Advances in post-thawing protocol of cryopreserved endangered Catalonian breed (Cabra Blanca de Rasquera) sperm doses towards improving their post-thawed fertility potential

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Doctoral Thesis

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Departament de Medicina i Cirurgia Animal

Universitat Autònoma de Barcelona

Bellatera, 2021

Universitat Autònoma de Barcelona

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Bellaterra (Cerdanyola del Vallés), 16 setembre de 2021.

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Dedicated to (Late) Mr Brendan Ohaneje

Forever in our thoughts

ACKNOWLEDGEMENTS

First and foremost, my sincere gratitude goes to the **Almighty God** for his sufficient grace, guidance and protection all through this programme.

My sincere appreciation goes to my amiable supervisor, **Professor Maria-Jesus Palomo,** Dept. de Medicina i Cirurgia Animals, Faculty of Veterinary Medicine for giving me the opportunity to be part of her team. Your selflessness and kind heart is second to none, you stood by me in my most difficult times, you were not only my supervisor, you were a confidant and a pillar of support (morally and financially). I am deeply grateful for all you have done for me and my family all through these years. Thank you for your patience and constructive criticisms throughout the period of this program, I learnt a lot under your watch. You are sincerely very much loved and appreciated.

I would love to appreciate **Alex Peña** of the Animal Reproduction Unit, **Manuela Costa** from cytometry unit of the university (UAB), and **Manuel Alvarez-Rodríguez** from the Department of Anatomy for helping me with the laboratory aspect of my work. I would also use this medium to appreciate **Lluís Sifuentes** from Mexico who assisted with some laboratory experiments during his short stay with us and **Iván Yánez-Ortiz** for assisting with the statistical analyses of this work

My sincere appreciation goes to the entire Professors of the Animal Reproduction Unit, **Joan-Enric Rodríguez-Gil**, **Jordi Miró Roig, Teresa Rigau**, **Teresa M. Mogas** and **Montserrat Rivera** for all your support.

I am also grateful to all my colleagues in the Reproduction unit, **Tània, Jaimen**, **Iris, Uche**, **Sabrina**, and **Ivan.** It was a great pleasure knowing and working with you all.

I would like to appreciate my dad, **(Late) Brendan Ohaneje**, even though you are no more, you were my provider, anchor, mentor and motivator to achieving my dreams, and you are still forever in my heart. To my lovely mum, **Mrs Benedette Chika Ohaneje**, your love, prayers, relentless efforts and encouragements brought me this far. You gave me hope when I was hopeless and taught me how to be positive in my difficult moments. I am grateful to God for keeping you alive to witness this day, and I hope I continue to make you proud every day of my life. I love you so much.

To my beautiful siblings, **Chidozie, Chinonso,** and my better half **Oluchi**. You all are the best siblings one could ever ask for. Thank you all for your support and encouragements all through, this is just our beginning of many heights to attain. I love you all deeply.

To my son, **Anselm,** thank you for your patience and understanding, even when I was too busy to play with you. I love you unconditionally.

To my in-laws **Mrs Norah Osuagwuh, Uchebuike, Ucheneake** and most especially my sister-in-law**, Uchebundu** and her husband **Ijeweme** for your generous support in my difficult times. Thank you all so much!

To the Love of my Life and best friend, **Ikeeh Osuagwuh,** I am eternally grateful for all the sacrifices you made on my behalf. You were my teacher, mentor, confidant, and pillar of support all through and during this period. You pushed and keep pushing me to be a better version of myself, and I will continue to be deeply grateful to you for being part of my life.

Finally, to my friends **Loveth, Esther**, **Nene**, **Naza**, **Blessing**, **Jecy**, **Chioma** to mention but a few. You guys are much appreciated and can never be forgotten. I appreciate your calls and messages all through.

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SUMMARY

The *Cabra Blanca de Rasquera* is a native Catalonian breed of goats from the southern region of Catalonia enlisted as an endangered breed at the verge of extinction. To maintain the existence of this breed, the Food and Agricultural Organization of the United Nation (FAO) endorsed the preservation of breeds in danger of extinction by establishing *in situ* and *ex situ* conservation programmes (FAO, 2010). *Ex situ* programmes such as semen cryopreservation and the development of a genetic sperm bank enables the storage of semen indefinitely through various processes such as semen collection, processing, and freezing. Furthermore, the utilization of this process towards Assisted Reproductive Technologies (ART) is of major importance in preserving the genetic diversity of this breed. However, improving the post thaw sperm quality of this species is still a major concern. Several technological strategies have been proposed in order to achieve a better post thaw sperm quality, using various techniques such as the computer assisted sperm analysis (CASA), Sperm Chromatin Structural Assay (SCSA) and other different analyses through flow cytometer to assess sperm quality. Therefore, the major goal of this doctoral thesis was to establish new approaches in the protocols after thawing of cryopreserved sperm bank doses of this local breed, *Cabra Blanca de Rasquera* in danger of extinction with the aim of improving their post thaw viability and fertility.

In this thesis, we evaluated the effect of Single Layer Centrifugation (SLC) technique using Bovipure® colloid on the quality of sperm after thawing in relation to various factors such as donor age, season of collection and the effect of melatonin implant on the males during the non-breeding season. The aim was to determine if centrifugation through Bovipure® colloid can select viable spermatozoa of good motile abilities, active mitochondria, intact DNA and membrane integrity from bad and moribund ones after thawing in order to mimic the same process seen during natural selection in the female reproductive tract. In this study, two ejaculates/male/day were collected (n = 6 days) via artificial vagina from 6-8 males during 2 consecutive years in the breeding and non-breeding periods, at 12 and 24 months old in autumn and at 18 and 30 months old in spring. Prior to semen collection in the non-breeding period, males were randomly divided into two groups: one group was implanted with melatonin, while the other was not. After thawing, results for the total viability, sperm with intact acrosome and active mitochondria showed significantly higher values in samples collected in autumn compared to those collected during spring regardless of melatonin implant. However, these differences

were not reflected after SLC. Regarding the DNA integrity, there were no major significant differences found after thawing and after SLC. However, after SLC, the High DNA Stainability (HDS) showed significantly higher values during autumn compared to those in spring with or without melatonin implant. In regards to the motion parameters, the progressive motility (PM) after thawing showed significantly higher values during autumn compared to samples collected during spring regardless of melatonin implant. Nonetheless, these significant values were not reflected after SLC. In relation to the distribution of the motile sperm subpopulation, no major significant differences were observed after thawing in relation to age, season, and melatonin treatment except the SP3 sperm (characterised by sperm with high velocities and low linearity) which were slightly higher in autumn compared to implanted males in spring. Also, after SLC, no significant improvements were observed in the sperm motile subpopulation distribution. This study demonstrates that SLC through Bovipure® colloid after thawing did not improve cryopreserved buck sperm quality regardless of the male age, semen collection season and melatonin implant of the donors.

In this thesis, the effect of incubating thawed sperm samples for 3 hours under capacitation conditions in order to mimic the survivability of thawed sperm for a period of time during active migration in the female reproductive tract, in relation to donor age, season of collection, and melatonin implants of the males in the non-breeding season was investigated. On the other hand, the role of seminal plasma has been extensively studied for different groups, and it has been found that it provides a source of nutrient to the spermatozoa, improves sperm motility, prevents premature activation during migration of spermatozoa in the female reproductive tract (Desnoyers & Manjunath, 1992; Rozeboom et al., 2000a). Therefore, the addition of seminal plasma to frozen-thawed sperm could be implemented as an alternative to improving sperm quality (Maxwell et al., 2007). In the present thesis, the addition of 20% seminal plasma (SP) in the incubation media for 3h under *in vitro* capacitation conditions in order to improve sperm quality after thawing was evaluated. Our results showed that a significant reduction on motility, viability, mitochondria activity and acrosome integrity were observed after 3 h incubation under *in vitro* capacitation conditions, regardless of the addition of SP in the media. Similarly, the addition of SP did not improve the distribution of the sperm motile subpopulation. While there

was no effect on the DNA integrity. Therefore, the addition of SP failed to improve post thaw buck sperm quality irrespective of male age, season of collection and melatonin implant.

