reproduction in Albacete (Spain).

Chemicals were of reagent grade and were purchased from Sigma (Madrid, Spain). Biladyl® and Andromed® were purchased from Minitüb (Tiefenbach, Alemania). Fluorescence probes were purchased from Invitrogen (Barcelona, Spain), except for propidium iodide (PI) which was purchased from Sigma. Chromatographically purified acridine orange was purchased from Polysciences Inc. (Warrington, PA, USA).

2.3.2 Semen collection

For each male, the collection of ejaculates was performed first using artificial vagina (AV) and later by electroejaculation (EE), both on the same day. Males were trained to conduct collections by AV. Ejaculates were routinely collected once per week. The procedure of EE was carried out using the protocol described by Garde *et al.* (2003). Males were sedated with xylacine (0.2mg/kg Rompun® 2% i.m.; Bayer S.A., Barcelona, Spain), then the rectum was cleaned of faeces and the prepucial area was shaved and washed with physiologic saline serum. For EE, we used a three electrode probe connected to a power source that allowed voltage and amperage control (P.T. Electronics, Boring, OR, USA). Probe diameter, probe length and electrode length was 3.2, 35.0 and 6.6 cm, respectively. The EE regime consisted of consecutive series of 5-s pulses of similar voltage, each separated by 5-s break. Each series consisted of a total of four pulses. The initial voltage was 1 V and was increased in each series until a maximum of 5 V. Two ejaculates per buck and collection method were obtained during the breeding (July to December) and non-breeding season (January to June), on a weekly basis.

2.3.3 Evaluation of ejaculates

Immediately after the collection of ejaculates, volume, sperm concentration, wave motion, subjective sperm motility (SM) and the percentage of spermatozoa with intact acrosomes (NAR) were determined. The volume of the ejaculates was measured in a conical graduated tube. Sperm concentration was calculated using a spectrophotometer. Spermatozoa total number (STN) was calculated with the volume and concentration (Volume × Concentration). Wave motion was assessed from fresh non-diluted semen and it was scored on a scale of 0 to 5 being 0: no movement and 5: strong wave movement. The percentage of motile spermatozoa was estimated from semen diluted (1:200) in a phosphate buffer saline (PBS) and evaluated subjectively using a phase-contrast microscope (100×) in samples previously incubated for 5 min at 37 °C. Samples fixed in buffered 2% glutaraldehyde solution were used to assess NAR by observing spermatozoa using phase-contrast microscopy (400×).

2.3.4 Semen cryopreservation

Semen from each male was aliquoted and used to conduct the different studies. One aliquot was washed with PBS (1:9) at 1200 × *g* for 10 min at room temperature and seminal plasma was removed. After that, washing and non-washing sperm samples were split in three aliquots and diluted with three different extenders: two commercial extenders, Biladyl® (20% egg yolk, using clarified egg yolk, and 7% glycerol) and

Andromed® (soybean-based extender with 7% glycerol) and a skim milk-based extender (7% glycerol) prepared as described by Corteel (1974).

Semen dilution was performed by the two-step dilution method for Biladyl® and skim milk-based extender. First, non-glicerolated fraction was added at 30 °C and the diluted semen was cooled to 5 °C for 2 h. Then, it was further diluted (v:v) with fraction containing glycerol. The diluted sperm samples were then held at 5 °C for 2 h more before freezing (total refrigeration time at 5 °C was 4 h). For Andromed®, samples were diluted in only one step at 30 °C and then cooled to 5 °C for 4 h. At the end of this time, the diluted semen was loaded into 0.25 mL plastic straws and frozen. Straws were frozen over nitrogen vapours for 10 min, 4 cm above the nitrogen level, plunged and stored in liquid nitrogen. Sperm samples were frozen to a final concentration of 140-200 × 10^6 spermatozoa/mL.

2.3.5 Thawed sperm evaluation

Thawing was performed by dropping the straws in a water bath at 37 °C for 30 s and pouring the semen in dry tubes and incubated for 5 min at the same temperature. For each male, season, method of semen collection, washing or non-washing process and freezing extender, one straw was thawed and analyzed.

Frozen-thawed spermatozoa were evaluated for SM and NAR, similar than for fresh semen. Motility was also evaluated by Computer Assisted Semen Analysis (CASA) using the Sperm Class Analyzer software (SCA® 2002, Microptic, Barcelona, Spain). Sperm samples were diluted (1:1) with TALP-HEPES medium (87 mM NaCl, 3.1 mM KCl, 2 mM CaCl$_2$, 0.4 mM MgCl$_2$, 0.3 mM NaH$_2$PO$_4$, 40 mM HEPES, 21.6 mM sodium lactate, 1 mM sodium pyruvate, 50 µg/mL kanamycin, 10 µg/mL phenol red, and 6 mg/mL bovine serum albumin (BSA) (pH 7.5)) and 5 µL were put on a Makler chamber recording the characteristics of sperm motility. The following motility parameters were assessed: velocity according to the straight path (VSL, µm/s), amplitude of lateral head displacement (ALH, µm) and linearity index (LIN, %).

Also, aliquots of thawed semen were used to conduct flow cytometry analysis. We assessed the membrane stability with YO-PRO-1, the mitochondrial membrane potential with Mitotracker Deep Red and the viability with propidium ioide (PI) (Martínez-Pastor *et al.*, 2008). A staining solution using TALP-HEPES was prepared by adding 50 nM YO-PRO-1 (stock: 100 µM in DMSO) and 15 µM propidium iodide (stock: 7.5 mM in Milli-Q water). We diluted 20 µL of sample in 0.5 mL of staining solution in polypropylene tubes for flow cytometry. The tubes were allowed to rest for 15 min in the dark. Mitochondrial membrane potential of the samples were

evaluated with 0.1 μM YO-PRO-1 and 100 nM Mitotracker Deep Red solution (stock: 1 mM in DMSO) in TALP-HEPES. Mitotracker Deep Red is a far red-fluorescent dye (abs/em ~640/662 nm) which stains mitochondria in live cells and its accumulation is dependent upon membrane potential. Sperm samples were diluted to 1×10^6 spermatozoa/mL, and 300 μL were transferred to a polypropylene tube and left in the dark for 30 min. After incubation times, sperm samples were analyzed using a Cytomics FC500 flow cytometer (Beckman coulter Inc. USA).

YO-PRO-1-/PI- spermatozoa were considered as intact spermatozoa (indicating live spermatozoa with intact plasmalemma), YO-PRO-1+/PI- as apoptotic spermatozoa (indicating live spermatozoa with altered plasmalemma) and Mitotracker+/YO-PRO-1- as viable spermatozoa with active mitochondria, as described by García-Álvarez *et al.* (2010).

Chromatin stability was assessed by using the SCSA® (Sperm Chromatin Structure Assay) technique (SCSA® Diagnostics Inc., Brookings, SD, USA) (Evenson and Jost, 2000). This technique is based in the susceptibility of the sperm DNA to acid-induced denaturation *in situ* and the metachromatic stainig Acridine Orange (AO). This stain fluoresces green when combined with double-stranded DNA, and red when combined with single-stranded DNA (denaturated). Thawed spermatozoa were diluted with TNE buffer to 2×10^6 cells/mL. Samples were flash frozen in LN_2 and stored at -80 °C until analysis. We calculated the DNA fragmentation Index (DFI) for each spermatozoon as the ratio of red fluorescence respect to total fluorescence (red + green). High values of DFI, indicates chromatin abnormalities. We calculated %DFI, as the percentage of spermatozoa with DFI > 25.

2.3.6 Statistical Analysis

The R statistical environmental (R development Core Team, 2010) was used. Numeric results are expressed as the mean ± standard error of the mean (SEM). Data were considered statistically significant when $P \leq 0.05$.

For the analysis, a mixed effects model with males as random effect was conducted. Effects of the method of collection (AV compared to EE) and season (breeding compared to non-breeding) on the quality of the ejaculates were studied. Differences on sperm quality after thawing between collection methods (AV compared to EE), seasons (breeding season compared to non-breeding season), freezing extenders (Biladyl®, Andromed® and skim milk-based extender) and pre-treatments before freezing (washing compared to non-washing), together with the analysis of interactions: season × pre-treatment, collection method × season, collection method ×

freezing extender, collection method × pre-treatment, freezing extender × pre-treatment were evaluated. When interactions resulted no significant, they were removed from the analysis. Multiple comparisons analyses were carried out using the Bonferroni adjustment.

2.4 Results

The method of collection influenced ($P \le 0.05$) the volume and sperm concentration of ejaculates, showing those collected by AV the lesser volume and greatest sperm concentration (Table 2.1). At thawing, those ejaculates collected by AV had greater values ($P \le 0.05$) for SM and NAR, and no significant differences were found between both methods of collection for any motility parameter evaluated by CASA (Table 2.2). Ejaculates collected by AV also had greater values ($P \le 0.05$) for the percentage of intact spermatozoa and the percentage of spermatozoa with active mitochondria (Table 2.3). However, % DFI was lower for sperm samples collected by AV in relation to those collected by EE.

On fresh semen, the season of collection had an effect on the percentage of motile spermatozoa, with greater values ($P \le 0.05$) on the non-breeding season (Table 2.1). After thawing, the season influenced the NAR and LIN values ($P \le 0.05$), showing those ejaculates collected during breeding season greater percentages of NAR and lesser values of LIN (Table 2.2). Moreover, the percentages of intact spermatozoa and spermatozoa with active mitochondria assessed by means of flow cytometry were greater ($P \le 0.05$) when ejaculates were obtained during this breeding season (Table 2.3).

Regarding to the extender used for cryopreservation, semen samples diluted with Biladyl® or Andromed® showed similar results on the quality of thawed semen. Thus, these samples had greater values ($P \le 0.05$) for the percentage of motile spermatozoa assessed subjectively, NAR, ALH, and the percentage of intact spermatozoa and spermatozoa with active mitochondria than for those samples diluted with skim-milk based extender (Tables 2.2 and 2.3). The latter samples had greater values ($P \le 0.05$) for the VSL and the LIN parameters.

Washing of sperm samples before diluting with freezing extenders did not influence ($P \ge 0.05$) on sperm quality after thawing (Tables 2.2 and 2.3).

Table 2.1 Effects of method of collection and season on the characteristics of ejaculates of the Blanca-Celtibérica bucks (n = 7).

	Ejaculate characteristics				
	Volume (mL)	Concentration (x 10^6 spermatozoa/mL)	STN (x 10^6 spermatozoa)	SM (%)	NAR (%)
Collection Method					
AV	1.1 ± 0.2^b	2487.2 ± 474.4^a	2498.0 ± 474.4	82.2 ± 1.7	88.2 ± 1.3
EE	2.0 ± 0.3^a	1163.0 ± 274.2^b	2055.5 ± 493.3	84.5 ± 1.7	89.6 ± 1.3
Season					
Breeding	1.2 ± 0.1	2024.0 ± 154.1	2334.0 ± 257.5	76.8 ± 1.8^b	93.4 ± 0.8
Non-Breeding	1.7 ± 0.2	2328.0 ± 230.1	2868.0 ± 290.4	86.9 ± 1.0^a	95.2 ± 0.8

Data are means ± SEM. For each factor, different superscripts within a column differ ($P \leq 0.05$). AV: artificial vagina; EE: electroejaculation; STN: total number of spermatozoa; SM: sperm motility; NAR: percentage of spermatozoa with intact acrosome.

Table 2.2 Effects of method of collection, season, freezing extender and pre-treatment on subjective sperm motility, acrosomal integrity and motility evaluated by CASA of thawed semen on the Blanca-Celtibérica bucks (n = 7).

	Sperm parameters				
	SM (%)	NAR (%)	VSL (µm/s)	LIN (%)	ALH (µm)
Collection Method					
AV	43.4 ± 3.9^a	65.1 ± 4.0^a	58.0 ± 2.2	55.4 ± 1.3	2.6 ± 0.1
EE	24.5 ± 4.2^b	56.4 ± 4.4^b	57.1 ± 3.0	56.9 ± 1.6	2.4 ± 0.1
Season					
Breeding	34.6 ± 4.1	86.0 ± 4.3^a	59.6 ± 2.7	54.5 ± 1.5^b	2.6 ± 0.1
Non-Breeding	33.3 ± 4.1	35.6 ± 4.3^b	55.4 ± 2.6	57.9 ± 1.5^a	2.4 ± 0.1
Freezing extender					
Biladyl®	45.3 ± 4.3^a	61.3 ± 4.5^{ab}	54.8 ± 2.6^b	55.4 ± 1.5^b	2.5 ± 0.1^a
Andromed®	38.8 ± 4.4^a	67.6 ± 4.4^a	52.1 ± 2.8^b	52.3 ± 1.6^b	2.8 ± 0.1^a
Skim milk	17.7 ± 4.4^b	53.5 ± 4.4^b	65.6 ± 3.3^a	60.9 ± 1.8^a	2.2 ± 0.1^b
Pre-treatment					
Washing	35.7 ± 4.1	58.9 ± 4.2	60.2 ± 2.6	56.5 ± 1.5	2.6 ± 0.1
Non-washing	32.1 ± 3.9	62.6 ± 4.2	54.8 ± 2.5	55.9 ± 1.4	2.4 ± 0.1

Data are means ± SEM. For each factor, different superscripts within a column differ ($P \leq 0.05$). AV: artificial vagina; EE: electroejaculation; SM: sperm motility; NAR: percentage of spermatozoa with intact acrosome; VSL: velocity according to the straight path; LIN: linearity index; ALH: amplitude of lateral head displacement.

Table 2.3 Effects of method of collection, season, freezing extender and pre-tratment on post-thaw membrane integrity and DNA fragmentation index evaluated by flow cytometry of thawed semen on the Blanca-Celtibérica bucks (n = 7).