Furthermore, the assessment of individual male factor on post-thawed buck sperm quality and on the effectiveness of SLC was carried out, since the ability of spermatozoa to survive in the female reproductive tract may vary according to different individual males. To this regard, semen was collected via artificial vagina from 6 male goats of the *Cabra Blanca de Rasquera* breed, approximately 2 years of age in early winter. After carrying out various analyses, few significant differences were observed immediately after thawing on total sperm viability, mitochondria activity and acrosome membrane integrity between individual males, while in regards to the DNA integrity, total and progressive motility, no significant differences were observed between donors after thawing. Similarly, in relation to the motile sperm subpopulation distribution, no significant differences were found. Furthermore, after SLC, only few individual differences related to the effectiveness of SLC were observed, where 2 males from the 6 tested males showed significantly reduced acrosome damage. In regards to the other sperm analysed parameters, no significant effect of SLC was observed between males.

Lastly, the variations that exist between males can also influence differences in the male freezabilities with respect to their survival in the female reproductive tract (Shannon, 1978). Some ejaculates may contain subpopulations of spermatozoa that vary in fertilizing capacity, and these characteristics are peculiar to each male (Gomes et al., 2020). Therefore, the last study was the assessment of individual male factor on post-thawed buck sperm quality after 3 h incubation under capacitation conditions with the aim to determine if individual male variability may be reflected on their response to *in-vitro* incubation under capacitation conditions with or without the presence of seminal plasma thus mimicking the survivability during migration in the female reproductive tract. We found that few individual differences were observed in the motility, viability, acrosome membrane integrity, and mitochondria function. Also few differences were observed in the sperm motile subpopulation distribution after 3hours incubation and the presence of seminal plasma significantly reduced these parameters in all the males without improving the overall quality of these parameters. However, in regards to the DNA integrity, individual differences were less manifested after 3h incubation in the different media, which were inexistent in the presence of SP.

From the results obtained in this study, we can conclude that the frozen-thawed seminal doses from *Cabra Blanca de Rasquera* semen bank collected during autumn showed better qualities than the thawed sperm doses obtained during spring or winter. Notwithstanding, other factors such as donor age and melatonin implant during the non-breeding season did not improve thawed sperm quality. Also Single layer centrifugation procedure through Bovipure® colloid did not improve the quality of thawed sperm samples from this breed and as such may not be useful in selecting spermatozoa of good quality after thawing towards its use for assisted reproductive technologies. Finally, the addition of seminal plasma after thawing, or during *in-vitro* incubation did not improve the overall quality of spermatozoa. Therefore, it does not seem to be an effective alternative to be used with the aim of improving the viability and fertility of frozen sperm doses from *Cabra Blanca de Rasquera* semen bank in artificial insemination programmes or towards its use for other assisted reproductive techniques.

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RESUMEN

La *Cabra Blanca de Rasquera* es una raza autóctona catalana en peligro de extinción de la zona sur de Cataluña. Para mantener su existencia, la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO) recomienda para la conservación de las razas catalogadas en riesgo de extinción establecer programas de conservación tanto *in situ* como and ex situ (FAO, 2010). Los programas de conservación *ex situ* como es la crioconservación espermática y la creación de bancos de germoplasma permiten el almacenamiento del semen indefinidamente a través de varios procesos como la recogida de semen y su congelación para su posterior utilización en las diferentes Tecnologías de Reproducción Asistida (ART), las cuales son de gran importancia en la conservación de la diversidad genética en estas razas. Sin embargo, mejorar la calidad de los espermatozoides descongelados en esta especie es todavía una gran preocupación. Diferentes estrategias tecnológicas han sido propuestos para lograr una mejor calidad seminal tras la descongelación, usando varias técnicas como el análisis informatizado de la motilidad espermática (*Computer Assisted Sperm Analysis,* CASA), ensayos sobre la estructura de la cromatina espermática (*Sperm Chromatin Structural Assay*, SCSA), así como otros análisis realizados a través de la utilización del citómetro de flujo para valorar las distintas características espermáticas. Por lo tanto, la principal meta de esta tesis doctoral ha sido establecer nuevos enfoques en los protocolos tras descongelación de las dosis espermáticas crioconservadas del banco de la raza autóctona Cabra Blanca de Rasquera en peligro de extinción con el objetivo de mejorar su viabilidad y fertilidad tras la descongelación.

En la presente tesis, se ha evaluado el efecto de la técnica de centrifugación en capa única (*Single Layer Centrifugation*, SLC) usando la solución de coloide comercial Bovipure®, sobre la calidad espermática tras la descongelación en relación a varios factores como la edad del donante, la estación de la recogida del semen y la administración de implantes de melatonina en los machos en la época no reproductiva. El objetivo fue determinar si la centrifugación a través del coloide Bovipure® puede seleccionar espermatozoides viables con buenas habilidades cinéticas, ADN intacto, mitocondrias activas e integridad de membranas plasmática y acrosomal, de los espermatozoides de mala calidad, muertos o moribundos tras la descongelación con la finalidad de mimetizar el mismo proceso observado en la selección natural que ocurre en el tracto reproductor femenino. Para ello, en este estudio, se recogieron 2 eyaculados/macho/día (n = 6 días) vía vagina artificial de 6-8 machos durante 2 años consecutivos en el periodo reproductivo y no reproductivo a la edad de 12 y 24 meses en otoño y de 18 y 30 meses de edad

en primavera. Previa a la recogida de semen en el periodo no reproductivo, los machos fueron repartidos al azar en 2 grupos (4 machos/grupo): a un grupo se les administró implantes subcutáneos de melatonina mientras que el otro grupo permaneció sin tratar. Tras descongelar, los resultados de viabilidad espermática total y el porcentaje de espermatozoides con acrosoma intacto y mitocondrias activas mostraron valores significativamente superiores en las muestras recogidas en otoño comparado con las muestras de primavera. Sin embargo, estas diferencias no se reflejaron tras realizar la técnica SLC. Respecto a la integridad del ADN, no se observaron diferencias significativas tras la descongelación, aunque la proporción de espermatozoides con HDS (Alta estabilidad del ADN) fue superior en las muestras seminales recogidas durante otoño. Referente a los parámetros cinéticos, la motilidad progresiva fue significativamente superior también durante otoño comparado con las muestras recogidas en primavera una vez descongeladas. No obstante, estas diferencias desaparecieron tras llevar a cabo la centrifugación (SLC) de las distintas muestras. Finalmente, no se observaron grandes diferencias significativas entre las muestras seminales no seleccionadas y las que fueron sometidas a la técnica SLC respecto a la motilidad total y progresiva, viabilidad, actividad mitocondrial el integridad acrosomal y de ADN, lo que demuestra que la centrifugación simple a través del coloide Bovipure® de los espermatozoides descongelados no mejoró su calidad, independientemente de la edad del macho, la estación durante la que se realizó la recogida del semen o la administración de implantes de melatonina a los machos.

Además, este trabajo ha evaluado el efecto de incubar las distintas muestras seminales descongeladas bajo condiciones capacitantes durante 3 horas para intentar simular la supervivencia de los espermatozoides descongelados durante la migración activa en el tracto reproductor femenino, en relación a la edad del donante, estación del año de la recogida del semen o la aplicación de implantes de melatonina en la época no reproductiva. Por otra parte, el papel del plasma seminal ha sido extensamente estudiado por diferentes grupos, el cual proporciona una fuente de nutrientes para los espermatozoides, mejora la motilidad espermática y previene la activación prematura del espermatozoide durante su migración por el tracto femenino (Desnoyers & Manjunath, 1992; Rozeboom et al., 2000a). Por lo que la adición de plasma seminal a los espermatozoides descongelados podría ser implementada como una alternativa para mejorar la calidad seminal (Maxwell et al., 2007). Así, en la presente tesis, también se ha evaluado la adición de un 20% de plasma seminal en el medio durante la

incubación de 3h bajo condiciones capacitantes con la finalidad de mejorar la calidad espermática tras la descongelación. Sin embargo, los resultados obtenidos mostraron una significativa reducción de la motilidad y viabilidad espermática y la actividad mitocondrial, así como también de la integridad del acrosoma tras 3 h de incubación bajo condiciones *in vitro* de capacitación, independientemente de la presencia de plasma seminal en el medio, mientras que la integridad del ADN no se vio afectada. Estos resultados parecen indicar que la adición de plasma seminal no es útil como estrategia para mejorar la calidad de los espermatozoides descongelados de macho cabrío, independientemente de los factores estudiados anteriormente citados.

Asimismo, el efecto del factor individual del donante sobre la calidad seminal post-descongelación y sobre la eficacia de la técnica de selección SLC fue evaluado, ya que la habilidad del espermatozoide a sobrevivir en el tracto genital femenino puede variar en función del macho. Con este objetivo, se recogieron a principios de invierno 2 eyaculados/macho/día (n = 6 días) vía vagina artificial de 6 machos de la raza Cabra Blanca de Rasquera de aproximadamente 2 años de edad. Tras llevar a cabo los diferentes análisis, pocas diferencias significativas fueron observadas tras la descongelación de las dosis individuales en la viabilidad espermática, actividad mitocondrial e integridad acrosomal entre los distintos machos, mientras que, respecto a la integridad del ADN, motilidad total y progresiva, ninguna diferencia fue detectada entre donantes. Además, tras realizar la técnica de la SLC, pocas diferencias individuales relacionadas con la efectividad de este método fueron observadas, únicamente 2 machos de los 6 testados mostraron una reducción en el daño acrosomal de las dosis, mientras que en el resto de parámetros seminales analizados no se observaron diferencias significativas entre individuos tras SLC.

Finalmente, las posibles variaciones entre machos pueden también producir diferencias en la congelabilidad del semen con respecto a su supervivencia en el tracto reproductivo femenino (Shannon, 1978). Algunos eyaculados pueden contener subpoblaciones de espermatozoides que difieran en su capacidad fecundante y cuyas características son peculiares a cada macho (Gomes et al., 2020). Por este motivo, el último estudio de la presente tesis se centró en la evaluación del factor individual-macho sobre la calidad espermática post-descongelación tras 3 horas de incubación bajo condiciones *in vitro* de capacitación con el objetivo de determinar si la variabilidad individual puede verse reflejada en su respuesta a estas condiciones en presencia o no de plasma seminal, intentando simular la supervivencia en condiciones in vivo.

Los resultados indicaron escasas diferencias individuales en la motilidad, viabilidad, integridad acrosomal y función mitocondrial tras la incubación y que la presencia de plasma seminal redujo significativamente estos parámetros en todos los machos, mientras que respecto a la integridad del ADN, diferencias individuales fueron menos evidentes tras 3 horas de incubación en los diferentes medios, siendo inexistentes en presencia de plasma seminal.

Así, a partir de los resultados obtenidos en la presente tesis, podemos concluir que las dosis seminales descongeladas de la raza Blanca de Rasquera a partir de eyaculados obtenidos en otoño presentan mejores cualidades que las obtenidas en primavera o invierno. A pesar de ello, otros factores como la edad del donante o la aplicación de implantes de melatonina durante la estación no reproductiva no mejoraron la calidad seminal post-descongelación, así como tampoco el hecho de someter las muestras descongeladas a un supuesto proceso de selección espermática mediante la técnica de SLC a través del coloide Bovipure®. Esta ausencia de mejora de la calidad de los espermatozoides descongelados sugiere que esta técnica de selección espermática no es útil para su aplicación en tecnologías de reproducción asistida en esta raza. Por último, la adición de plasma seminal en el medio durante la incubación de las dosis descongeladas en condiciones *in vitro* de capacitación tampoco mejoró la calidad espermática, por lo que no parece ser una alternativa efectiva para ser usada con el objetivo de incrementar la viabilidad y fertilidad de las dosis crioconservadas del banco de semen de la raza Cabra Blanca de Rasquera en programas de inseminación artificial o en otras tecnologías de reproducción asistida.

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Chapter I

INTRODUCTION

Few years ago, various surveys reported that several animal species have suffered a sharp decline in population, and approximately 20% are in danger of extinction due to the discontinuity and low generational changes associated with farmers (Jordana et al., 2010). According to the International Union for Conservation of Nature (IUCN) of the recent FAO report 2020, out of the 120,372 species currently tracked by the IUCN, there are 6,811 species that are considered to be critically endangered. However, most domestic species such as birds, pigs, and milk cattle reared intensively are not exempted and most local and native breeds are included. In Catalonia, most native breeds are at the verge of extinction (Parés et al., 2006).

In recent times, the *Cabra Blanca de Rasquera* native breed of Catalan goats, located in the southern region of Catalonia has been enlisted in danger of extinction in relation to the last population census whereby only 6,000 out of 30,000 were counted in the early fifties. Furthermore, the restoration of this breed is quite difficult because most farmers cannot guarantee the continuity of this breed due to lack of interest by their new generations (Jordana et al., 2010). The *Cabra Blanca de Rasquera* breed is of utmost importance, due to it possess high valued traits such as small land productivity with low cost resources, best economic use, and maintenance of ecosystems of great landscape and environmental value (Torres, 2007).

To preserve this breed, a new approach in accordance with the FAO was established towards the characterization of this type of breeds which consists of various phases: racial characterization, general description of the population, *in situ* and *ex situ* conservation, and genetic improvement programmes. Furthermore, *ex situ* programmes such as assisted reproductive techniques (ART) which includes semen cryopreservation and the development of a semen bank has also been reported to help in the conservation of this breed (Tabarez, 2014).

Few years ago, a study investigating the ideal goat sperm freezing protocols, which includes the choice of cryoprotectants, effect of seminal plasma and antioxidants, factors affecting fresh and frozen semen quality in relation to donor age, time of the year, and influence of melatonin during the positive photoperiod was carried out by our unit (Tabarez, 2014). Despite the above study, a more detailed investigation is required to evaluate sperm parameters in order to ascertain its quality after cryopreservation/post thaw and also to predict its fertilizing ability (Gadea et al., 2005; Rodriguez-Gil, 2006).

Therefore, several strategies have been implemented to improve the post thaw semen quality and the survivability in the female reproductive tract. With the onset of technological advancement, sperm analyses techniques such the CASA system and flow cytometer have been implemented, due to the accuracy and detailed information on sperm motile/kinematic characteristics, viability, functional integrity, and DNA integrity. With the aforementioned techniques, detailed evaluations of post thaw semen quality can be assessed and thus the survival of the *Cabra Blanca de Rasquera* breed can be explored (Tabarez, 2014). Furthermore, different opinions on the idea and knowledge of sperm quality have been sampled, while some have been described in terms of number of spermatozoa, motility, and normal morphology (Gadea et al., 2005), others have included parameters such as sperm membrane and DNA integrity (Graham, 2001; Rodríguez-Martínez, 2003) and mitochondria function (Srivastava & Pande, 2016). Furthermore, sperm selection can be used to improve the quality of spermatozoa after cryopreservation (Morrell & Rodriguez-Martinez, 2009). With the onset of sperm selection techniques, the Density Gradient Centrifugation (DGC) and Single Layer Centrifugation (SLC) have been proposed to mimic the natural selection in the female reproductive tract thereby separating normal motile spermatozoa with a good and intact chromatin from the rest of the sperm sample (Morrell, 2006). However, SLC provides a more practical approach compared to DGC for selection of viable, potentially fertile, frozen-thawed spermatozoa (Macías García et al., 2009).