	Sperm parameters			
	YO-PRO-1/PI- (%)	YO-PRO-1+/PI- (%)	Mitotracker+/YO-PRO-1- (%)	% DFI
Collection method				
AV	22.4 ± 2.7ᵃ	8.8 ± 0.9ᵃ	20.0 ± 2.7ᵃ	3.2 ± 0.5ᵇ
EE	14.9 ± 2.8ᵇ	4.4 ± 0.9ᵇ	9.6 ± 2.8ᵇ	6.1 ± 0.6ᵃ
Season				
Breeding	23.0 ± 2.8ᵃ	4.4 ± 0.9ᵇ	19.0 ± 2.8ᵃ	4.8 ± 0.6
Non-breeding	14.3 ± 2.7ᵇ	8.9 ± 0.9ᵃ	10.6 ± 2.8ᵇ	4.5 ± 0.6
Freezing extender				
Biladyl®	25.6 ± 2.8ᵃ	6.2 ± 0.9ᵇ	20.0 ± 2.8ᵃ	4.7 ± 0.6
Andromed®	20.2 ± 2.8ᵇ	8.4 ± 1.0ᵃ	16.0 ± 2.9ᵃ	3.8 ± 0.6
Skim milk	10.1 ± 2.8ᶜ	5.2 ± 0.9ᵇ	8.4 ± 2.9ᵇ	5.5 ± 0.7
Pre-treatment				
Washing	17.3 ± 2.7	7.2 ± 0.9	13.5 ± 2.8	4.8 ± 0.6
Non-washing	20.0 ± 2.7	6.1 ± 0.9	16.1 ± 2.8	4.5 ± 0.5

Data are means ± SEM. For each factor, different superscripts within a column differ ($P \leq 0.05$). AV: artificial vagina; EE: electroejaculation; YO-PRO-1-/PI-: live spermatozoa with intact plasmalemma; YO-PRO-1+/PI-: apoptotic spermatozoa; Mitotracker+/PI-: viable spermatozoa with active mitochondria; %DFI: DNA fragmentation index.

The interaction, season × pre-treatment was significant for SM, VSL and LIN (Fig. 2.1). Thus, the washing procedure before freezing improved these sperm parameters when the semen was collected during non-breeding season. No other interactions resulted significant for any of the sperm parameters studied.
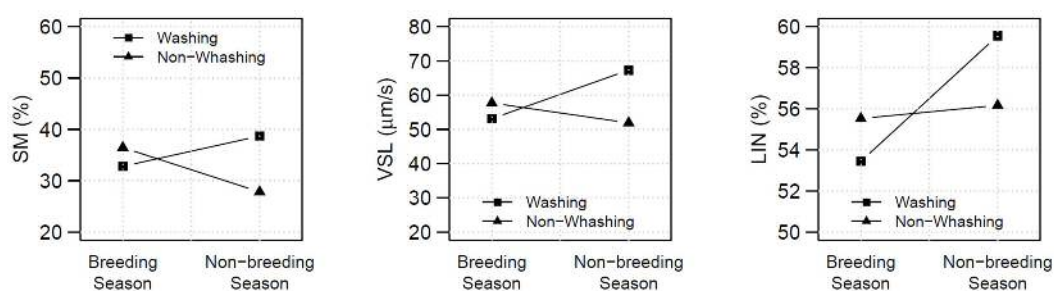
Figure 2.1 Mean values for SM (sperm motility, %), VSL (velocity according to the straight path, μm/s) and LIN (linearity index, %) in thawed smen for the interaction between pre-treatment (Washing *vs.* non-washing before dilution) and season in the Blanca-Celtibérica bucks (N = 7).

2.5 Discussion

In the present research, effects of semen collection method, season in which the semen was collected, freezing extender and pre-treatment before cryopreservation, on

post-thaw motility and membrane integrity evaluated by CASA and flow cytometry, respectively, were studied in Blanca Celtibérica bucks.

Electroejaculation allows collect ejaculates in animals whose training for AV is difficult. However, this method could lead to different ejaculates from those obtained by physiological way and could influence on the response of spermatozoa to different procedures such as cryopreservation. Results from the present study showed that ejaculates obtained by EE had a greater volume and lesser concentration in relation to those collected by AV, while no significant differences were found for SM and NAR between both collection methods for fresh semen. These results are similar to those to reported by Memon *et al.* (1986) and Marco-Jiménez *et al.* (2005, 2008) in bucks and rams, respectively. A greater volume for the ejaculates collected by EE could be due to contribution of accessory sex glands because of electrical stimuli as suggested by Mattner and Volgmayr (1962). In general, no significant differences were observed on the characteristics of the ejaculates regardless of the season of collection. These results agree with Pérez and Mateos (1996) in Malagueña bucks. Only those ejaculates collected during the non-breeding season had a greater percentage of motile spermatozoa, although differences among seasons were low and in both seasons the percentage of motile spermatozoa was high.

After freezing, results from the present study indicated semen samples from ejaculates collected by AV had greater values for most assessed sperm parameters than those obtained by EE, except for % DFI. These results agree with those reported by Quinn *et al.* (1968) where ram spermatozoa collected by AV were more resistant to cold shock than those obtained by EE. However, other studies provided evidence that sperm quality after freezing was greater for those sperm samples obtained by EE in the same species (Marco-Jiménez *et al.*, 2005). It is possible that the EE procedure changes the secretory function of one or more accessory glands so that could influence the amount of fluid produced and the chemical composition of the seminal plasma. This event could modify the survival to freezing of sperm samples obtained by this method. Marco-Jiménez *et al.* (2008) showed that seminal plasma from ram sperm samples obtained by EE was different for the Na^+ concentration in relation to AV, with greater values of this ion for semen collected by EE. Also, in this previous study there were differences in the protein profile in samples obtained by both methods (Marco-Jiménez *et al.*, 2008). Protein composition of seminal plasma has an important function for preventing the cold-shock sperm membrane damage (Barrios *et al.*, 2005). In addition, in the seminal plasma there are substances that protecting to spermatozoa against to oxidative stress (Wat-sum *et al.*, 2006). It is possible, that the concentration of these substances are altered in semen obtained by EE and it could be the reason for

greater DNA damage in these samples because of the relation between oxidative stress and sperm DNA damage (Aitken and Koopers, 2011).

Regarding to season of collection of the ejaculates, those samples which were obtained during the breeding season had more positive results for some parameters evaluated after thawing. These results agree with other studies conducted with other species of small ruminants (D´Alessandro and Martemucci, 2003). Coloma *et al.* (2011) showed that spermatozoa from Spanish Ibex collected during the winter (January to March) survived to freezing-thawing to a greater extent in relation to those collected in spring (April to June). The improved fertilisation potential of the frozen-thawed ram spermatozoa collected during the breeding season has been related to changes in total protein and protein composition of seminal plasma (Smith *et al.*, 1999). This suggests that seminal plasma proteins may influence seasonal resistance of the spermatozoa to freeze-thaw damage. Thus, La Falci *et al.* (2002) showed that sperm samples supplemented with proteins from buck seminal plasma obtained during the non-breeding season led to deterioration of sperm quality, while other studies have demonstrated the positive effect on sperm quality after thawing when the semen was supplemented with proteins or seminal plasma collected during breeding season (Leahy *et al.*, 2010).

The extender composition is crucial for spermatozoa protection against cryopreservation. In the present study, three extenders were compared: two commercial extenders, Biladyl® (egg-yolk based extender) and Andromed® (soybean based extender), and a skim milk-based extender. Results of the present study indicated greater values for SM and NAR when the semen was cryopreserved with Biladyl® or Andromed®. These results are consistent with those obtained by Sariözkan *et al.* (2010) who found similar values for SM and NAR in sperm samples cryopreserved with Triladyl® (egg-yolk based extender) or Bioxcell® (soybean based extender). Also, Chauhan and Anand (1990) found that semen from bucks frozen with a Tris-egg yolk based extender had greater values of sperm quality in relation to that frozen with a skim milk-egg yolk based extender. In relation to sperm parameters evaluated by flow cytometry in the present study, lower values of intact spermatozoa and spermatozoa with active mitochondria were found in samples cryopreserved in milk-based extender in relation to that cryopreserved with Biladyl® or Andromed® with the greatest value for intact spermatozoa for the Biladyl®. Similar results were reported by Chauhan and Anand (1990) when the viability was subjectively evaluated in samples frozen in egg-yolk and skim milk based extenders. However, in our study there were greater values for VSL and LIN in sperm samples cryopreserved in skim-milk based extender. Dorado *et al.* (2007) found greater values for objective motility

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parameters in samples frozen in an egg-yolk based extender in relation to a milk-based extender, except for LIN which had a greater value in a milk based extender as was found in the present study. Results from our study could be due to the lesser viscosity of the skim milk-based extender, which could offers less resistance to the movement of spermatozoa. Viscosity in the medium surrounding the spermatozoa affects the pattern of sperm motion (Rikmenspoel, 1984).

In the present study there were not differences for DNA stability between the three extenders used. However, in other species such as cattle, an egg-yolk based extender offered more protection against sperm DNA damage in relation to a skim milk based extender (Waterhouse *et al.*, 2010).

Results from the present study, therefore, provide evidence that sperm quality was affected less when buck ejaculates were cryopreserved with an extender with egg yolk in relation to soybean or skim-milk based extenders. It is possible, that the detrimental effect of the egg yolk based extender was less because, clarified egg yolk was used instead of whole egg yolk. The procedure of centrifugation used for obtaining clarified egg yolk might remove substances harmful for spermatozoa frozen in egg yolk based extenders. Another possibility is that phospholipids of egg yolk were not hydrolyzed by enzymes of seminal plasma (phospholipase A_2 and lysophospholipase), as has been shown by Chauhan and Anand (1990).

In our study effects of seminal plasma removal (washing) on sperm parameters at thawing was also studied. In several previous studies, it has been suggested the washing procedure of goat semen is a beneficial process because it avoids that EYCE and protein SBUIII acts (Kozdrowski *et al.*, 2007; Machado and Simplicio, 1995). In the present study there were no differences between washing and non-washing samples. Moreover, washing is a complex-time consuming process, and also causes some loss of spermatozoa (Ritar, 1993). Nevertheless, the positive effect of washing can depend on the season of the year and the freezing extender used (Cabrera *et al.*, 2005; Sariözkan *et al.*, 2010). We did not find interaction between pre-treatment (washing or non-washing) and the freezing extender for all sperm parameters assessed, being independent the washing effect from the diluents of cryopreservation used. However, in the present study there was a significant interaction between pre-treatment and season for SM, VSL and LIN. Thus, values for these sperm parameters were greater when samples were washed in the non-breeding season. Cabrera *et al.* (2005) observed similar results, showing that samples collected during spring (non-breeding season) had greater cryosurvival when the washing procedure was performed and samples frozen during winter (breeding season) had greater cryosurvival when the

washing procedure was not performed. Phospholipase activity is greater during non-breeding season (Ritar and Salamon, 1991) and it is possible that the washing procedure removes proteins which have a detrimental activity.

In conclusion, results of the present study suggest that the success of the semen cryopreservation in Blanca-Celtibérica goat depends on the semen collection method, breeding season in which the ejaculates are collected and extender used. Sperm quality after cryopreservation is greater when ejaculates were obtained by AV and are collected during the breeding season. Also, there are more desirable results for most sperm parameters assessed after thawing when Biladyl® or Andromed® are used as freezing extenders as compared to a skim milk based extender, irrespective of semen collection method. Moreover, the washing procedure did not show any improvement on post-thawing sperm quality.

CHAPTER 3

Single layer centrifugation (SLC) improves sperm quality of cryopreserved Blanca-Celtibérica buck semen.

3.1 Abstract

The aim of the present study was to evaluate the effect of sperm selection by means of single layer centrifugation (SLC) on sperm quality after cryopreservation, either when SLC is used before freezing or after thawing, using Blanca-Celtibérica buck semen collected by electroejaculation (EE). Ejaculates from six bucks were collected by EE and divided into two aliquots. One of them (unselected) was diluted with Biladyl® by the two-step method and frozen over nitrogen vapor. The other aliquot was selected by the SLC technique and subsequently frozen in the same way as the unselected samples (SLC before freezing). In a further treatment, two unselected straws were thawed and SLC was carried out (SLC after thawing). At thawing, sperm motility of all samples ((i) unselected; (ii) selected before freezing and (iii) selected after thawing) was evaluated by CASA. In addition, integrity of the plasma membrane, mitochondrial membrane potential, ROS production and DNA fragmentation index were assessed by flow cytometry. Most of the sperm parameters were improved ($P \leq 0.001$) in samples selected by SLC after thawing in relation to unselected or selected by SLC before freezing. The percentage of progressive motile spermatozoa was greater (86%) for sperm samples selected after thawing compared with unselected (58%) or selected before freezing (54%). Moreover, percentages of spermatozoa with intact plasma membrane and spermatozoa with high mitochondrial membrane potential (hMMP) were also greater for sperm samples selected after thawing compared to sperm samples unselected or selected before freezing (spermatozoa with intact plasma membrane: 80% *vs.* 32% *vs.* 12%; spermatozoa with hMMP: 54% *vs.* 1% *vs.* 15%; respectively). Therefore, sperm quality after cryopreservation is improved in Blanca-Celtibérica buck ejaculates collected by EE when a sperm selection technique such as SLC is carried out after thawing.

Keywords: Blanca-Celtibérica buck; Electroejaculation; Single layer centrifugation; Cryopreservation.

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3.2. Introduction

The Blanca-Celtibérica goat is an autochthonous breed from Spain considered to be endangered. Endangered breeds must be preserved by conservation methods, one of which is the development of genetic resource banks. In genetic resource banks, gametes and embryos are cryopreserved allowing genetic resources to be stored indefinitely (Watson and Holt, 2001).

Semen from domestic males is usually collected by artificial vagina (AV) (Leboeuf *et al.*, 2000), but this technique requires a previous training period (Wulster-Radcliffe *et al.*, 2001). Electroejaculation (EE) is another method of semen collection which is an alternative when males are not trained for an AV, or for wild species, an may be a viable method of repeatedly collecting semen from the same individuals without resulting in the death of the animal (Santiago-Moreno *et al.*, 2009).

The Blanca-Celtibérica goat breed is bred and reared in extensive systems. In the case of animals breeding in the field, different strategies are needed instead of routine reproductive techniques. In these cases, EE could be used as an alternative collection method to obtain semen because of the difficulty of training animals to use an AV under field conditions. To date, the majority of studies carried out for cryopreserving buck sperm have used samples collected by AV; few of them have used other semen collection methods in this species. Some authors have reported, in various species, higher volume and lower concentration when ejaculates are collected by EE (Marco- Jiménez *et al.*, 2005; Marco- Jiménez *et al.*, 2008; Memon *et al.*, 1986), which could be due to an increased contribution from the accessory sex glands because of electrical stimuli (Mattner and Voglmayr, 1962). Moreover, the EE procedure changes the secretory function of one or more accessory glands modifying the composition of the seminal plasma, as has been demonstrated in other species (Marco- Jiménez *et al.*, 2008). It is possible that this fact could affect the cryosurvival of sperm samples obtained by EE. The only study in goats which compared the effect of semen collection method (AV *vs.* EE) on sperm cryopreservation demonstrated lower sperm quality after cryopreservation for samples obtained by EE in relation to those obtained by AV (Jiménez-Rabadán *et al.*, 2012a).