After cryopreservation, the maintenance of frozen thawed spermatozoa over an extended period of time in the presence of seminal plasma has been indicated to improve sperm survival (Rebolledo et al., 2009; Maxwell et al., 2007). Also several factors such as donor age (Mandiki et al., 1998), season (Zarazaga et al., 2012), individual male factor (Curry, 2000) and melatonin treatment (Casao et al., 2010) could affect the quality of spermatozoa after thawing. Furthermore, evaluation of individual variability after thawing and during 3h incubation period under capacitation condition, thus mimicking the environment in the female reproductive tract could provide an understanding of the differences in sperm survival, and fertilizing ability after artificial insemination (Shannon, 1978).

With the above knowledge, we hypothesised that implementing different sperm techniques and methods in the post thaw processes after cryopreservation would enhance the survival and fertility of the *Cabra Blanca de Rasquera* breed.

Chapter II

OBJECTIVES

The main goal of this Doctoral Thesis has been the establishment of new approaches in the protocols after thawing of cryopreserved sperm doses from the semen bank of the autochthonous breed *Cabra Blanca de Rasquera* in danger of extinction with the aim of improving their post thaw viability and fertility.

Therefore, the following specific objectives were suggested:

1. To investigate the efficiency of Single Layer Centrifugation (SLC) through Bovipure® colloid as sperm selection method, taking into consideration different factors such as donor age, season of semen collection and melatonin treatment of males.

2. To study the behaviour of washed thawed buck sperm after 3h incubation period under *in vitro* capacitation conditions mimicking the female reproductive tract, with regards to donor age, seasons and melatonin treatment.

3. To assess the effect of adding seminal plasma (SP) to different incubation media on thawed sperm quality with regards to donor age, seasons, and melatonin treatment.

4. To assess the effect of individual male factor on the efficiency of Single Layer Centrifugation as post thaw buck sperm selection method.

5. To determine the existence of individual differences in the freezability of males and in their response to *in vitro* incubation with or without seminal plasma (SP) under capacitation conditions.

HYPOTHESIS

1. Single Layer Centrifugation method may improve cryopreserved buck sperm quality, by selecting spermatozoa of better quality irrespective of individual male factor, donor age, season of collection, and melatonin treatment.

2. That subjecting seminal doses after thawing to incubation with seminal plasma (20%) under capacitation conditions mimicking the female tract could improve the quality of thawed buck sperm.

3. Thawed buck sperm quality after 3h incubation under capacitation conditions will better predict the freezability of spermatozoa in the female reproductive tract.

Chapter III

LITERATURE REVIEW

3.1. Brief history and description of the *Cabra Blanca de Rasquera* breed

The *Blanca de Rasquera* goats are said to be one of the relevant breed of goats since the mid-20th century (Parés et al., 2006), located in the southern region of Catalonia. They have a characteristic white and black fur colour. The white colour of their fur usually predominates over the black colour, hence the name "*white of Rasquera*", although in recent times, goats with a predominantly black coloured fur have been seen (Francesch et al., 2002). The origin of this breed is still uncertain, although Jordana et al. (2010) postulated an ancestral linkage between "*capra prisca*" and "*capra aegagrus*"of the *pyranean* form. The *Blanca de Rasquera* breed is particularly known for their great meat quality and taste weighing between 7-10kg. They are seasonal breeders and are slaughtered at two months of age especially during the Christmas season, hence an increase in consumption during this period. They have an extensive breeding ability primarily for the production of kids which is of economic advantage to farmers (Parés, 2017). Due to their great rusticity, they are usually found around the mountainous environment where they feed on shrubs and bushes which enhance their adaptive abilities to the geographical location of southern Catalonia (Vidal, 2010).

With regards to its extinction, the racial classification of this breed of goat in several southern regions of Catalonia is important for the maintenance and conservation of this genetic line (Francesch et al., 2002). A conservation programme which kick started in 2004 sponsored by the *Departament d'Agricultura, Ramaderia, Pesca, Alimentació* (DARP) *de la Generalitat de Catalunya* in collaboration with the Autonomous University of Barcelona (UAB), was established to propose a standard for characterization of this indigenous Catalonian breed in order to initiate the publication of the genealogical book (Carné et al., 2007). Later in 2010, the *Instituto Nacional de Investigaciones Agroalimentaria* (INIA) alongside the Programme of Preservation of Zoogenetic Resources financed two projects of UAB researchers with the objective of creating a genetic resource bank for this breed presumed to be endangered.

3.2 Creating a semen bank of *Cabra Blanca de Rasquera*

The cryopreservation of semen has become an important aspect of reproductive technology, and the creation of a sperm bank was necessary to prevent the loss of genetic resource materials from animals going into extinction. This allows the storage of genetic materials such as semen for a long period of time (Holt & Pickard, 1999; Roldan et al., 2006).

However, to create a successful sperm cryobank, an effective collection, freezing and evaluation technique is required, therefore understanding the basic reproductive physiology of these endangered breed is important.

However, sperm cryopreservation remains challenging despite the fact that all sperm cells undergo similar physical stress during cryopreservation, there are still differences in size, shape, and lipid composition. For instance, buck sperm requires exceptional attention to enhance post thaw viability due to the deleterious interaction between egg yolk and the bulbourethral gland secretions which does not exist in other species (Purdy, 2006). Despite the similarities in most sperm cryopreservation protocols used in small ruminants, some differences still exist between and within species due to breed or species characteristics and response of sperm to cryopreservation protocols (Jiménez-Rabadán et al., 2016).

3.3. Factors affecting buck semen production and its preservation

One of the first pioneer work on buck semen cryopreservation was carried out by Smith & Polge, (1950). Furthermore, due to the low fertility rates observed with the use of frozen – thawed goat semen, necessary steps were adopted to improve buck sperm freezing by establishing basic cryopreservation protocols such as collection and extension of semen, addition of cryoprotectant, cooling above 0 ºC, cooling below 0 ºC, storage and thawing (Curry, 2007).

Furthermore, goat semen cryopreservation is distinctly different from processing of other semen from cattle or buffalo, and hence remains a challenge because of the peculiarity observed in this species. The seminal plasma of goat semen contains Egg Yolk Coagulating enzymes, EYCE (phospholipase A2) and SBUIII from the secretions of the bulbourethral glands that interacts with the egg yolk and skimmed milk, an essential component of semen extender thus producing toxic substances that affects sperm cells (Gangwar et al., 2016; Purdy, 2006) and reduces its freezing ability (Leboeuf et al., 2000). Therefore, the removal of seminal plasma before sperm freezing is recommended in buck to avoid these adverse interactions (Kozdrowski et al., 2007).

Nevertheless, the success of semen preservation and the subsequent artificial insemination are also dependent on semen production and quality thus to meet the recent demand for assisted reproductive techniques (ART). However, many factors may be responsible for the quality of produced semen such as individual male factor, testicular size,

donor age, breed, nutrition, method, frequency and season and frequency of collection or seminal plasma composition., affecting cryopreservation which in turn influences reproductive outcome (Purdy, 2006). Also, the manipulation of the semen samples, the extender composition, freezing and thawing rates could affect the quality of the thawed sperm and subsequently the success of ART (De Leeuw et al., 1993; Salamon & Maxwell, 1995).

3.3.1. Individual male factor

Individual male factor has shown to affect cryopreservation of semen due to the composition of the lipid content of their cell membrane. For instance, some males are regarded as 'good or bad freezers' depending on the ability of their semen to withstand freezing (Barbas & Mascarenhas, 2009). This factor has been also reported in goats (Dorado et al., 2010), stallions (Yeste et al., 2015) and boars (Yeste et al., 2013). Furthermore, genetics have also shown to play a major role in the ability of semen to withstand freezing as seen in boars and goats, which can be determined by analysing the chromatin structure (Casas et al., 2009; Sundararaman & Edwin, 2008).

3.3.2. Donor age

The quality and quantity of semen produced is strongly influenced by age. Mandiki et al. (1998) reported an increase in semen parameters such as volume, concentration and motility and a decrease in sperm abnormalities with the age of the animal. Similar reports have been demonstrated in the Alpine and Damascus goats (Al-Ghalban et al., 2004) with both producing good quality semen at younger (6 months) and older age (2-4 years) respectively. Furthermore, the effect of age has shown to influence thawed sperm quality, and this has been demonstrated in boars with the highest post thaw ratings seen in younger boars (< 2years) compared to the older ones (>2years) (Joyal et al., 1986).

3.3.3. Testicular size

The testicular size can be ascertained by measuring the scrotal circumference, since sperm production is directly related to the circumference of the testicles. Literature has shown that more semen is produced by bucks with greater scrotal circumference (Langford et al., 1987). This suggests that a matured buck with a scrotal circumference of at least 25cm may be regarded as a good choice of selection, as well as a good indicator for semen production (Rege et al., 2000). To this regard, a study has shown that scrotal circumference though

depending on season of semen collection is another critical factor affecting the success of cryopreservation, thus affecting post-thawed semen quality (Kulaksiz et al., 2020).

3.3.4. Breed

Literature has shown that certain breed of males tend to affect the production and quality of semen (Leboeuf et al., 2000). Breeds like the Creole or Payoya goat showed better semen characteristics compared to the Black Bengal breed (Delgadillo et al., 2004; Islam et al., 2008). Also, to the best of our knowledge, results obtained by our previous team indicated that *Blanca de Rasquera* breed showed better semen characteristics compared to the *Blanca Celtibérica* goat breed (Jiménez Rabadán, 2013). However, there are limited data on the effect of buck breed on frozen-thawed semen quality and most of the studies report results after the comparison between only two breeds on fresh semen quality. Nonetheless, a study showed that breed influenced post-thaw sperm quality in pigs, with the ejaculates of Landrace and Pietrain boars having higher post-thaw sperm motility and viability compared to Duroc, Large White, Yorkshire and cross-bred boars (Roca et al., 2006).

3.3.5. Season

Seasonal variations and photoperiod is an important factor affecting goat semen production at high or mid-levels (Gordon, 1997; Shackell et al., 1977). Furthermore, majority of male goats found in the temperate regions are seasonal breeders. An increase in reproductive activity have been demonstrated during the autumn/winter period known as the breeding season (Coloma et al., 2011b; Mandiki et al., 1998; Pérez & Mateos, 1996). Other environmental factors such as social stimuli, feed availability, ambient temperature, and daylength are also regarded as important regulators of seasonality in buck reproduction (Ungerfeld & Bielli, 2012). Decrease in daylength increases testosterone secretion to stimulate reproductive activity (Chemineau et al., 1992), while morphological abnormal semen parameters increases when males are exposed to high temperatures/humidity (Marai et al., 2007). In sexually matured males, heat stress as a result of excessive hot temperatures tends to affect their reproductive ability which in turn affects semen production and fertilizing rate (Daader et al., 1985). Equally, seasonal effect has been reported to affect the ability of spermatozoa to withstand freeze-thawing process in most domestic species (Coloma et al., 2011; Janett et al., 2003; Medrano et al., 2010).

3.3.6. Male treatments

With the onset of assisted reproductive techniques (ART), semen availability along the year is desirable also for seasonal breeders. In small ruminants, sperm production can be manipulated to meet the demand of farmers especially outside the breeding season with subcutaneous melatonin implants (Casao et al., 2008). Melatonin implants has been demonstrated to improve reproductive abilities in and out the breeding period (Palacín et al., 2008). A higher amount of melatonin is secreted during the dark phase of the total daylength, therefore decreasing daylight enhances the reproductive ability in bucks/does and vice versa (Delgadillo & Chemineau, 1992). Reports have shown that the use of subcutaneous melatonin implants is stimulatory and mimics the positive effects of short photoperiod in rams and buck reproduction (Cortez, et al., 2004). Furthermore in bucks, sexual behaviour and testicular activity are influenced by photoperiodic change. Also photo stimulation treatment of bucks with electric lamps (lamps of 65 W with a light intensity of 300 lx at the level of the buck eyes) showed a high variation of sexual behaviour when exposed to ovariectomized females (Sifuentes et al., 2020). Treatment of the Merino rams with melatonin implants during the non-breeding produced spermatozoa with improved progressive motility and DNA integrity after thawing (Pool et al., 2020).

3.3.7. Semen collection method

Semen collection is best performed during the natural breeding phase (autumn/early winter) to achieve the best semen sample for processing and storage. Consequently, sperm production and quality has been reported to be affected by the method of collection. The use of an effective semen collection method is an important factor towards achieving a successful cryopreservation. The most commonly used methods are the artificial vagina and electro ejaculator even though the artificial vagina has been reported to give better results (Leboeuf et al., 2000). Semen collection may require an expertise especially in cases of collecting semen from wild ruminants and untrained males (Jiménez-Rabadán et al., 2012; Wulster-Radcliffe et al., 2001).

However, either methods of collection have been reported to affect the quality of fresh and post-thawed semen (Greyling & Grobbelaar, 1983). Previously, ejaculates with greater volume and lower concentrations were obtained with EE as opposed to AV, and this has been reported in various species (Austin et al., 1968; Dziuk et al., 1954; Mattner & Voglmayr, 1962). Production of a larger amount of seminal plasma has been associated with semen

collection via EE due to the electric stimulation of the accessory glands compared to ejaculates collected via AV (Jiménez-Rabadán et al., 2012). However, The AV method of collecting semen is the closest to natural service; it is the fastest and most hygienic of the methods available, but requires training of the buck (Wulster-Radcliffe et al., 2001).

3.3.8. Seminal plasma composition

Seminal plasma is a complex fluid secreted by the seminiferous tubules of the testes and accessory glands which serves as a medium for protection, transport, and nourishment for sperm cells and also regulates sperm function (Maxwell et al., 2007; Mendoza et al., 2013). Seminal plasma is composed of proteins, electrolytes, lipids, and other factors which are vital for metabolic regulation of sperm cells (Mendoza et al., 2013). Reports have demonstrated that semen with low seminal plasma ration is associated with poor semen quality (Ashworth et al., 1994; Pérez-Pé et al., 2001). Also decrease in sperm motility and concentration has been related to low seminal plasma lipids (Taha et al., 2000).

In buck sperm cryopreservation, due to the harmful interaction between seminal plasma and cryoprotectants containing egg yolk, it has been suggested that the removal of seminal plasma by centrifugation has shown to improve post thawed semen quality (Kozdrowski et al., 2007). While other studies have shown no effect in the quality of thawed semen after removal of seminal plasma (Morrier et al., 2011) or detrimental effect (Moore et al., 2005; Rovegno et al., 2013). These discrepancies may be due to the complex nature of seminal plasma in various species (Mandiki et al., 1998).

Buck seminal plasma contains enzymes such as the egg yolk coagulating enzyme (EYCE) and bulbourethral III secretion (SBUIII) which are secreted by bulbourethral glands and responsible for phospholipase activity. EYCE hydrolyzes egg yolk lecithin contained in freezing extenders into fatty acids and lysolecithin (Iritani & Nishikawa, 1964; Roy, 1957). Similarly, SBUIII hydrolyzes residual triglycerides in the skim milk from freezing extenders resulting in fatty acids which are toxic to spermatozoa (Pellicer-Rubio & Combarnous, 1998). Therefore removal of seminal plasma by centrifugation is suggested to prevent this harmful effect, thus increasing sperm motility, integrity of the membrane after freeze-thawing (Kozdrowski et al., 2007). Although it has been reported that the addition of seminal plasma after thawing improve semen penetration through the cervical mucus especially during cervical insemination in ewes (Maxwell & Johnson, 1999; Swelum et al., 2018) which could

be a potential strategy to be used after thawing in order to improve buck thawed sperm quality.