In addition, there is a specific problem for buck semen cryopreservation when samples are extended in diluents containing egg yolk. Seminal plasma has been shown to contain a phospholipase secreted by the accessory bulbourethral gland which catalyses the hydrolysis of lipids in egg yolk to fatty acids and lysophospholipids, which are toxic to spermatozoa (Aamdal *et al.*, 1965; Iritani and Nishikawa, 1963; Sias *et al.*, 2005). Many studies have reported that a washing procedure for removing the seminal

plasma from goat ejaculates is necessary to increase sperm quality after freezing and thawing (Kozdrowski *et al.*, 2007; Machado and Simplicio, 1995), although Jiménez-Rabadán *et al.* (2012a) showed no improvement on sperm quality after cryopreservation in Blanca-Celtibérica buck when semen samples were washed.

Sperm selection methods have been used in order to remove seminal plasma or extenders from the spermatozoa, and also to enrich the amount of cells with normal morphology and/or motility for their later use in the different reproductive techniques (Hollinshead *et al.*, 2004; Mortom *et al.*, 2006). Recently, a sperm selection technique using single layer centrifugation (SLC) has been reported, with the ability to select effectively good quality spermatozoa with a shorter preparation time and less complicated process than the conventional density gradient centrifugation (DGC) (Morrell and Rodríguez-Martínez, 2009). In this SLC technique, spermatozoa are centrifuged through a column (single layer) of glycidoxypropyltrimethoxyl silane-coated silica in a species-specific formulation (Androcoll®), resulting in the selection of motile, morphologically normal spermatozoa with intact membranes and good chromatin integrity (Morrell *et al.*, 2009a; Morrell *et al.*, 2009b). SLC selected a sperm sub-population with high quality and fertility from frozen-thawed stallion and bull semen (Macías- García *et al.*, 2009; Thys *et al.*, 2009). Since semen collected by EE is considered to have a higher amount of seminal plasma and with a different composition compared to semen collected by an AV (Marco-Jiménez *et al.*, 2005; Marco-Jiménez *et al.*, 2008), and if seminal plasma from buck males is detrimental in egg-yolk based freezing extender, the use of SLC to separate the spermatozoa from seminal plasma, in semen collected by EE could improve the sperm parameters after cryopreservation.

With this background, the effect of sperm selection by means SLC through Androcoll-B (a colloid for bull spermatozoa) on goat sperm quality after cryopreservation was studied, either when the SLC was used before freezing or after thawing for semen samples collected by EE.

3.3. Materials and Methods

3.3.1 Animals

All animal procedures were performed in accordance with Spanish Animal Protection Regulations, RD 1201/2005, which conforms to European Union Regulation 2010/63. Six males of Blanca-Celtibérica goat breed (age > 1.5 years) were used. Males were maintained and managed at the Regional Center of Animal Selection and Reproduction located in Valdepeñas (Spain).

3.3.2 Semen Collection

Ejaculates from 6 males were collected by EE. The EE procedure was carried out using the protocol described by Garde *et al.* (2003). Males were anaesthetized with xylacine (0.2mg/kg Rompun® 2% i.m.; Bayer S.A., Barcelona, Spain), the rectum was cleaned of faeces, and the preputial area was shaved and washed with physiologic saline serum. A three electrode probe connected to a power source that allowed voltage and amperage control was used (P.T. Electronics, Boring, OR, USA). Probe diameter, probe length and electrode length were 3.2, 35.0 and 6.6 cm, respectively. The EE regime consisted of consecutive series of 5-s pulses of similar voltage, each separated by 5-s break. Each series consisted of a total of four pulses. The initial voltage was 1V which was increased in each series until a maximum of 5V. Contamination for urine was tested and ejaculates where urine was present were discarded. Ejaculates were collected weekly during the breeding season (September-December) with three ejaculates per buck being obtained for the present study.

3.3.3 Evaluation of ejaculates

Immediately after semen collection, the volume of the ejaculate (measured in a conical graduated tube), sperm concentration (by spectrophotometer) and wave motion (scored on a scale of 0-5) were evaluated. The percentage of motile spermatozoa (SM) was evaluated subjectively in aliquots of semen diluted (1:200) in a phosphate buffer saline (PBS) and incubated for 5 min at 37 °C, using a phase-contrast microscope (× 100). The percentage of spermatozoa with intact acrosomes (NAR) was determined in samples fixed in buffered 2% glutaraldehyde using phase-contrast microscopy (× 400), based on an evaluation of 100 spermatozoa.

3.3.4 Semen cryopreservation

After initial sperm evaluation, semen from each male was divided into two aliquots. One of them was used as control (unselected) and was diluted by the two-step dilution method with Biladyl® (Minitüb, Tiefenbach, Germany). First, the non-glycerolated fraction was added at 30 °C to $200\text{-}400 \times 10^6$ spermatozoa/mL and the diluted semen was cooled to 5 °C for 2 h, followed by addition of the fraction containing glycerol (v:v). The diluted sperm samples were then held at 5 °C for a further 2 h before freezing (total refrigeration time at 5 °C was 4 h). The diluted semen was loaded into 0.25 mL plastic straws and was frozen in nitrogen vapor for 10 min, by placing the straws 4 cm above the surface of liquid nitrogen. The straws were subsequently plunged into liquid nitrogen and stored. For the other aliquot, SLC technique was carried out (SLC before freezing). For this, 4 mL Androcoll-B® (Patent applied for;

SLU, Sweden) were pipetted into a centrifuge tube and an aliquot of extended semen (4 mL of semen diluted in PBS containing approximately 100×10^6 spermatozoa/mL) was layered on top. After centrifugation at 300 x g for 20 min, the supernatant and most of the colloid were discarded and the sperm pellet was transferred to a clean centrifuge tube and diluted for cryopreservation as described above for control samples. Sperm samples for both treatments (unselected and SLC before freezing) were frozen at a final concentration of $100\text{-}200 \times 10^6$ spermatozoa/mL (see Purdy, 2006). The straws remained in liquid nitrogen for a minimum period of 6 months before thawing was carried out.

3.3.5 Thawed semen evaluation

Thawing was performed by placing the straws in a water bath at 37 °C for 30 s, emptying the contents into dry tubes and incubating for 5 min at the same temperature. For the third treatment, two straws from control samples were thawed and used for the SLC technique, as described above (SLC after thawing). In addition, an unselected straw and a straw from the SLC before freezing were thawed. Thus, three different treatments were evaluated after thawing: (i) unselected samples (control); (ii) SLC before freezing; and (iii) SLC after thawing. The treatment SLC before freezing and after thawing yielded a low recovery rate (data not shown), thus the inclusion of this treatment was not considered to be practical in its present form and for this reason was not incorporated in this study. Modifications of SLC will be investigated in future studies for this treatment.

Frozen-thawed sperm samples from each treatment were evaluated for the parameters below described.

3.3.5.1 Sperm recovery after SLC

Different recovery rates were calculated to establish the effect of SLC. Thus, recovery yield and motile sperm recovery rate for each treatment were calculated as follows:

$$Recovery\ yield = \frac{C_{afterSLC} \times V_{afterSLC}}{C_{beforeSLC} \times V_{beforeSLC}} \times 100$$

$$Motile\ sperm\ recovery\ rate = \frac{C_{afterSLC} \times V_{afterSLC} \times TM_{afterSLC}}{C_{beforeSLC} \times V_{beforeSLC} \times TM_{beforeSLC}} \times 100$$

where,

C: concentration.

V: volume

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TM: percentage of motile spermatozoa.

3.3.5.2 Acrosome integrity (NAR)

The percentage of spermatozoa with intact acrosomes (NAR) was determined in samples fixed in buffered 2% glutaraldehyde using phase-contrast microscopy ($\times$ 400), based on an evaluation of 100 spermatozoa.

3.3.5.3 Computer Assisted Sperm Analysis (CASA)

CASA was performed using the SpermVision (Minitüb, Tiefenbach, Germany) on aliquots (6µL) of sperm samples. The aliquots were pipetted on to a warm glass slide (38 °C) and a cover slip was placed on top. The measurement was carried out on the warm stage (38 °C) of a microscope fitted with a video camera. Sperm motility was analysed using the Sperm Vision software and the following motility parameters were recorded: percentage of motile spermatozoa (TM, %), percentage of progressive motile spermatozoa (PM, %), straight line velocity (VSL, µm/sec), linearity index (LIN, %) and amplitude of lateral head displacement (ALH, µm).

3.3.5.4 Assessment of sperm membrane integrity

Sperm membrane integrity was evaluated using a combination of the fluorochromes SYBR-14 and propidium iodide (PI) (Live-Dead Sperm Viability Kit L-7011; Invitrogen, Eugene, OR, USA). Sperm samples were diluted with Buffer B (patent applied for; SLU, Uppsala) to 2×10^6 spermatozoa/mL and 0.6 µL of SYBR-14 (1:50 in Buffer B) and 3 µL of PI were added. The tubes were incubated in the dark at 38 °C for 10 min. Samples were analysed using a LSR flow cytometry (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with standard optics. From each sample, a total of 50,000 events were collected and quantified as percentages.

Three categories of spermatozoa could be described: live spermatozoa with an intact membrane (SYBR-14+/PI−), moribund (SYBR-14 intermediate/PI intermediate) and dead (SYBR-14+/PI+), according to the degree of intactness of the plasma membrane as described by Johannisson *et al.* (2009).

3.3.5.5 Assessment of sperm mitochondrial status

The lipophilic cation JC-1 was used to assess the mitochondrial status of spermatozoa. According to the manufacturer (Molecular Probes, Eugene, OR, USA), JC-1 reversibly changes its fluorescence from green (monomeric status) to orange (multimeric status) when mitochondrial membrane potential is high. Sperm samples were diluted with Buffer B to a concentration of 2×10^6 spermatozoa/mL and 1.2 µL of JC-1 stock

solution (3mM JC-1 in DMSO) were added. The tubes were incubated in the dark at 38 °C for 40 min. Samples were analysed on a LSR flow cytometry (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), equipped with standard optics. The proportion of orange-stained cells was recorded, being considered as a population of cells with high mitochondrial membrane potential (hMMP).

3.3.5.6 Analysis of ROS

For assessing intracellular ROS, spermatozoa were diluted in Buffer B to 2×10^6 spermatozoa/mL and 9 µL Hoechst 33258 (Sigma-Aldrich, stock solution 40µM in distilled water), 9 µL Hydroethidine (Invitrogen, stock solution 40µM in DMSO), 9 µL dichlorodihydrofluorescein diacetate H_2DCFDA (Invitrogen, stock solution 2 mM in DMSO) and 3 µL Menadione (Sigma-Aldrich, stock solution 20 mM in DMSO) were added. The H_2DCFDA is cleaved by cellular esterases being thus retained within the spermatozoa. Once oxidised by H_2O_2, it acquires an intense fluorescence, indicating presence of intracellular ROS. Hydroethidine is freely permeable to cells and is oxidized by $-O_2$ to ethidium which binds to DNA with fluorescent emission, thus providing a measurement of the superoxide radical in living and dead spermatozoa in the HE:HO dotplot. Spermatozoa were incubated for 30 min in the dark at 37 °C before being analysed. Samples were analysed on a LSR flow cytometry (Becton Dickinson Inmunocytometry Systems, San Jose, CA, USA). From each sample, a total of 50000 events were collected and quantified as percentages in the following seven categories: living superoxide negative; living superoxide positive; dead superoxide positive; living hydrogen peroxide negative; living hydrogen peroxide positive; dead hydrogen peroxide negative; dead hydrogen peroxide positive.

3.3.5.7 Assessment of sperm chromatin stability

Chromatin stability was assessed by metachromatic staining with Acridine Orange (AO), based on the susceptibility of the sperm DNA to acid-induced denaturation in situ. The metachromatic fluorescent dye AO shift from green (dsDNA) to red (ssDNA) fluorescence depending on the degree of DNA denaturation (Evenson and Jost, 2000).

Aliquots (50 µL) of the thawed sperm samples were mixed with an equal volume of Tris-sodium chloride-ethylene diamine tretraacetic acid buffer (TNE buffer; 0.01M Tris-HCl, 0.15M NaCl, 1mM EDTA; pH 7.4), followed by snap-freezing in liquid nitrogen and were then transferred to a -80 °C freezer, where they were stored until further processing and flow cytometry analysis.

Samples were thawed on crushed ice and were further diluted with TNE buffer (1:9, v/v). Acid-induced denaturation of DNA in situ was attained adding 0.2 mL of a low pH detergent solution containing 0.17% Triton X-100, 0.15 mol/L NaCl and 0.08 mol/L HCl, pH 1.2. After 30 s the cells were stained by adding 0.6 mL of AO (6 µg/mL in 0.1 mol/L citric acid, 0.2 mol/L Na_2HPO_4, 1 mmol/L EDTA, 0.15 mol/L NaCl, pH 6.0). Measurements were made on a FACStar Plus flow cytometer (Becton Dickinson Inmunocytometry Systems, San José, CA, USA) equipped with standars optics. AO was excited with an argon ion laser (Innova 90; Coherent, Santa Clara, CA, USA) at 488 nm, running at 200mW. The fluorescence stability of the flow cytometer was monitored daily using standard beads (Fluoresbrite plain YG 1.0 µmol/L; Polysciences, Warrington, PA, USA). Equivalent instrument settings were used for all samples. Data collection was carried out using CellQuest, version 3.3 (Becton Dickinson). Further calculations of the sperm chromatin structure assay parameters, DNA fragmentation index (DFI, %), calculated as the ratio of single-stranded DNA in the population (Evenson et al., 2002), were performed using FCS Express version 2 (De novo software, Thornhill, ON, Canada).

3.3.6 Statistical analysis

The statistical analysis was performed using SPSS for Windows v. 19 (SPSS Inc., Chicago, IL). Numeric results are expressed as the mean ± standard error of the mean (SEM). Data were considered statistically significant when $P \leq 0.05$.

For the analysis, a mixed effects model with males and replicate within male as random effects was carried out. We studied the effect of different treatments: i) unselected (control); ii) SLC before freezing; and iii) SLC after thawing, on sperm quality after cryopreservation. Multiple comparisons analyses were carried out using the Bonferroni adjustment.

3.4. Results

The sperm parameters evaluated in ejaculates showed an average volume of 1.28 ± 0.60 mL and a sperm concentration of 1380 ± 509 x 10^6 spermatozoa/mL. The values for SM and NAR were 70 ± 10% and 91 ± 4%, respectively.

The recovery rates after SLC are shown in Table 3.1. SLC after thawing showed a lower sperm recovery yield and motile sperm recovery rate than SLC before freezing.

Sperm motility parameters assessed by CASA and NAR are shown in Table 3.2 for different treatments. Differences ($P \leq 0.001$) among treatments were found for all

evaluated parameters except for ALH. We observed greater values ($P \leq 0.001$) when SLC after thawing was carried out in relation to unselected samples or when SLC before freezing was used.

Table 3.1 Sperm recovery rates after SLC in Blanca-Celtibérica buck semen.

	Recovery yield (%)	Motile sperm recovery rate (%)
Sperm selection method		
SLC before freezing	49.70 ± 6.01	52.68 ± 6.77
SLC after thawing	13.61 ± 2.62	17.60 ± 3.30

Data are means ± SEM. Recovery yield = (after SLC concentration x after SLC volume) / (before SLC concentration x before SLC volume) x 100; Motile sperm recovery rate: (after SLC concentration x after SLC volume x after SLC percentage of motile spermatozoa) / (before SLC concentration x before SLC volume x before SLC percentage of motile spermatozoa)x 100.

Table 3.2 Effects of sperm selection method on motility parameters assessed by CASA and NAR in frozen-thawed Blanca-Celtibérica buck semen.

	Sperm parameters					
	TM (%)	PM (%)	NAR (%)	VSL (µm/s)	LIN	ALH (µm)
Sperm selection method						
Unselected	67.46 ± 4.61[b]	58.19 ± 4.89[b]	85.12 ± 3.06[a]	55.61 ± 2.50[b]	0.57 ± 0.01[b]	3.15 ± 0.19
SLC before freezing	62.18 ± 4.40[b]	54.05 ± 4.68[b]	65.33 ± 2.97[b]	57.08 ± 2.42[b]	0.61 ± 0.01[b]	3.23 ± 0.18
SLC after thawing	86.45 ± 4.61[a]	80.08 ± 4.89[a]	93.05 ± 3.25[a]	76.64 ± 2.50[a]	0.66 ± 0.01[a]	2.81 ± 0.19

Data are means ± SEM. Different superscripts within a column differ significantly ($P = 0.001$). TM: percentage of motile speramatozoa; PM: percentage of progressive motile spermatozoa; NAR: intact acrosome; VSL: straight line velocity; LIN: linearity index; ALH: amplitude of lateral head displacement.

The results from flow cytometric analysis are shown in Fig. 3.1. The greatest proportion of spermatozoa with intact plasma membrane ($P \leq 0.001$) was for SLC carried out after thawing whereas the least was observed when SLC was carried out before freezing (Fig. 3.1A). The proportion of spermatozoa with hMMP was greater ($P \leq 0.001$) for samples which underwent SLC after thawing in relation to unselected samples or SLC before freezing (Fig. 3.1B). Intact spermatozoa containing greater ($P \leq 0.001$) SO- levels were found in samples treated with SLC after thawing or unselected compared with SLC before freezing (Fig. 3.1C), whereas no difference was found for H_2O_2 production among treatments (Fig. 3.1D).

Regarding DNA fragmentation index, a greater value ($P \leq 0.001$) for %DFI was found in SLC after thawing or unselected samples compared to SLC before freezing (Fig. 3.1E).
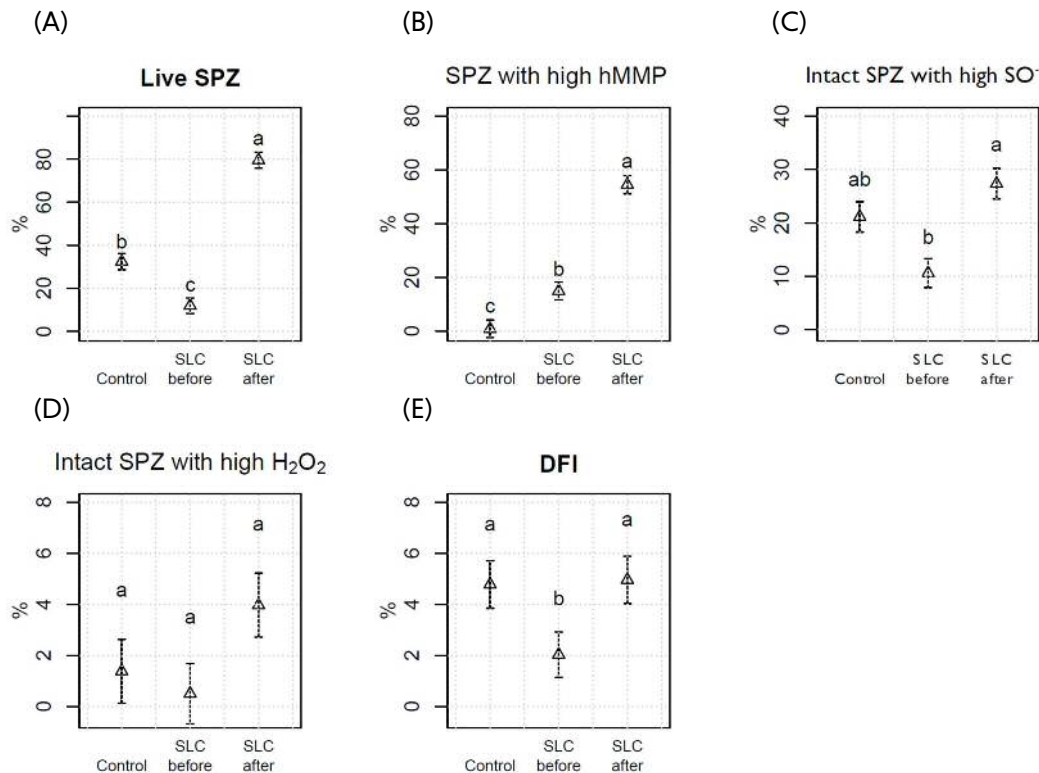
Figure 3.1 Effects of sperm selection method on parameters assessed by flow cytometry in frozen-thawed buck semen. Data are means ± SEM. Different superscripts on histograms show significant differences (P=0.001). hMMP: high mitochondrial membrane potential; DFI: DNA fragmentation index.

3.5. Discussion

The aim of this research was to improve the quality of cryopreserved spermatozoa in Blanca Celtibérica bucks by removing seminal plasma using a sperm selection technique (SLC) from ejaculates collected by EE. The amount and composition of the seminal plasma changed when EE was used for collecting semen (Marco-Jiménez *et al.*, 2005; Marco-Jiménez *et al.*, 2008). It has been showed that Blanca-Celtibérica buck ejaculates obtained by EE contained higher amount of seminal plasma than when collected by AV. Moreover, a detrimental effect on sperm quality was noticeable after cryopreservation for samples collected by EE, although no improvement was found when sperm samples were washed before freezing (Jiménez-Rabadán *et al.*, 2012a). It is possible that EE could modify sperm cryosurvival. We suggest that sperm selection techniques could efficiently remove the seminal plasma and thereby, the harmful effects when the semen is frozen in egg-yolk based diluents since the seminal plasma contain a phospholipase which catalyses the hydrolysis of lipids in egg yolk to fatty acids and lysophospholipids which are toxic to spermatozoa.

Sperm selection techniques through colloids separate spermatozoa from seminal plasma and select the sub-population of spermatozoa with good motility,

viability and chromatin integrity. During the process, extended semen is centrifuged through layers of colloids and cells move to the point in the gradient which matches their own density (Pertoft, 2000). By altering the centrifugation force and time, and the physical properties of the colloid, the spermatozoa with good quality are allowed to pellet in the bottom of the tube. Seminal plasma is retained on top of the colloid while the spermatozoa move to the bottom during centrifugation. Comparisons of some density gradient products have been made in several species with variable results (Samardzija *et al.*, 2006; Valcárcel *et al.*, 1996). A recent method, SLC, employs only one layer of colloid with species-specific formulations and produces sperm samples of equivalent quality to conventional DCG. The main advantages of SLC in relation to DGC are the shorter preparation time, the less complicated process and the possibility of processing larger volumes of semen (Morrell and Rodríguez-Martínez, 2009).

In the present study, the sperm recovery yield was greater for samples using SLC before freezing than SLC after thawing. These results agree with Chatdarong *et al.* (2010) who found greater sperm recovery using SLC before freezing than SLC after thawing although in their case, the sperm yield was less than in our results. The cryopreservation process caused loss of spermatozoa (Watson, 1979) and although it has been reported that about 40-60% of ram spermatozoa preserve their motility after thawing, only about 20-30% remain biologically undamaged (Salamon and Maxwell, 2000). The lower sperm yield found for SLC after thawing demonstrated that SLC successfully removed cryoinjured spermatozoa.

In this study, post-thaw sperm quality was evaluated and compared between treatments (unselected, SLC before freezing, and SLC after thawing). Better sperm quality after cryopreservation (greater values for motility, NAR and parameters evaluated by flow cytometry) was found when SLC was done after thawing. In contrast, Chatdarong *et al.* (2010) found a lower proportion of motile spermatozoa when SLC was used after thawing in relation to use it before freezing for cat epididymal spermatozoa. During cryopreservation, spermatozoa undergo ultrastructural and biochemical changes which could be responsible for a decrease in their functional integrity, *in vitro* survival and fertilising ability (Salamon and Maxwell, 2000). From results found in this work, it is suggested that SLC after thawing removed seminal plasma, debris and cryoinjured spermatozoa which did not withstand the cryopreservation procedure, or those that swim slowly and were unable to pass through the colloid layer. Thus, the quality of these selected spermatozoa is higher than unselected spermatozoa or those selected before freezing. On the other hand, several studies have reported that seminal plasma proteins are absorbed on to cold-

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shocked ram sperm surface and this adsorption is able to partially repair membrane alterations induced by cold shock (Barrios *et al.*, 2000; García-López *et al.*, 1996; Pascual *et al.*, 1994; Pérez-Pe *et al.*, 2001a; Pérez-Pe *et al.*, 2001b). It could explain low results found when SLC is carried out before freezing. It is possible that SLC removed seminal plasma but also some useful proteins in order to help spermatozoa to withstand cold shock (Barrios *et al.*, 2005). Thus, seminal plasma may contain beneficial as well as harmful substances, which may contribute to sperm cryosurvival. The beneficial/deleterious effects may be changed in samples collected by EE since this procedure modifies the secretory function of accessory sex glands. As an example, the beneficial effect of some proteins could overcome the harmful effects of other substances present in the seminal plasma, which could be the reason for a lower sperm quality in separating spermatozoa from seminal plasma before freezing.

Regarding ROS production, although no differences were observed for H_2O_2 production between treatments, a greater proportion of SO^--producing intact spermatozoa was found for unselected samples or in samples selected by SLC after thawing. A fundamental aspect of sperm physiology is their ability to generate ROS (Aitken et al., 2010). ROS are produced as by-products of metabolism and increased levels could be an indicator of high sperm metabolism. At physiological levels, free radicals such as H_2O_2 and SO^- control sperm maturation, capacitation and hyperactivation, the acrosome reaction and sperm-oocyte fusion (Kothari *et al.*, 2010). However, when ROS levels overwhelm the antioxidant defence system, pathological effect results (Kothari *et al.*, 2010). Thus, relationships between oxidative stress and loss of motility (Agarwal *et al.*, 2008; Aitken and Fisher, 1994; Tremellen, 2008) as well as DNA damage (Aitken *et al.*, 2010) have been described. The fact that no motility loss was observed in this study for samples selected after thawing suggests that ROS are generated at suitable levels and that the metabolically active spermatozoa were selected.

Analysis of DNA fragmentation revealed lower values for %DFI when SLC before freezing was carried out compared to SLC after thawing or unselected samples. Contrary to these results, Chatdarong *et al.* (2010) did not found differences for %DFI between samples selected before or after cryopreservation and an improvement was observed on %DFI in stallion using SLC after cryopreservation compared to unselected samples (Macías-García *et al.*, 2009). It should be pointed out that the %DFI values found in our study were quite low. Some authors (Didion *et al.*, 2009; Evenson and Wixon, 2006; Love, 2005) have reported %DFI thresholds that impact fertility in different species, although in all case the values were greater than those observed in our study.

We can conclude that for buck semen obtained by EE and submitted to sperm selection with SLC before freezing, the sperm quality after thawing is not different to unselected samples. However, when SLC is carried out after thawing, sperm quality is better than in unselected samples. Further studies are required in this species to determine the function of seminal plasma during freezing for samples obtained by EE and its impact on fertility.

CHAPTER 4

Improved cryopreservation protocol for Blanca-Celtibérica buck semen collected by electroejaculation

4.1 Abstract

The collection of sperm samples by electroejaculation (EE) leads to an increase of the production of seminal plasma which could modify the tolerance of spermatozoa to the cryopreservation procedure. This study aims to compare a standard sperm cryopreservation protocol for samples collected by artificial vagina (AV) with the same protocol and modifications to this for samples obtained by EE. Semen from six males of Blanca-Celtibérica goat breed was collected by AV (control) and EE, and three experiments were conducted. In Experiment 1, it was examined the effects of egg yolk concentration contained in freezing extender (0%, 1.5%, 10% and 20% of egg yolk); in Experiment 2, it was evaluated the cooling rate from 30 °C to 5 ° C (*fast*: 10 min and *slow*: 90 min) and the temperature of glycerol addition (30 °C and 5 °C); and in Experiment 3, it was examined the time of equilibration at 5 °C (0, 1, 2 or 3 hours). A heterologous *in vitro* fertilization test was carried out in order to compare the fertility of control samples with that resulting from the EE protocol which showed the highest sperm quality. Results showed greater sperm motility parameters after thawing for control samples cryopreserved in standard conditions in the three experiments. For samples collected by EE, extender with 20% egg yolk, a slow cooling rate and a longer equilibration time (3 h) provided higher sperm quality, and no differences were observed between temperatures of glycerol addition. Samples collected by EE and cryopreserved with the protocol which yielded the best sperm quality after thawing showed higher fertility compared to AV.

Keywords: Electroejaculation, Egg yolk, Cooling rate, Glycerol, Equilibration, Blanca-Celtibérica buck

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4.2. Introduction

The Blanca-Celtibérica goat is an autochthonous breed from Spain considered to be endangered (RD 2129/2008) and its conservation by genetic resource banks through semen cryopreservation is a key tool to ensure the long-term availability of genetic resources. Hence, the importance of defining optimal cryopreservation protocols in order to obtain the best post-thaw sperm characteristics.