3.3.9. Cryoprotectants, diluents, and freezing extenders

Due to the susceptibility of sperm cells to extreme temperatures and cryo-damages during freezing, maintenance of a suitable environment is required to ensure sperm survival (Purdy, 2006). Glycerol has been reported as the frequently used cryoprotective agent during cryopreservation in most species due to its ability to reduce bacteria growth during cryopreservation as well as reduces the damage caused by the formation of ice crystals in the cytoplasm of the sperm cell (Holt, 2000; Salamon & Maxwell, 1995).

In goats the most common non-penetrating cryoprotectant used during cryopreservation is egg yolk (Ritar & Salamon, 1982; Tuli & Holtz, 1994). However, the harmful interaction between egg yolk and goat semen containing seminal plasma has shown to be detrimental due to and the presence of egg yolk coagulating enzyme (EYCE) secreted by the bulbourethral gland (Roy, 1957). Also, skim milk has demonstrated similar interactions like egg yolk due to the presence of SBUIII enzyme from buck seminal plasma (Leboeuf et al., 2000). Therefore, removal of the seminal plasma in goat semen is necessary to prevent these cryogenic damages. However in recent years, the use of cryoprotectants such soy lecithin has been implemented in goat sperm cryopreservation (Roof et al., 2012; Vidal et al., 2012). This process occurs by two processes: replacement of the some of the phospholipids of the sperm membrane to maintain the structure and function of the plasma membrane, and secondly by forming a protective film around the cell to prevent the formation of intracellular ice crystals which may lead to sperm damage during freeze-thawing.

Tris –citric acid has been regarded as the most preferred used buffer and diluent for the freezing of goat spermatozoa (Mishra et al., 1970). Changes in semen pH can result in cellular and sub-cellular damages to the sperm cell, thus affecting the viability and fertilizing ability. However most diluents contain compounds with buffering capacity necessary to maintain suitable environment by controlling pH fluctuations in the cryopreservation media, stability of the plasma membrane and neutralizes acids generated during in-vitro storage (Purdy, 2006). Also Tris based extenders should contain carbohydrates (glucose, lactose,

raffinose, and saccharose) to provide nutrients for the extender (Barbas & Mascarenhas, 2009).

3.3.10. Freezing and thawing rate

Freezing and thawing process is an important aspect of the post-thawed survivability of goat sperm after cryopreservation (Evans & Maxwell, 1987). However, most cells cannot withstand the freezing process in the absence of a cryoprotectant. An average of 50% of goat sperm cells survive after cryopreservation due to thermal, biochemical and mechanical stress encountered during the process (Gangwar et al., 2016). Freezing of sperm cells rapidly under low temperatures can result in the formation of intracellular ice crystals, a phenomenon known as re-crystallization during freezing and subsequently cell injury and death (Watson, 1995). Slow cooling of buck semen has been reported to increase sperm survival rate compared to fast cooling which has an adverse effect on buck sperm freezability (Purdy, 2006).

The thawing of sperm samples is determined by the method used to freeze the semen. The optimal thawing speed depends on the cooling pattern, however the ultimate goal is to minimise damages caused by either the formation of intracellular ice-crystals or rapid transport of solute through the cell membrane (Hammerstedt et al., 1990). Sperm cells encounter damages during freezing, therefore adequate handling must be ensured during the defrosting process to prevent further damages.

Evans & Maxwell, (1987) suggested that buck sperm straws should be thawed at a temperature not below 37^{o}C. Thawing rates should be indirectly proportional to the applied temperature; thawing sperm cells above the required temperature can be fatal to the sperm cells (Tuli et al., 1991).

3.4. Sperm selection techniques or how to improve thawed sperm quality

In an ideal situation *in vivo*, potentially fertile spermatozoa undergo natural selection in the female genital tract, during the process of migration through the cervical mucus whereby motile spermatozoa are separated from immotile spermatozoa, debris, and seminal plasma (Henkel & Schill, 2003). During this process, motile spermatozoa also undergo physiological changes called capacitation which primes spermatozoa prior to acrosome reaction (Bedford, 1983). However, with the introduction of assisted reproductive techniques (ART), various methods of selection have been developed to mimic the selection that takes

place in the female reproductive tract (Morrell & Rodriguez-Martinez, 2009). Apart from the selection of robust spermatozoa with good quality and fertilizing ability, sperm selection has also been used to remove seminal plasma from the rest of the ejaculate.

These techniques can be categorized based in sperm selection techniques according to certain sperm characteristics and/or in sperm washing techniques on the basis of the removal of seminal plasma and/or cryoprotectants and debris. The methods that select spermatozoa on the basis of certain characteristics includes sperm migration (based on sperm motility), filtration (based on membrane integrity), and colloid centrifugation (based on sperm motility, morphology, viability and chromatin integrity) (Morrell & Rodriguez-Martinez, 2011).

3.4.1. Migration Technique

The self-propelled movement of spermatozoa is a crucial pre-requisite since this technique relies on the ability of spermatozoa to move from one medium to another and does not depend on morphology, acrosome or chromatin integrity (Mortimer, 2000). The migration techniques can again be subdivided into swim-up, under-lay and migration-sedimentation (Bedford, 1983).

In the migration techniques, spermatozoa with tail abnormalities are prevented from migrating into the swim-up medium. Furthermore, some studies have shown that adding hyaluronic acid to the migration medium may select spermatozoa with intact membrane (Shamsuddin & Rodriguez-Martinez, 1994). However, low recovery rate has been demonstrated as a major disadvantage of these techniques which makes them non-practical during sperm preparation for A.I., especially in cryopreserved sperm samples.

3.4.2. Filtration

This method of selection is based on the ability of spermatozoa to move, their retention and ability to adhere to filtration medium such as glass fibres, membrane pores or Sephadex beads (Mogas et al., 1999). Dead and immotile spermatozoa adhere to Sephadex particles due to changes in the surface proteins after capacitation which allows for sperm binding (Samper et al., 1995). Filtration technique has been reported to improve the post thawed viability of bovine and canine spermatozoa compared to the unfiltered sample (Januskauskas et al., 2005; Mogas et al., 1999). As opposed to the migration technique, fewer spermatozoa are lost during the process.

3.4.3. Sperm washing

This process involves the centrifugation of the extended ejaculate, such that the supernatant is removed and the sperm pellets are re-suspended in an extender. During this process, spermatozoa are thus separated from most of the seminal plasma (Björndahl et al., 2005). However, sperm washing does not improve the quality of the sperm samples since no selection occurs with this process and bulk of moribund or dead spermatozoa are retained in the process leading to the production of ROS which may be detrimental to spermatozoa (Hallap et al., 2004). In the equine breeding industry, sperm washing is mostly used to remove the bulk of seminal plasma prior to freezing (Morrell et al., 2010).

3.4.4. Colloid Centrifugation

This method involves the centrifugation of spermatozoa through a colloid which can be grouped into: density gradient centrifugation, and single layer centrifugation.

3.4.4.1. Density Gradient Centrifugation (DGC)

DGC is a conventional method of selection and has demonstrated to be a commonly used separation technique in practise due to its flexibility and ability to separate good sperm from bad or moribund sperm (Henkel & Schill, 2003). This method basically involves the use of colloids such as Androcoll® or Percoll® to separates sperm cells based on their densities. Since normal and abnormal sperm cells have different densities, morphologically normal sperm cells concentrate at the bottom of the tube, while moribund ones float at the top along with other cells (Henkel & Schill, 2003; WHO, 2010). DGC has been reported to improve sperm survival, total motility, and chromatin integrity (Morrell et al., 2004; Pertoft, 2000).

3.4.4.2. Single layer centrifugation (SLC)

Single layer colloid centrifugation is a more recent sperm selection technique whereby spermatozoa are centrifuged through a colloid known as silane-coated silica in a specie-specific formulation usually at low g force which results in the selection of a morphological intact spermatozoa, with an intact membrane and good chromatin integrity (Morrell et al., 2009).

Although a lot of sperm selection techniques are employed in ART, the SLC techniques has been reported to be more practical and easier to use especially for AI purposes

where semen can be scaled up to produce large volume of semen for stallions (Morrell et al., 2009) or smaller volume for boars (Morrell et al., 2011). SLC technique has also been indicated as a good sperm preparation method for ICSI from poor quality sperm samples (Morrell et al., 2010) and also for bovine IVF (Thys et al., 2009) by enhancing the rate of zygote to blastocyst development phase. Other major indication for SLC includes the improvement of post-thawing survivability of sperm cells after cryopreservation (Macías García et al., 2009b), removal of cryoprotectants and pathogens and improving the shelf-life of samples stored for *in vitro* fertilization in stallions (Morrell et al., 2009). Furthermore, in bucks, SLC has shown to improve post thawed semen quality (Jiménez-Rabadán et al., 2012).

3.5. Addition of seminal plasma after thawing as a potential strategy to improve sperm quality

Seminal plasma (SP) contains proteins that may influence cryosurvival and prevent capacitation-like changes. The role of Seminal plasma on spermatozoa spermatozoa have been widely studied and a various results have been found which includes: motility activation, maintenance of osmotic pressure and source of nutrients, prevents premature activation during migration in the female reproductive tract and plasmmalema stabilization by capacitation inhibitors (Desnoyer & Manjunath, 1992; Villemure et al., 2003). A beneficial effect of seminal plasma after thawing have been demonstrated by various authors, such an increase in sperm motility (White et al., 1987), reverts membrane damages (Domínguez Rebolledo et al., 2009), and overall sperm quality parameters (Maxwell et al., 2007). However, some other authors did not observe any effect of seminal plasma, while others found harmful effect on the sperm motility, and viability (Graham, 1994; Moore et al., 2005). The amount of seminal plasma protein may be affected by some factors such as season of collection, stress, temperature, feeding and collection method (Marco-Jiménez et al., 2008; Perez-Pe et al., 2001). Furthermore, seminal plasma contains some proteins which are secretions from epididymis and accessory sex glands (Chandonnet et al., 1990). The addition and removal of some these proteins during the process of sperm maturation in the epididymis and during sperm ejaculation plays an important role on the motility, and stability of plasmalemma (Desnoyers & Manjunath; Henricks et al., 1998).

Seminal plasma contains other compounds such as enzymes. For instance, an enzyme which plays an important role during acrosome reaction and spermatozoa-oocyte fusion is the phospholipase A2 (Yuan et al., 2003). In caprine, Egg yolk Coagulating Enzyme (EYCE) and

bulbourethral III secretion (SBUIII) are secreted by bulbourethral glands and have phospholipase activity. EYCE hydrolyses egg yolk lecithin contained in freezing extenders into fatty acids and lysolecithin, which is toxic to spermatozoa (Iritani & Nishikawa, 1961). Similarly, SBUIII hydrolyses residual triglycerides in the skim milk from freezing extenders that result in fatty acids which are toxic to spermatozoa (Pellicer-Rubio et al., 1997), and this causes 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).

3.6. How and when to assess thawed sperm quality?

The general assessment of semen quality is an important aspect in predicting the fertilizing ability of cryopreserved spermatozoa, since increasing interest in assisted reproduction techniques (ARTs) has led to a major improvement in animal reproduction (Kordan et al., 2013). Manual or visual semen analyses can be performed by placing a fixed and stained semen sample on a microscope slide by optical examination using specific criteria. However, major disadvantages of the manual analyses are that they are subjective to human bias (Graham & Mocé, 2005).

3.6.1. Sperm parameters analyses (How)

Different methods have been established in the last decades, however only few have been implemented for research purposes (Gadea et al., 2005). Therefore, the ideal sperm parameters are necessary to evaluate these functions.

3.6.1.1. Computer Assisted Sperm Analyses (CASA)

Sperm motility has been reported to be one of the easiest methods used to determine the quality and fertilizing ability of spermatozoa (Rodríguez-Martínez, 2006). Hence the use of a CASA system was developed as a method to analyse sperm samples with a much more degree of accuracy, hence errors associated with human interference have been reduced (Jane et al., 1996). This system uses computer technology to instantaneously track individual sperm cells, while simultaneously evaluating various parameters of their movements like the velocity, direction, angle of curvature between the head and tail with a much more objective and accurate measure of sperm count and motility. CASA also provides different programmes with a precise and useful information regarding various sperm with descriptive and motion parameters like progressive motility, total motility, linear velocity, curvilinear

velocity, straightness coefficient, linear coefficient, and beat cross frequency. Benefits abound with this system due to its level of accuracy in semen evaluation, time saving ability, and reduction of sperm wastage.

Different sperm subpopulations have been identified in mammalian ejaculates based on the motility characteristics exhibited by individual spermatozoa. With the CASA system, it is possible to identify and quantify different sperm subpopulation with specific movement pattern within an ejaculate (Quintero-Moreno et al., 2004). However, due to the variability of mean values and the different types of analysis available in statistical packages, the approach to evaluating mean data makes the interpretation of results obtained with CASA a little bit challenging.

The FASTCLUS procedure, a clustering analysis that examines the heterogeneity of sperm swimming characteristics, has been previously used to define subpopulations of spermatozoa based on motion variables in bucks (Dorado et al., 2010), stallions (Quintero-Moreno et al., 2003), boar (Quintero-Moreno et al., 2004), and deer (Martinez-Pastor et al., 2005).

3.6.1.2. The use of Flow Cytometer for sperm analyses

On the other hand, flow cytometry has been recognised as a powerful tool for the evaluation of sperm cells. This technique involves a process whereby sperm cells previously stained with fluorescent dyes travel individually through a cell flow at a high speed where they are illuminated by lasers, causing light scattering and fluorescence excitation which can be detected by photomultiplier tubes, and sent to a computer program. According to Martínez-Pastor et al. (2010), the computer programme presents this information in relative fluorescent intensity units which are displayed as scattered plots or histograms and grouped into sperm population with a defined characteristic. The intensity of this equipment is such that approximately 50,000 sperm cells can be counted in a minute. A variety of sperm parameters can be measured rapidly such as viability, acrosome integrity, mitochondrial function and DNA integrity (Kordan et al., 2013). The technique involves the combination of various fluorochromes to assess the functionality of spermatozoa.

From the different sperm parameters, the study of the mitochondria is of utmost importance in spermatology. The mitochondria is located in the midpiece of a spermatozoon and is responsible for ATP production, that moves sperm flagellar motion to their site of fertilization as well as cytoplasmic glycolysis required for psychological sperm function and

cell metabolism (Aitken et al., 2004). During cryopreservation, the mitochondria initiate the mechanism of apoptosis due to its sensitivity to freezing and thawing (Rasola & Bernardi, 2007), due to damages to the membrane potential uncovering membrane lipids to oxidative stress. One of the fluorescent probes commonly used to detect mitochondria membrane damages after cryopreservation is Mitotracker® (Peña et al., 2009). The Mitotracker molecules undergo diffusion through the plasma membrane and bind to specific membrane lipids of functional mitochondria but they are not retained by mitochondria with altered membrane potential (Fraser et al., 2001). Due to advantages of using the Mitotracker probes such as its great photostability, specificity, and availability, it has been used in double, triple, and quadruple staining techniques (Osuagwuh & Palomo, 2017; Sutovsky et al., 1999; Tabarez et al., 2017).

Acrosome integrity is also one of the determinant factors of a successful fertilization (Rodríguez-Martínez, 2006). Furthermore, during cryopreservation, a process known as cryocapacitation and spontaneous acrosome reaction occurs due to damages related to the acrosome membrane which alters membrane lipids and fluidity (Duru et al., 2001; Sion et al., 2004), thus affecting sperm functionality (Plant & Zeleznik, 2014). Over the years, acrosome integrity was assessed *in vitro* using the phase contrast microscope, light microscope, and differential interference-contrast microscope (Rodríguez-Martínez, 2006). However, plant lectins such as *pisum sativum agglutinin* (PSA) or *peanut agglutinin* (PNA) are the most commonly used probes conjugated with fluorescent probes for evaluating acrosome integrity (Kumar et al., 2019) and allows the use of the flow cytometer to analyse thousands of sperm in few minutes.