It is important to note that the first step in the sperm cryopreservation is the collection of ejaculates. The electroejaculation (EE) is an alternative collection method to obtain semen in species or breeds which are bred and reared in extensive systems being necessary using different strategies instead of routine reproductive techniques. To date, the majority of studies carried out for cryopreserving buck semen have used samples collected by AV and few of them have used other semen collection methods. Differences on characteristics of ejaculates obtained by different collection methods have been observed in various species (Jiménez-Rabadán *et al.*, 2012a; Marco-Jiménez *et al.*, 2005; Marco-Jiménez *et al.*, 2008; Memon *et al.*, 1986) and it has been suggested that the EE varies from physiological ejaculation (Álvarez *et al.*, 2012). Thus, the electrical stimuli during the EE artificially stimulate the accessory glands causing an increase of volume (Mattner and Voglmayr, 1962), which results in greater amount of seminal plasma. In addition, differences on seminal plasma composition have been demonstrated between semen obtained by AV and EE in rams (Marco-Jiménez *et al.*, 2008) and it could affect to sperm membranes being spermatozoa more or less vulnerable to cryopreservation procedure (Álvarez *et al.*, 2012). One of the few studies which compared the effect of semen collection method (AV *vs.* EE) on sperm cryopreservation in goats reported lower quality after thawing for samples obtained by EE, suggesting a detrimental effect of seminal plasma on cryopreservation (Jiménez-Rabadán *et al.*, 2012a). This could be due to the presence in the seminal plasma of a phospholipase secreted by bulbourethral gland which catalyzes the hydrolysis of lipids in the egg yolk to fatty acids and lysophospholipids which are toxic to buck spermatozoa (Iritani and Nishikawa, 1963; Sias *et al.*, 2005) and this effect may be more marked in samples collected by EE. Hence, sperm samples collected by EE could require different freezing extenders and protocols to carry out a successful cryopreservation.

Although the sperm cryopreservation using different freezing extenders for samples from different origin has been studied for buck semen (Jiménez-Rabadán *et al.*, 2012a), few studies have been carried out on the effect of modifying other factors involved during the cryopreservation procedure. In order to avoid the negative effect

of seminal plasma in the buck sperm cryopreservation, most of studies made changes on the concentration of egg yolk contained in the freezing extender with different results, although most of them were conducted in samples collected by AV and few studies used the EE as method of semen collection (Aboagla and Terada, 2004; Cabrera *et al.*, 2005; Daskin and Tekin, 1996; Ritar and Salamon, 1991). In addition, other factors such as reducing the period of contact between spermatozoa and seminal plasma during the cooling and equilibration steps could report a beneficial effect on sperm cryopreservation for samples collected by EE.

With this background, the objective of this study was to examine the effect of different factors involved during the cryopreservation process, namely, egg yolk concentration, temperature of glycerol addition, cooling rate and equilibration time in samples collected by EE from Blanca-Celtibérica bucks to achieve samples with similar sperm quality than those obtained by AV. In addition to this, a heterologous *in vitro* fertilization test was carried out to evaluate the fertility of the control (collected by AV) and EE samples frozen with the best cryopreservation protocol.

4.3. Materials and Methods

4.3.1 Animals and Reagents

Animal handling was performed in accordance with Spanish Animal Protection Regulation, RD 1201/2005, which conforms to European Union Regulation 2010/63. Six males of Blanca-Celtibérica goat breed (age > 1.5 years) were used. All males were maintained and managed at the Regional Center of Animal Selection and Reproduction (CERSYRA) located in Valdepeñas (Spain). The thawing procedure was conducted at laboratories from SaBio group in Albacete (Spain).

Chemicals were of reagent grade and were purchased from Sigma (Madrid, Spain). Biladyl® was purchased from Minitüb (Tiefenbach, Alemania). Fluorescence probes were purchased from Invitrogen (Barcelona, Spain), except for propidium iodide (PI) which was purchased from Sigma. Chromatographically purified acridine orange was purchased from Polysciences Inc. (Warrington, PA, USA).

4.3.2 Semen Collection

All experiments were carried out during breeding season, from September to December. For each male, the collection of ejaculates was performed using AV and EE, both on the same day. Males were trained to conduct collections by AV and ejaculates were routinely collected once per week. The procedure of EE was carried out using the protocol described by Garde *et al.* (2003). Males were anesthetized with xylacine

(0.2mg/kg Rompun® 106 2% i.m.; Bayer S.A., Barcelona, Spain), the rectum was cleaned of faeces and the prepucial area was shaved and washed with physiologic saline serum. A three electrode probe connected to a power source that allowed voltage and amperage control was used (P.T. Electronics, Boring, OR, USA). Probe diameter, probe length and electrode length were 3.2, 35.0 and 6.6 cm, respectively. The EE regime consisted of consecutive series of 5-sec pulses of similar voltage, each separated by 5-sec break. Each series consisted of a total of four pulses. The initial voltage was 1 V which was increased in each series until a maximum of 5 V. Urine contamination was tested and ejaculates with urine were rejected. Three ejaculates per male and collection method were obtained for all experiments.

4.3.3 Evaluation of ejaculates

Immediately after the collection of ejaculates, the volume of ejaculate (measured in a conical graduated tube), sperm concentration (by spectrophotometer) and wave motion (scored on a scale of 0 to 5) were evaluated. Spermatozoa total number (STN) was calculated with the volume and concentration (Volume × Concentration). The proportion of motile spermatozoa (SM) was evaluated subjectively in aliquots of semen diluted (1:200) in a phosphate buffer saline (PBS) and incubated for 5 min at 37 °C, using a phase-contrast microscope (× 100).

4.3.4 Semen cryopreservation

Sperm samples were collected for each male by AV (control samples) and by EE from six males of Blanca Celtibérica breed in each experiment. For all experiments, the commercial extender Biladyl® was used. Egg yolk added to the extender was clarified as described by Holt *et al.* (1996b). Control samples were frozen by the standard conditions usually used for buck semen cryopreservation (Cseh et al., 2012). These conditions were: dilution in extenders containing 20% egg yolk, cooling to 5 °C for 90 min, addition of glycerolated fraction of extender at 5 °C and equilibration time for 2h.

4.3.4.1 Experiment 1: Effect of the concentration of egg yolk contained in freezing extender on sperm quality at thawing

Each ejaculate was divided into 4 aliquots and diluted with four extenders containing different egg yolk concentration: 0%, 1.5%, 10% and 20%. The dilution was performed by two-step method. Each aliquot was diluted with the Fraction A of diluent at 30 °C and cooled to 5 °C for 90 min. Then, it was further diluted (v:v) with Fraction B containing glycerol (7%) and maintained at 5 °C for 2 h more before freezing. At the end of this time, the diluted semen was loaded into 0.25 mL plastic straws and frozen in nitrogen vapours for 10 min by placing the straws 4 cm above the surface of liquid

nitrogen. The straws were subsequently plunged into liquid nitrogen and stored. Sperm samples were frozen at a final concentration of 100-200 × 10⁶ spermatozoa/mL.

Since results from this experiment showed Biladyl® with 20% egg yolk as the best extender to buck sperm cryopreservation, it was used in Experiments 2 and 3.

4.3.4.2 Experiment 2: Effect of cooling rate and temperature of glycerol addition on sperm quality at thawing

In the Figure 4.1 is showed the experimental design for experiment 2. Briefly, each ejaculate was diluted with the non-glycerolated fraction of Biladyl® (Fraction A) with 20% egg yolk at 30 °C and divided into four aliquots in order to evaluate two cooling rates: a *slow cooling rate* in which temperature dropped from 30 °C to 5 °C in 90 min and a *fast cooling rate* in which temperature dropped from 30 °C to 5 °C in 10 min, and two temperatures of glycerol addition (Fraction B) at 30 °C and at 5 °C. Samples in which the glycerolated fraction was added at 30 °C were cooled to 5 °C by means of fast and slow cooling rates, respectively. For samples which were cooled to 5 °C before adding glycerol, using both fast and slow cooling, the glycerol was added at 5 °C after finishing the cooling time. All aliquots were maintained at 5 °C for 2 hours more (equilibration time) and then the diluted semen was loaded into 0.25 mL plastic straws and frozen over nitrogen vapours as described in Experiment 1. Sperm samples were frozen at a final concentration of 100-200 × 10⁶ spermatozoa/mL.
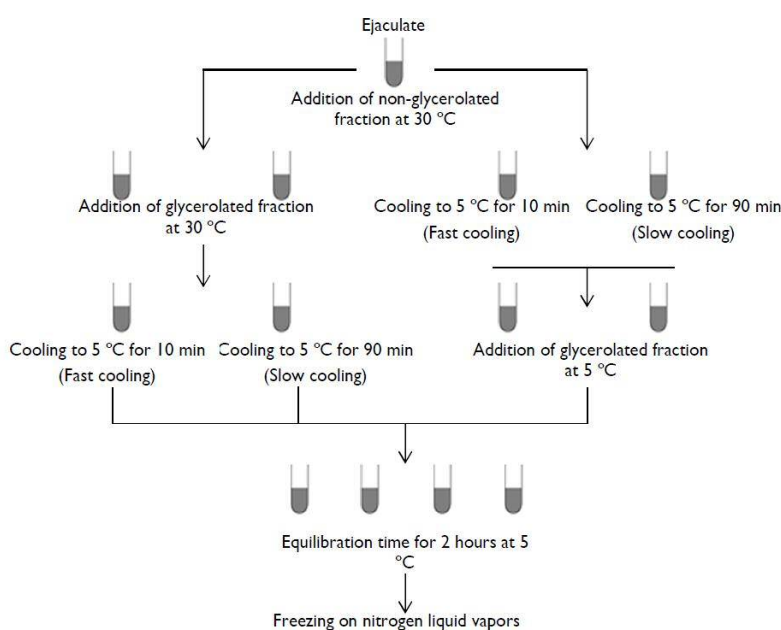
Figure 4.1 Design of the Experiment 2

4.3.4.3 Experiment 3: Effect of equilibration time on sperm quality at thawing

Since results in Experiment 2 showed that slow cooling rate reported better results at thawing, it was used in this experiment. Regarding temperature of glycerol addition, we used the temperature of 5 °C for convenience, due to this temperature was used for the control samples and there were no differences between adding glycerol at 30 °C or 5 °C for samples collected by EE.

Each ejaculate was diluted by the two-step method with Fraction A of Biladyl® with 20% egg yolk at 30 °C and cooled to 5 °C for 90 min. Then, it was further diluted with Fraction B containing glycerol at 5 °C and the diluted ejaculates were divided into 4 aliquots and maintained at 5 °C for 0, 1, 2 and 3 h (equilibration time), respectively. After equilibration time, samples were loaded into 0.25 mL plastic straws and frozen over nitrogen vapours as described in Experiment 1. Sperm samples were frozen at a final concentration of $100\text{-}200 \times 10^6$ spermatozoa/mL.

4.3.5 Thawed semen evaluation

Thawing was performed by placing the straws in a water bath at 37 °C for 30 sec, and then emptying the content into dry tubes and incubating for 5 min at the same temperature.

For thawed sperm samples, samples were fixed in buffered 2% glutaraldehyde solution to assess NAR by observing spermatozoa using phase-contrast microscopy (400×). Motility was evaluated by Computer Assisted Semen Analysis (CASA) using the Sperm Class Analyzer software (SCA® 2002, Microptic, Barcelona, Spain) with the following software settings adjusted to goat spermatozoa: 25 frames/s, 20–70 μm^2 for head area, velocity limit for slow sperm: 30 µm/s, velocity limit for medium sperm: 45 µm/s, velocity limit for fast sperm: 75 µm/s and minimal straightness for progressive spermatozoa: 80%. Sperm samples were diluted (v:v) with TALP-HEPES medium (87 mM NaCl, 3.1 mM KCl, 2 mM CaCl2, 0.4 mM MgCl2, 0.3 mM NaH2PO4, 40 mM HEPES, 21.6 mM sodium lactate, 1 mM sodium pyruvate, 50 µg/mL kanamycin, 10 µg/mL phenol red, and 6 mg/mL bovine serum albumin (BSA) (pH 7.5)) and 5 µL were put on a Makler chamber. At least 200 spermatozoa were counted with SCA® using a phase contrast microscope (Nikon Eclipse 80i; Nikon; Tokyo, Japan) at ×100 magnification and the following motility parameters were assessed: percentage of motile spermatozoa (TM; %), percentage of progressive motile spermatozoa (PM; %), straight line velocity (VSL; µm/sec), linearity index (LIN; %), and amplitude of the lateral displacement of the sperm head (ALH; µm).

Also, aliquots of thawed semen were used to carry out flow cytometry analyses. It was assessed the membrane stability with YO-PRO-1, the mitochondrial membrane potential with Mitotracker Deep Red and the viability with Propidium Iodide (PI) (Martínez-Pastor *et al.*, 2008). A staining solution using TALP-HEPES was prepared by adding 50 nM YO-PRO-1 (stock: 100 µM in DMSO) and 15 µM propidium iodide (stock: 7.5 mM in milli-Q water). We diluted 20 µL of sample in 0.5 mL of staining solution in polypropylene tubes for flow cytometry. The tubes were kept in dark for 15 min. Mitochondrial membrane potential of the samples was evaluated with 0.1 µM YO-PRO-1 and 100 nM Mitotracker Deep Red solution (stock: 1 mM in DMSO) in TALP-HEPES. Sperm samples were diluted to 1×10^6 spermatozoa/mL, and 300 µL were transferred to a polypropylene tube and left in the dark for 30 min. After incubation times, sperm samples were analyzed using a Cytomics FC500 flow cytometer (Beckman coulter, Inc. USA) and the following parameters were taken into account: YO-PRO-1-/PI- spermatozoa were considered as intact cells (indicating live spermatozoa with intact plasmalemma), YO-PRO-1+/PI- as apoptotic spermatozoa (indicating live spermatozoa with altered plasmalemma) and Mitotracker+/YO-PRO-1- as viable spermatozoa with high mitochondrial membrane potential.

Chromatin stability was assessed by using the SCSA® (Sperm Chromatin Structure Assay) technique (SCSA® Diagnostics, Inc., Brookings, SD, USA) (Evenson and Jost, 2000). This technique is based on the susceptibility of the sperm DNA to acid-induced denaturation *in situ* and the metachromatic staining Acridine Orange (AO). This stain fluoresces green when combined with double-stranded DNA, and red when combined with single stranded DNA (denatured). Thawed spermatozoa were diluted with TNE buffer to 2×10^6 cells/mL. Samples were flash frozen in LN$_2$ and stored at -80 °C until analysis. We calculated the DNA fragmentation index (DFI) for each spermatozoon as the ratio of red fluorescence respect to total fluorescence (red + green). High values of DFI indicate chromatin abnormalities. The %DFI was calculated as the percentage of spermatozoa with DFI > 25.

4.3.6 In Vitro Fertilization Test (IVF)

Control samples (those collected by AV) and samples collected by EE and with best sperm quality after different modifications of cryopreservation protocol (those freezing with 20% egg yolk, temperature of glycerol addition 30 °C or 5 °C, slow cooling rate and long equilibration time) were also used to perform a heterologous *in vitro fertilization test* (IVF) to assess the fertilization ability. A pool of three males with similar sperm quality belong to the same treatment (electroejaculation or artificial vagina) was used for IVF.

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Heterologous IVF was carried out three times for each treatment and thirty oocytes per well and replicate were used (n=180). Ovaries were collected at slaughterhouse from prepubertal sheep and transported to our laboratory in saline solution (25-30 °C) between 1-2 h after removal. Ovaries were sliced using a micro-blade and the follicle content was released in TCM 199 medium supplemented with HEPES (2.38 mg/mL), heparin (2μL/mL) and gentamycin (40μg/mL). Cumulus oocyte complexes (COC) were washed in TCM 199-gentamycin (40 μg/mL), and those dark homogeneous cytoplasm and surrounded by tightly packed cumulus cells were selected and randomly placed in four-well plates containing 500 μL of TCM 199 supplemented with cysteamine (100 μM), FSH/LH (10 μg/mL), FCS (10%) and gentamycin (40 μg/mL), and matured at 38.5 °C in 5% CO_2. After 24 h, COC were washed in fertilization medium (synthetic oviduct fluid (SOF) supplemented with gentamycin (40 μg/mL), 20% of oestrous sheep serum and heparin (1 μL/mL)) and cumulus cells were removed by gentle pipetting. Oocytes were transferred into four-well plates containing 450 μL of fertilization media under mineral oil.

Thawed spermatozoa were selected on a Percoll® discontinuous density gradient (45/90) and were capacited in the fertilization medium for 1 h. Finally, spermatozoa were co-incubated with oocytes at a final concentration of 1 x 10^6 spermatozoa/mL for 18 h at 38.5 °C, 5% CO_2, 5% O_2 and 90% N_2 humidified atmosphere. Presumptive zygotes were cultured at 18 h of insemination in SOF supplemented with BSA (Gardner et al., 1994) for 48 h at 38.5 °C in 5% CO_2, 5% O_2 and 90% N_2.

Fertility (cleavage rate) was assessed at 48 h post insemination by phase-contrast microscopy.

4.3.7 Statistical analysis

The R program (R Core team, 2012) was used to perform statistical analysis. Numeric results are expressed as the mean ± standar error of the mean (SEM). Data were considered statistically significant when $P \leq 0.05$.

The mixed effects models used in the analyses were the following:

Exp 1: y = EYconcentration + male + replicate(male) + ε

Exp 2: y = COOLINGrate + Tªglycerol + COOLINGrate×Tªglycerol + male + replicate(male) + ε

Exp 3: y = EQtime + male + replicate(male) + ε

where,

y represents the different sperm quality parameters at thawing,

EYconcentration is the egg yolk concentration (0, 1.5, 10 and 20 %),

COOLINGrate is the cooling rate (slow=90 min *vs.* fast=10 min),

Tªglycerol is the temperature of glycerol addition (30 °C *vs.* 5 °C),

COOLINGrate x Tªglycerol is the interaction between cooling rate and temperature of glycerol addition,

EQtime is the length of equilibration step (0, 1, 2 and 3 hours),

male is the effect of male (*n*=6), treated as random, and

replicate(male) is the effect of replicate for each experiment (*n*=3) within male, treated as random.

When necessary, multiple comparisons were performed using the Bonferroni adjustment. A GLM (General Lineal Model) ANOVA tested the effect of collection method (electroejaculation *vs.* artificial vagina) on *in vitro* fertility.

4.4. Results

4.4.1 Sperm characteristics for ejaculates obtained by AV and EE

Ejaculates collected by AV and EE showed great values of sperm viability, motility and NAR. Differences ($P \leq 0.05$) between collection methods were observed for sperm concentration, STN and SM, showing lower values for samples obtained by EE, except for SM which was higher for this kind of samples (Table 4.1).

Table 4.1 Effect of semen collection method on fresh sperm characteristics in Blanca-Celtibérica bucks.

	Sperm Characteristics					
	Volume (mL)	Concentration (x10⁶spz/mL)	STN (x10⁶spz)	SM (%)	NAR (%)	Viability (%)
Semen Collection Method						
AV	1.16±0.12	2845.42±142.64[a]	3328.03±319.27[a]	71.92±2.09[b]	95.21±0.75	79.02±3.03
EE	1.15±0.13	1334.35±143.35[b]	1423.36±322.05[b]	77.52±2.12[a]	95.80±0.76	79.70±0.85

Data are means ± SEM. Different superscripts within a column differ significantly ($P \leq 0.05$). AV: artificial vagina; EE: electroejaculation; STN: spermatozoa total number; SM: sperm motility; NAR: spermatozoa with intact acrosomes.

4.4.2 Experiment 1: Effect of the concentration of egg yolk contained in freezing extender on sperm quality at thawing.

Control samples showed higher values ($P \leq 0.05$) for TM and PM compared to EE samples frozen with the different extenders. However, there were no differences ($P >$

0.05) for the other sperm parameters between control and EE samples frozen with 10 or 20% egg yolk except for Mitotracker+/YOPRO- with greater values for AV samples compared to EE samples frozen with 10% egg yolk (Tables 4.2 and 4.3). Nevertheless, those EE samples frozen with 0 or 1.5% egg yolk displayed lower values ($P \leq 0.05$) for the most sperm parameters assessed in relation to those frozen with 10 or 20% egg yolk and control samples (Tables 4.2 and 4.3).

4.4.3 Experiment 2: Effect of cooling rate and temperature of glycerol addition on sperm quality at thawing

Regarding the cooling procedure, control samples showed higher values ($P \leq 0.05$) for TM compared to EE samples and no differences ($P > 0.05$) for other parameters were found between control samples and EE samples cooled slowly (Tables 4.2 and 4.3). There were no differences ($P > 0.05$) for the most sperm parameters for EE samples cooled slowly or fast except for TM with higher values ($P > 0.05$) for those processed slowly (Table 4.2).

In relation to the temperature of glycerol addition (Tables 4.2 and 4.3), control samples showed higher TM in relation to those collected by EE ($P \leq 0.05$). No differences ($P > 0.05$) between both temperatures of glycerol addition were found on sperm quality after thawing in samples obtained by EE.

The interaction glycerol temperature × cooling rate was no significant ($P > 0.05$).

4.4.4 Experiment 3: Effect of equilibration time on sperm quality at thawing

No differences ($P > 0.05$) were observed between control samples and samples collected by EE and equilibrated for 3 h, except for TM with higher values for control samples.

For sperm samples collected by EE, the equilibration time for 3 h provided better results on sperm quality after cryopreservation compared to 0 and 1 h. Thus, YO-PRO-1/PI- and Mitotracker+/YO-PRO-1- were higher ($P \leq 0.05$) when samples were equilibrated for 3 h. There were no differences between equilibration times for TM, NAR and velocity parameters and %DFI (Tables 4.2 and 4.3).

Table 4.2. Effect of concentration of egg yolk, cooling rate, temperature of glycerol addition and equilibration time on motility parameters and acrosome integrity after thawing in cryopreserved Blanca-Celtibérica buck ejaculates obtained by EE.

	Sperm parameters					
	TM (%)	PM (%)	VSL (µm/sec)	LIN (%)	ALH (µm)	NAR (%)
Egg yolk concentration						
0%	3.52±4.36[c]	1.68±2.12[c]	77.74±8.77	65.00±3.86	2.89±0.27	67.75±3.62[b]
1.5%	10.59±4.55[c]	5.32±2.21[c]	80.04±5.47	63.19±2.49	3.18±0.18	70.55±3.80[ab]
10%	29.12±4.36[b]	41.08±2.12[b]	83.37±4.92	60.28±2.28	3.51±0.16	74.74±3.80[ab]
20%	35.71±4.36[b]	16.54±2.12[b]	84.11±4.92	61.53±2.27	3.46±0.16	77.13±3.71[ab]
Control	61.43±4.55[a]	27.15±2.21[a]	74.01±4.70	58.53±2.18	2.98±0.16	80.36±3.80[a]
Cooling Rate						
Slow (90 min)	52.71±5.28[b]	21.64±2.60[ab]	75.03±4.87	57.33±1.80	3.44±0.12[ab]	81.18±2.59
Fast (10 min)	38.36±5.51[c]	18.27±2.72[b]	71.49±5.01	55.49±1.89	3.67±0.13[a]	78.56±2.76
Control	69.96±5.93[a]	27.19±2.94[a]	71.86±5.06	54.45±1.94	3.33±0.13[b]	78.86±2.80
Glycerol temperature						
30°C	46.46±5.32[b]	21.17±2.62[ab]	74.10±4.87	57.49±1.80	3.49±0.12[ab]	80.37±2.63
5°C	44.62±5.43[b]	18.74±2.68[b]	72.42±4.99	55.33±1.88	3.67±0.13[a]	79.37±2.67
Control	69.96±5.93[a]	27.19±2.94[a]	71.86±5.06	54.45±1.94	3.33±0.13[b]	78.86±2.80
Equilibration time						
0h	16.51±5.84[b]	7.88±3.31[c]	70.55±9.14	58.20±4.03	3.49±0.21	60.98±4.79
1h	17.60±5.84[b]	9.38±3.31[bc]	74.80±9.40	57.51±4.19	3.29±0.22	64.30±4.29
2h	35.48±6.13[ab]	18.59±3.46[ab]	76.93±9.20	59.26±4.04	3.17±0.21	58.73±4.52
3h	29.79±6.30[b]	15.48±3.54[ac]	83.54±9.64	59.79±4.35	3.25±0.22	73.23±4.79
Control	49.75±5.49[a]	22.65±3.12[a]	76.52±8.12	58.40±3.32	3.02±0.18	72.50±3.92

Data are means ± SEM. For each factor, different superscripts within a column differ significantly ($P \leq 0.05$). TM: percentage of motile spermatozoa; PM: percentage of progressive motile spermatozoa; VSL: straight line velocity; LIN: linearity index; ALH: amplitude of lateral head displacement; NAR: percentage of spermatozoa with intact acrosome; Control: samples collected by AV and cryopreserved with 20% egg yolk, slowly cooled, glycerol added at 5 °C, and equilibrated time for 2 h.

Table 4.3 Effect of concentration of egg yolk, cooling rate, temperature of glycerol addition and equilibration time on sperm parameters evaluated by flow cytometry after thawing in cryopreserved Blanca-Celtibérica buck ejaculates obtained by EE.

	Sperm parameters			
	YO-PRO-1-/PI- (%)	YO-PRO-1+/PI- (%)	Mitotracker+/YO-PRO-1- (%)	DFI (%)
Egg yolk concentration				
0%	6.22±2.30[c]	1.75±0.75[c]	2.79±3.36[c]	1.52±0.38
1.5%	18.54±2.40[b]	4.89±0.79[b]	13.04±3.49[c]	1.91±0.39
10%	30.80±2.30[a]	7.52±0.75[ab]	30.54±3.36[b]	2.21±0.38
20%	37.60±2.30[a]	8.53±0.75[a]	36.35±3.36[ab]	2.77±0.39
Control	39.52±2.40[a]	9.12±0.79[a]	45.02±3.49[a]	1.59±0.39
Cooling Rate				
Slow (90 min)	30.60±2.37	11.84±1.09	25.25±3.48[ab]	1.24±3.37
Fast (10 min)	34.46±2.61	10.04±1.13	18.49±3.68[b]	1.06±3.63
Control	33.82±3.14	11.55±1.19	32.37±4.01[a]	1.52±3.63
Glycerol temperature				
30°C	31.73±3.23	11.13±1.10	23.37±3.53[ab]	1.26±3.42
5°C	33.33±2.53	10.74±1.12	20.37±3.60[b]	1.05±3.56
Control	33.82±3.14	11.55±1.19	32.37±4.01[a]	1.52±3.63
Equilibration time				
0h	17.23±3.23[b]	7.20±1.37[b]	7.74±2.92[c]	1.85±0.47
1h	19.00±3.23[b]	9.15±1.37[ab]	9.42±2.92[bc]	1.98±0.47
2h	24.96±3.42[ab]	10.17±1.43[ab]	11.94±2.98[bc]	2.03±0.49
3h	32.90±3.53[a]	12.21±1.47[a]	14.11±3.01[ab]	1.90±0.50
Control	30.00±3.01[a]	11.84±1.29[a]	19.15±2.82[a]	1.86±0.44

Data are means ± SEM. For each factor, different superscripts within a column differ significantly ($P \leq 0.05$). YOPRO-/PI-: intact spermatozoa; YOPRO+/PI-: apoptotic spermatozoa; Mitotracker+/YOPRO-: viable spermatozoa with high mitochondrial membrane potential; DFI: DNA fragmentation index; Control: samples collected by AV and cryopreserved with 20% egg yolk, slowly cooled, glycerol added at 5 °C, and equilibrated time for 2 h.

4.4.5 In vitro fertilization test

Sperm samples collected by EE and frozen with the best sperm cryopreservation protocol (20% egg yolk, slow cooling rate, glycerol addition at 5 °C and equilibration for 3h) provided higher fertility ($P \leq 0.05$) than control samples, (35 ±1.81% vs. 28 ± 1.81%) (Figure 4.2).
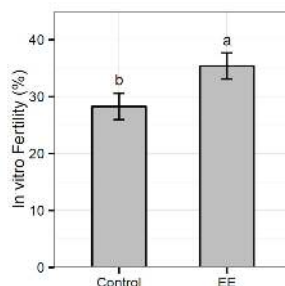

Figure 4.2 *In vitro* fertility for sperm samples collected by AV, control samples, and EE, those frozen with the best cryopreservation protocol. Data are means ± SEM. Different superscripts differ significantly ($P \leq 0.05$). Control: samples collected by AV (artificial vagina) and frozen by standard cryopreservation protocol: 20% egg yolk, slow cooling to 5 °C, glycerol addition at 5 °C and equilibration time for 2 h; EE: samples collected by electroejaculation and frozen with 20% egg yolk, slow cooling to 5 °C, glycerol addition at 5 °C and equilibration time for 3h.

4.5. Discussion

The correct design and optimization of the sperm cryopreservation protocol is a key step for the creation and maintenance of a cryobank. Semen collection is performed traditionally by artificial vaginal (AV), although alternative methods exist. The electroejaculation (EE) is a substitute to collect semen from untrained or wild males whose training to AV is not possible, being a suitable method for Blanca-Celtibérica bucks due to its production system. However, this collection method could lead to different ejaculates from those obtained by physiological way and could influence on the response of spermatozoa to different procedures such as cryopreservation.

The aim of this study was to assess modifications in the cryopreservation protocol in Blanca-Celtibérica buck semen collected by EE for improving sperm quality after thawing, achieving samples with similar quality to those obtained with AV. The collection method had an effect on sperm production parameters such as the concentration and STN, with higher values for AV samples. These results were similar to those found previously by our group (Jiménez-Rabadán *et al.*, 2012a) and in the same line that those reported by Memon *et al.* (1986) and Marco-Jiménez *et al.* (2005, 2008) in bucks and rams, respectively. During the EE procedure, sexual glands are artificially stimulated and the volume of seminal plasma varies in relation to physiological ejaculate (Marco-Jiménez *et al.*, 2005; Marco-Jiménez *et al.*, 2008).

Although initially no differences were found in sperm quality for the most sperm parameters assessed in ejaculates collected by AV and EE, after sperm cryopreservation the AV samples showed higher values for motility parameters than those obtained by EE. However, there were no differences between AV samples and those from EE frozen with the best cryopreservation protocol. Our results agree with those reported by Álvarez *et al.* (2012) who observed lower values of motility for EE samples and similar values for the percentage of normal acrosomes and viability compared to AV samples. Nevertheless, in a study conducted previously by our group, we found a worse sperm quality for the most of sperm parameters assessed in samples collected by EE compared to those obtained by AV (Jiménez-Rabadán *et al.*, 2012b). This fact could be due to the individual variability observed in the resistance to cryopreservation for EE samples. Thus, some males showed frequently low values of sperm quality because of freezing extender is coagulated. However, the same males displayed acceptable values of sperm quality when samples were collected by AV. A specific problem for buck semen cryopreservation has been described when extenders containing egg yolk are used due to the presence of a phospholipase enzyme in the seminal plasma (Iritani and Nishikawa, 1963; Sias *et al.*, 2005). Seminal plasma from EE samples have a different composition from that collected by AV (Marco-Jiménez *et al.*, 2005; Marco-Jiménez *et al.*, 2008), affecting possibly the response of spermatozoa to cryopreservation procedure and this effect may be more noticeable in some males.

The best cryopreservation protocol reported for EE samples involved changes in the concentration of egg yolk, cooling rate and equilibration time. Regarding to studies about egg yolk, 20% rendered the best sperm quality being our results similar to a study conducted by Daskin and Tekin (1996), although other authors found that lower egg yolk concentrations preserved better the sperm quality during cryopreservation than the greater concentrations (Santiago-Moreno *et al.*, 2009). This could be due to differences in the cryopreservation extender, in the species or even to the season of semen collection. Besides, it is important to highlight that the egg yolk used in our experiments was clarified (Holt et al., 1996b) and this type of egg yolk could provide lesser sperm toxicity than whole egg yolk during the freezing-thawing process.

In relation to cooling rate, our results suggested that slow cooling was better and agree with the study carried out in bucks by Memon *et al.* (2013), although in that study the semen was collected by AV. However, for other species a fast cooling rate yield a higher sperm quality (Fernández-Santos *et al.*, 2006) which could be due to the different origin of spermatozoa. It is known that a too rapid cooling of ungulates semen from 30 to 0 °C induces a lethal stress on spermatozoa proportional to the rate of

cooling, the temperature interval and the temperature range (Watson, 1981). In addition, it would be possible to add the glycerol at 30 °C since there were no differences between adding glycerol at 30 or 5 °C, making easier the work in field conditions. These results agree with those from Coloma *et al.* (2010) in bucks, who reported no effect of the glycerolization temperature.

Finally, the effect of different times of equilibration (0, 1, 2 and 3 h) at 5 °C was studied in this work. The best sperm quality was obtained when samples were equilibrated for 3 h in relation to shorter times. Our results agree with Deka and Rao (1986) who found greater progressive motility when the equilibration period increased from 1 to 5 h in samples collected by AV. On the other hand, some authors have frozen buck semen collected by AV without equilibration time with good results (Ritar and Salamon, 1983; Ritar *et al.*, 1990b). The exposure of spermatozoa to subphysiological temperatures prior freezing can induce alterations in the organization of the lipid bilayer of the plasma membrane, particularly in species with high concentrations of polyunsaturated fatty acids (Holt, 2000). The better results obtained when longer equilibration periods are used could be due to an improvement on the organization of the cell membrane during this transition phase, thereby minimizing cryoinjury during semen processing.

The fertility assessed by IVF for those samples collected by EE and cryopreserved with the best protocol was higher than for AV samples, although the motility values were higher for the latter. Although this fact seems surprising, we have to consider that spermatozoa were selected by means of density gradients for using them in IVF test. Jiménez-Rabadán *et al.* (2012b) showed that sperm quality of thawed semen improved after selecting by single layer centrifugation (SLC). Thereby, the differences observed in the motility parameters between AV and EE samples after cryopreservation might disappear, showing similar values and comparable fertility rates. In addition, Álvarez *et al.* (2012) found acceptable fertility values for ram sperm samples collected by EE being similar to those obtained by AV, although AV samples showed higher values of motility.

In conclusion, the protocol which renders best sperm quality after cryopreservation for samples collected by EE was that which included a 20% of egg yolk, with a slow cooling rate, with glycerol added at 30 °C or 5°C and an equilibration time equals to 3 h. Under such conditions, sperm samples collected by EE showed a sperm quality at thawing quite similar to those collected by AV and cryopreserved using the standard protocol.

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GENERAL DISCUSSION

The development of Germoplasm Banks, by gamete cryopreservation, allows us the storage of genetic resources indefinitely, being a key tool to preserve endangered species (Watson and Holt, 2001). In order to obtain the best sperm quality at thawing, it is importat to improve the knowledge of factors involved during the cryopreservation procedure.

This Doctoral Thesis has been aimed to develop an appropiate methodology to collect and cryopreserve semen from Blanca-Celtibérica bucks, which allow us to store seminal doses in a Germoplasm Bank. For this purpose, it has been evaluated the thawed sperm quality of samples collected by different methods (artificial vagina and electroejaculation) and subjected to different treatments during the cryopreservation process by means of routine analyses, objective analyses of motility parameters (CASA) and flow cytometry analyses.

In small ruminant species, semen from live animals is usually collected by artificial vagina (AV) or electroejaculation (EE). The electroejaculation is an alternative to obtain semen from animals when males can not be trained to AV. The production of Blanca-Celtibérica goat breed is orientated to meat productions by means of extensive systems. Therefore, the semen collection by AV is not a useful method in this breed. Nevertheless, sperm characteristics are different depending on the collection method used and this fact may influence the resistance to freezing (Álvarez et al., 2012; Marco-Jiménez et al., 2005). Hence, Chapter 1 of this Thesis evaluated the influence of collection method (AV and EE) on thawed sperm quality of semen obtained from Blanca-Celtibérica bucks. Results were compared with those obtained in another close-related small ruminant species, the ovine.

In this work, higher volume and lower concentration were observed in samples collected by EE for both species, with the lowest concentration values for buck samples collected by EE. Electrical stimuli on accessory sex glands during the EE procedure increase the contribution of these glands to ejaculates, resulting in an increase of volume (Mattner and Volgmayr, 1962). In our study, no significant differences were found on sperm quality parameters such as motility, notwithstanding the fact that differences on productive parameters were observed. In the same line, Marco-Jiménez et al. (2005) and Álvarez et al. (2012) did not find differences on motility between sperm samples collected by AV and EE in rams. However, after thawing, the collection method and the species had an effect on sperm quality parameters, especially on those related with membrane integrity and mitochondrial activity. Thus, buck sperm samples collected by EE had lower quality than those collected by AV. Contrary, no differences between collection methods were found in

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rams, being some parameters similar than those obtained from buck sperm samples collected by AV. It should be highlighted a specific problem during buck sperm cryopreservation due to the presence of a phospholipase called egg yolk coagulating enzyme (EYCE) in seminal plasma. This enzyme is secreted by accessory sex glands and interacts with egg yolk contained in the freezing extender resulting in toxic compounds to spermatozoa (Iritani and Nishikawa, 1963; Sias *et al.*, 2005). Higher volume found in samples collected by EE in this breed could be related to higher amount of seminal plasma and therefore, related to higher level of EYCE. In this way, it has been reported in other species that sperm collection method modifies the ionic and protein seminal plasma composition (Marco-Jiménez *et al.*, 2008). On the other hand, epididymal plasma contains a protein factor (sperm motility inhibiting factor, MIF-II) which is able to inhibit the spermatozoa motility (Das *et al.*, 2010). It is possible that electrical stimuli caused by EE provoke an increase of this protein resulting in a decrease of seminal quality, related specially with motility and mitochondrial activity. Moreover, seminal plasma also contains a high concentration of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). The epididymal enzyme CAT protects spermatozoa from oxidative damage in the epididymal lumen, whereas SOD and GP secreted from seminal vesicle protect sperm after ejaculation (Zubkova and Robaire, 2004). Levels of antioxidant defensive systems vary with species, season and type of ejaculation (Ollero *et al.*, 1996). The collection method could also have an effect on levels of antioxidant enzymes, being possible to find a variation of concentration in samples collected by EE, producing damage on DNA (Aitken and De Iuliis, 2010) and mitochondrial membrane.

In this first study, no differences on thawed sperm quality between collection methods were found in rams. There are controversy results about the effect of sperm collection method on resistante to cryopreservation in this species. Thus, Quinn *et al.* (1968) and Álvarez *et al.* (2012) observed that spermatozoa collected by AV were more resistant to freezing than those obtained by EE. However, Marco-Jiménez *et al.* (2005) reported higher sperm quality at thawing in sperm samples collected by EE in relation to AV samples. These differences between studies could be due to different methodologies used to collect and cryopreserve semen and to a variety of extenders used by different authors.

It could be concluded in this Chapter that the sperm quality after thawing of sperm samples collected by EE from Blanca-Celtibérica bucks is lower than those obtained in samples collected by AV. The collection method seems to have no influence on thawed sperm quality in ovine species.

On the basis of what is described above, the second experiment of this Thesis (Chapter 2) was planned. This Chapter was aimed to improve the sperm quality of samples collected by EE from Blanca-Celtibérica bucks. The thawed sperm quality was evaluated in sperm samples collected by AV and EE and diluted in three different freezing extenders, two commercial extenders: Biladyl® (egg yolk) and Andromed® (soy lecithin) and a non-commercial extender based on skim milk. Addicionally, the effects of season, washing of sperm samples in order to remove the seminal plasma before freezing and interactions between factors were studied.

Results demonstrated that sperm samples collected by EE showed lower thawed sperm quality than samples obtained by AV. Moreover, no interactions between collection method and season, removal of seminal plasma or extender used were found. Therefore, these results suggest that samples collected by AV and EE show similar response to the factors evaluated.

Regarding season of ejaculates collection, no differences on productive parameters were found between ejaculates collected during breeding season in relation to those obtained during non-breeding season, whereas motility was higher in samples collected during breeding season. Nevertheless, season had an effect on resistance to cryopreservation procedure. Thus, semen cryopreserved during breeding season had higher sperm quality at thawing. These results agree with other studies performed in other small ruminant species (Coloma *et al.*, 2011; D´Alessandro and Martemucci, 2003). An improvement in fertilization during breeding season has been associated to changes in protein composition of seminal plasma (Smith *et al.*, 1999). This fact suggests that some seminal plasma proteins could influence on resistance to spermatozoa to cryopreservation procedure. Thus, La Falci *et al.*, (2002) showed that supplementation of semen samples with seminal plasma proteins obtained during non-breeding season resulted in a reduction of sperm quality during the freezing-thawing process, whereas other researchers reported a beneficial effect of the supplementation with proteins from breeding-season (Leahy *et al.*, 2010).

In relation to the study of extenders should be highlighted that the widest used extenders in buck sperm semen cryopreservation have been those based on egg yolk (Salamon y Ritar, 1982) and skim milk (Corteel, 1974; Corteel, 1975; Leboeuf *et al.*, 2000). However, due to negative interactions between these compounds and seminal plasma during sperm cryopreservation in this species, it has been proposed the use of extenders based on soy as an alternative. Results from this study showed higher sperm quality at thawing in samples extended with Biladyl® or Andromed®. These results agree with Sariözkan *et al.* (2010), who observed similar values for buck sperm samples

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cryopreserved in Triladyl® (based-egg yolk extender) or Bioxcell® (soy-based extender). Other researchers also observed that buck semen frozen in egg yolk-based extenders had higher seminal quality at thawing than that frozen in extenders based on skim milk (Chauhan and Anand, 1990). Therefore, our results suggest that sperm quality is less affected when egg yolk-based extenders are used. This fact could be due to egg yolk used in our work was clarified egg yolk. The procedure of centrifugation performed for obtaining clarified egg yolk might remove harmful substances which have a negative effect on spermatozoa during the cryopreservation process and even, egg yolk phospholipides are not hydrolyzed by seminal plasma enzymes as suggested other authors (Chauhan and Anand, 1990). In any case, the beneficial effect of egg yolk as cryoprotector during the cryopreservation surpasses the negative effects. Finally, no interactions between extenders and collection method and seminal plasma removal and season were found.

In addition to this, the effect of seminal plasma removal on sperm quality at thawing was also studied in this Chapter. Several studies have suggested that removal of seminal plasma by washing/centrifugation is a useful procedure in order to avoid EYCE and SBUIII action (Kozdrowski *et al.*, 2007; Machado and Simplicio, 1995). In the present study, differences between removing or not the seminal plasma before freezing on thawed sperm quality were not found, as was reported by other authors (Azeredo *et al.*, 2001; Deka and Rao, 1988). Some researchers have reported that the positive effect of removing seminal plasma depends on season and freezing extender (Cabrera *et al.*, 2005; Sariözkan *et al.*, 2010). In our work, no interaction between washing and freezing extender was observed while washing and season of semen collection interaction was observed for some sperm characteristics. Thus, percentage of motile spermatozoa and some velocity parameters were improved when seminal plasma was removed before freezing during non-breeding season. These results are similar to those obtained by Cabrera *et al.* (2005) who observed that samples collected during spring (non-breeding season) showed higher cryosurvival when they were washed, whereas during winter (breeding season) no washed samples withstanded better this procedure. It has been described higher phospholipase activity during non-breeding season (Ritar and Salamon, 1991) and washing may remove some subtances with this activity, showing lesser harmful effect on spermatozoa.

Therefore, results from this Chapter suggest that sperm quality at thawing of samples collected by AV or EE from Blanca-Celtibérica bucks depends on season of collection of ejaculates and freezing extender used, being the breeding season the best season to collect and cryopreserve semen and Biladyl® (egg yolk-based extender) the best extender. Moreover, removal of seminal plasma before freezing during non-

breeding season offers an improvement on sperm quality at thawing, whereas the seminal removal makes no difference during breeding season.

Due to the importance of EE as semen collection method in bucks of Blanca-Celtibérica breed, Chapters 3 and 4 in this Doctoral Thesis were aimed to improve the sperm quality at thawing of samples collected by EE during breeding season and frozen in Biladyl®. For this, modifications on different stages involved during the cryopreservation procedure were carried out.

In Chapter 3, it was studied the effect of seminal plasma removal and enrichment of the sperm sample before freezing or after thawing by means of single layer centrifugation (SLC) on sperm quality at thawing. Sperm selection techniques separate spermatozoa from seminal plasma and allow us obtaining a sperm subpopulation with high motility, viability and good chromatin integrity (Morrell and Rodríguez-Martínez, 2009). The technique used in the present study (SLC) has some advantages in relation to conventional density gradient centrifugation (DGC): shorter preparation time, less complicated procedure and it is able to process large volume of semen (Morrell and Rodríguez-Martínez, 2009). In this study, the sperm recovery yield was greater in samples using SLC before freezing in relation to SLC after thawing. These results agree with Chatdarong et al. (2010), although percentage of recovery found by these authors was lower than in our study. The cryopreservation causes loss of spermatozoa (Watson, 1979) and although it has been suggested that around 40-60% of spermatozoa preserve their motility, only about 20-30% remain biologically undamaged (Salamon and Maxwell, 2000). Due to the number of damaged spermatozoa is higher after thawing, results are reasonable since lower sperm recovery was observed using SLC after thawing. Besides, sperm quality at thawing was evaluated and compared between 3 treatments: no selected samples, samples selected by SLC before freezing and samples selected by SLC after thawing. Results showed lower sperm quality in sperm samples selected before freezing, being the best sperm quality for samples selected after thawing. Contrary, Martínez-Alborcia et al. (2012) reported that boar sperm samples selected before freezing showed higher quality in relation to unselected samples and other authors found lower percentage of motile spermatozoa when SLC was performed after thawing in epididymal cat spermatozoa (Chatdarog et al., 2010). Our results suggest that SLC after thawing removed seminal plasma, debris and those damaged spermatozoa which did not withstand the cryopreservation proccess or those that swimmed slowly and were unable to pass through the colloid layer. For this reason, the sperm quality of spermatozoa selected after thawing would be higher than quality of unselected or selected before freezing spermatozoa. On the other hand, several studies have reported that some proteins

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from seminal plasma are absorbed onto sperm surface and this absorption is able to partially repair membrane alterations induced by cold shock (Barrios *et al.*, 2000; García-López *et al.*, 1996; Pascual *et al.*, 1994; Pérez-Pe *et al.*, 2001a; Pérez-Pe *et al.*, 2001b). Due to SLC removes seminal plasma, it is posible that this procedure applied before cryopreservation, avoids its negative effects but also may remove some useful proteins to withstand cold shock (Barrios *et al.*, 2005). This fact could explain low results found when SLC was performed before freezing. The seminal plasma may contain beneficial as well as harmful substances which may contribute to sperm survival. The beneficial/deleterious effects may be changed in samples collected by EE since this procedure modifies the secretory function of accessory sex glands. Thus, the beneficial effects of some proteins could overcome the harmful effects of other substances present in the seminal plasma, which could be the reason for a lower sperm quality when spermatozoa are separated from seminal plasma before freezing.

It could be concluded in this Chapter that buck spermatozoa collected by EE and selected by SLC before freezing show similar sperm quality at thawing than unselected samples. However, SLC after thawing improves the sperm quality. Nevertheless, although sperm quality was improved in this kind of samples, the recovery yield was very low and therefore, other studies were proposed as an alternative and were aimed to improve the sperm quality at thawing in samples collected by EE (Chapter 4).

In the Chapter 4, modifications on the cryopreservation procedure in semen samples collected by EE were studied. This work was aimed to reach similar semen quality and fertility than that observed in samples collected by AV and frozen by means of a standar protocol (control samples). For this, it was studied the effect of egg yolk concentration contained in the freezing extender (0, 1.5, 10 or 20%), the cooling rate from 30 °C to 5 °C (slowly in 90 min or fast in 10 min), the temperature of glycerol addition (5 °C or 30 °C) and the equilibration time (0, 1, 2 or 3 h) on sperm quality at thawing in samples collected by EE. In addition to this, a heterologous *in vitro* fertilization test (IVF) was carried out in samples collected by EE and frozen with the protocol which best results reported; *in vitro* fertility was compared with that obtained in samples collected by AV and frozen by the standard protocol.

Sperm samples collected by AV showed higher motility at thawing than those obtained by EE in all experiments carried out in this Chapter. For the rest of parameters, no differences were found between samples collected by AV and samples obtained by EE and frozen with the protocol that best results reported. These results are similar to those observed by Álvarez *et al.* (2012), who showed lower motility for

samples collected by EE and similar values in both collection methods (AV or EE) for other parameters such as percentage of spermatozoa with intact acrosome and viability.

In relation to the study carried out in this Chapter about the effect of egg yolk concentration contained in the freezing extender, 20% egg yolk rendered the best sperm quality, being our result similar to that observed by Daskin and Tekin (1996). However, other authors reported that lower egg yolk concentration preserve better the sperm quality during the cryopreservation process (Santiago-Moreno *et al.*, 2009). Differences between studies could be due to differences on extender composition, origin of spermatozoa, species or even season of semen collection. In this way, Álvarez *et al.* (2012) demonstrated that the origin of spermatozoa had an effect on the extender composition which best sperm quality reported, being 20% egg yolk the best concentration in samples collected by EE. On the other hand, it is important highlight that egg yolk used in our experiments was clarified (Holt *et al.*, 1996b). As it was said before, this kind of egg yolk could have lower toxicity than whole yolk during the freezing-thawing procedure.

Regarding the cooling rate, our results suggested that slow cooling rates preserve better sperm quality at thawing than the fast ones. These results agree with a study carried out in bucks by Memon *et al.* (2013), although that study was performed on samples collected by AV. However, for other species a fast cooling rate provided higher sperm quality (Fernández-Santos *et al.*, 2006), which could be due to sperm samples were collected from epididymis. A too rapid cooling of ungulates semen from 30 °C to 0 °C induces a lethal stress on spermatozoa proportional to the rate of cooling and temperature range (Watson, 1981). For this reason, it would be logical to think that slow cooling rates lead to higher sperm quality. Otherwise, glycerol could be added at 30 °C because of no differences were found between the glycerol addition at 30 °C or 5 °C. The addition of glycerol at 30 °C makes the work in field conditions easier. Our results are similar to those found by Coloma *et al.* (2010), who did not find an effect of glycerolization temperature on sperm quality at thawing.

In this Chapter was also studied the effect of equilibration time on sperm quality at thawing. Values of sperm quality were higher in samples equilibrated at 5 °C for 3 h. These results agree with Deka and Rao (1986), who observed higher progressive motility when equilibration period was increased from 1 to 5 h in samples collected by AV. However, some authors have frozen semen collected by AV without equilibration period with good results (Ritar and Salamon, 1983; Ritar *et al.*, 1990b). The exposure of spermatozoa to subphysiological temperatures prior freezing can

induce alterations in the organization of the lipid bilayer of the plasma membrane, particularly in species with high concentrations of polyunsaturated fatty acids (Holt, 2000). The better results obtained when longer equilibration periods are used could be due to an improvement on the organization of the cell membrane during this transition phase, thereby minimizing cryoinjury during semen processing.

Finally, in this Chapter was also evaluated the fertility of samples collected by AV and EE by means of a heterologous *in vitro* fertilization test. The fertility of samples collected by EE and cryopreserved by the protocol which best results reported in the different experiments performed through this Chapter was compared with the fertility of control samples (collected by AV and frozen by the standard protocol). The fertility was higher for samples collected by EE in spite of motility parameters were lower for this kind of samples in relation to AV. Although this fact seems surprising, we should consider that spermatozoa were selected by means of density gradients for using them in IVF test. Jiménez-Rabadán *et al.* (2012b) showed that sperm quality at thawing improved after selection by SLC. Therefore, differences observed in motility parameters between samples collected by AV and EE might disappear after sperm selection, showing similar values and comparable fertility rates. In addition, Álvarez *et al.* (2012) found acceptable *in vivo* fertility values for ram sperm samples collected by EE, being similar to those obtained by AV, although AV samples had higher values of motility than EE samples.

In conclusion, results from this Chapter show that the best sperm cryopreservation protocol in Blanca-Celtibérica buck samples collected by EE is that which includes freezing extender with 20% egg yolk, slow cooling rate (from 30 °C to 5 °C in 90 min), temperature of glycerol addition at 30 °C or 5 °C and equilibration time for 3 h. Under such conditions, sperm samples collected by EE showed similar sperm quality at thawing than those obtained by AV and frozen using the standard protocol.

CONCLUSIONS

1. The semen samples from Blanca-Celtibérica bucks collected by electroejaculation show lower sperm quality at thawing than samples obtained by artificial vagina, whereas no differences on sperm quality were observed between both collection methods in Manchego rams.

2. Blanca-Celtibérica buck ejaculates obtained during breeding season show higher sperm quality at thawing than those obtained during non-breeding season, for samples collected by artificial vagina as well as by electroejaculation.

3. The sperm quality after thawing of samples from Blanca-Celtibérica bucks is affected by the freezing extender used. Thus, the commercial extender Biladyl® (based on egg yolk) provides the best results for samples collected by artificial vagina as well as by electroejaculation.

4. The removal of seminal plasma before freezing of Blanca-Celtibérica buck ejaculates by centrifugation/washing improves the sperm quality at thawing when ejaculates are collected during non-breeding season, whereas the seminal plasma removal have no effect during the breeding season, for samples collected by artificial vagina as well as by electroejaculation.

5. The sperm selection by single layer centrifugation (SLC) after thawing improves the sperm quality in samples collected by electroejaculation from Blanca-Celtibérica bucks. Nevertheless, the sperm recovery is too low.

6. Concentrations of 20% egg yolk in the freezing extender reports higher sperm quality at thawing in samples collected by electroejaculation from Blanca-Celtibérica bucks than lower ones.

7. Slow cooling rates (from 30 °C to 5 °C in 90 minutes) provide higher sperm quality at thawing than fast rates (10 minutes) in sperm samples collected by electroejaculation from Blanca-Celtibérica.

8. The temperature of glycerol addition (30 °C or 5 °C) to the freezing extender had no effect on sperm quality at thawing in Blanca Celtibérica buck semen collected by electroejaculation.

9. Equilibration time at 5 °C for 3 hours improves the sperm quality at thawing in the sperm samples collected by electroejaculation from Blanca-Celtibérica buck.

10. The *in vitro* fertilty of ejaculates collected by EE and cryopreserved by the protocol which reported the best sperm quality at thawing is higher than for samples obtained by AV and cryopreserved by the standard protocol.

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ABBREVIATION INDEX

AND: *Ácido Desoxirribonucleico*

ALH: *Amplitude of Lateral Head displacement*

AO: *Acridine Orange*

AV: *Artificial Vagina*

BRG: *Bancos de Recursos Genéticos*

BSA: *Bovine Serum Albumin*

C: *Concentration*

CASA: *Computer Assisted Sperm Analysis*

CAT: *Catalase*

DFI: *DNA Fragmentation Index*

DNA: *Desoxirribonucleic Acid*

DGC: *Density Gradient Centrifugation*

EE: *Electroejaculation*

EQ: *Equilibration*

EY: *Egg yolk*

EYCE: *Egg Yolk Coagulating Enzyme*

FAO: *Food and Agriculture Organization of the United Nations*

GPx: *Glutathione Peroxidase*

GRB: *Genetic Resource Banks*

H$_2$DCFDA: *dichlorodihydrofluoresceindiacetate*

hMMP: *high Mitochondrial Membrane Potential*

IVF: *In Vitro Fertilization test*

LIN: *Linearity index*

MIF-II: *Sperm Motility Inhibiting Factor*

Mitotracker+/YO-PRO-1-: *viable spermatozoa with active mitochondria*

NAR: *spermatozoa with intact acrosomes*

PBS: *Phosphate Buffer Saline*

PI: *Propidium Iodide*

PM: *Progressive Motile spermatozoa*

ROS: *Reactive Oxigen Species*

SBUIII: *Bulbourethral III Secretion*

SCSA: *Sperm Chromatin Structure Assay*

SM: *Motile Spermatozoa*

SEM: *Standard Error of the Mean*

SLC: *Single Layer Centrifugation*

SM: *Sperm Motility*

SPZ: *Spermatozoa*

SOD: *Superoxide Dismutase*

SOF: *Synthetic oviduct fluid*

STN: *Spermatozoa Total Number*

SYBR-14+/PI-: *live spermatozoa with intact membrane*

SYBR-14 intermediate/PI intermediate: *moribund spermatozoa*

SYBR-14+/PI+: *dead spermatozoa*

Tª: *Temperature*

TM: *motile spermatozoa*

TNE: *Tris-NaCl-EDTA*

V: *Volume*

VSL: *Velocity according to the straight path*

YO-PRO-1-/PI-: *intact spermatozoa*

YO-PRO-1+/PI-: *apoptotic spermatozoa*

TABLE INDEX

FIGURE INDEX