The plasma membrane provides structure to the spermatozoa thereby protecting intracellular components, aids permeability, and maintains sperm homeostasis and survival within the female reproductive tract. Due to the susceptibility of the sperm membrane to freezing and thawing temperatures, damages to the sperm membrane can lead to loss of metabolites and other vital components of the sperm cell which may impair its functionality (Vazquez et al., 1997). The structural integrity of the plasma membrane can be analysed by conventional stains such eosin/negrosin, or blue tripan, whereby these stains penetrate damaged or ruptured cells thus changing their colour to purple (Rodriguez-Martinez, 2003). However, one disadvantage of using these stains is that errors may occur during preparation leading to poorly stained sperm cells which interfers with accurate interpretation of the sperm status (Watson, 1990).

In the past years, the fluorescent techniques with a better objectivity and a wide variety of fluorochromes were introduced to evaluate the integrity of the plasma membrane (Garner et al., 1986; Matyus et al., 1984). However recently, the most commonly used method for the plasma membrane integrity is the combination of SYBR-14 and propidium iodide (PI) (Garner et al., 1994; Yániz et al., 2013). The SYBR-14 which is permeable rapidly penetrates viable spermatozoa with an intact membrane, while the PI penetrates non-viable spermatozoa with a damaged membrane (Gillan et al., 2005; Yániz et al., 2013). Furthermore, when SYBR-14 and PI are combined with fluorescently labelled plant lectins, it can be used to evaluate both plasma and acrosome integrity (Flajšhans et al., 2004).

The DNA is the store house of genetic information in every living cell. Due to its composition, molecular structure and chemical characteristics, it is prone to damages from external environment (Shamsi et al., 2011). Therefore, its integrity is essential for proper functionality. Sperm DNA integrity is an essential component of fertility assessment and predictions of reproductive outcomes which may not be evaluated by the standard semen analysis (Evenson, 2016). Sperm DNA damage can be categorized into the type of damage associated with the DNA strand break or the susceptibility of the sperm DNA to fragmentation. The susceptibility of sperm DNA to damages often occurs at the initial stage of spermatogenesis due to poor or partial packing of the chromatin (Haaf & Smith, 1998). Also, DNA damages can be caused by either intrinsic factors such as oxidative stress, cell apoptosis, or extrinsic factors like temperature, semen extenders used, semen handlers, infection, and many others (Bilodeau et al., 2000). Some sperm selection techniques have been demonstrated to have an effect on the DNA integrity, although these reports are still controversial with some authors demonstrating low levels of DNA fragmentation after the separation process while others showed different levels of DNA fragmentation depending on the separation technique used (Gandini et al., 2004; Zini et al., 1999, 2000).

Various techniques are currently used to assess sperm DNA fragmentation. According to González-Marín et al. (2012), the most commonly used techniques are the TUNNEL assay, and the Sperm Chromatin Structural Assay (SCSA). The TUNNEL Assay uses a fluorescent deoxyribonucleotide triphosphate (dNTPs) to measure the extent of DNA breakage between DNA strands in the presence of a coenzyme, deoxynucleotidyl transferase (González-Marín et al., 2012). The SCSA which was first described by Evenson et al. (1980) was based on the extent of cell DNA denaturation which can be measured by the metachromatic shift of acridine orange from green to red induced by an acid treatment. This

technique has been demonstrated in various species (Rybak et al., 2004). Also, the SCSA technique identifies High DNA satiability (HDS), which is supposed to reflect the nuclear compaction of spermatozoa, although studies on the HDS is still controversial (Mohammadi et al., 2020).

3.6.2. Post-thaw Incubation (When and the conditions)

In vitro incubation (thermal resistance test) is a technique used to predict the capacity of a spermatozoa to survive in the female reproductive tract during migration while maintaining their fertilizing ability (Fiser et al., 1991). Also, the success of artificial insemination technique is associated with the effective prolongation of the fertile life of spermatozoa under *in vitro* storage. Therefore, Aisen et al. (2000) reported that the subjection of frozen-thawed sperm to *in vitro* incubation may reveal damages due to temperature variation, ultra-structural and biochemical changes during cryopreservation which were not discovered immediately after thawing. Thus, frozen spermatozoa are often assessed to estimate their resilience and functionality over a stipulated period of time (Osuagwuh, 2017). Presently, several media additives, either animal or synthetic origin, have been successfully used to support *in vitro* capacitation of mammalian spermatozoa as well *in vitro* fertilization (IVF) media have been developed to mimic the uterine and oviductal fluid composition to a closely natural environment (Parrish, 2014), in order to better analyse thawed sperm viability and fertility.

Chapter IV

MATERIALS AND METHODS

4.1. Animals and location of the study area

Experimental animals were housed at *Caldes de Montbui* in the *Institut de Recerca i Tecnologia Agroalimentàries* (IRTA, Spain), located in the *Vallès Oriental* shire, in the province of Barcelona, at 203 meters of altitude, 41º 37'55.77" of north latitude and 2º 10'3.12" of eastern longitude. The annual mean temperature is 14, 1ºC with rain precipitation mean of 678.9 mm. Feeding, management, and semen collection were carried out at this location, while the freezing protocol and semen analysis were carried out at the Autonomous University of Barcelona (UAB) located in Bellatera, 25 Km away from IRTA facilities. Animal handling was performed in accordance to Spanish Animal Protection Regulation, RD 53/2013, which conforms to European Union Regulation 2010/63.

For this study, 8 bucks from *Cabra Blanca de Rasquera* breed were selected. Criteria for selection were conducted on the basis of their ability to contribute to valuable genetic resources and diversity. Bucks were trained for semen collection through artificial vagina by using a doe in oestrus as a teaser female. Training was considered completed when males mount and ejaculate regularly when in close contact with the female teaser.

4.2. Reagents and Media

All chemicals and reagents used for this experiment were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). The powdered egg yolk was obtained from NIVE (Nunspeet Holland Eiproducten). The fluorescence probes and analysis kits ((LIVE/DEAD® sperm viability kit (L-7011; PI and SYBR-14) and Mitotracker deep red (M22426)) were all purchased from Invitrogen SA (Barcelona, Spain). All fluorochromes were stored at -20°C in the dark. The washing media used for single layer centrifugation (SLC) was Bovipure® (Nidacon, Mölndal, Sweden) while the control incubation media used was a modified phosphate buffer solution (PBS solution supplemented with 36 μg/mL pyruvate and 0.5 mg/ml BSA with an osmolarity of 280-300 mOsm/kg and pH of 7.3-7.4) and an *in vitro* fertilization commercial media (BO-IVF, IVF Biosciences, UK).

4.3. Preparation of cryopreservation extenders

The basic extender used in this study was Tris hydroxymethylaminoethane-citric acid glucose (TCG) solution, composed of Tris (0.3M), citric acid anhydrous (94.7 mM), and D (+)-

glucose (27.75 mM) (Salamon & Maxwell, 2000). The pH and osmolarity were adjusted to 7.25 ± 0.05 and 333.0 ±2.8 mOsm/kg respectively. Then, glycerol (5% v/v, final concentration) and antibiotics (1000 IU/mL sodium penicillin, 1.0 mg/mL streptomycin sulphate) were subsequently added, the pH and osmolarity were finally adjusted to 7.0 ± 7.17 and 1327 ± 234 mOsm/kg. Finally, 15% of powdered egg yolk (Nunspeet Holland Eiproductep) was added. Previously, powdered egg yolk was diluted (1:1.25) with Milli Q water and stirred for 20 min as published elsewhere (Tabarez et al., 2017).

4.4. Semen collection

Semen collection was routinely performed via artificial vagina twice per day and 2 days a week from all the males used in our study (Tabarez, 2014). All fresh ejaculates were evaluated macroscopically for volume using a transparent graduated tube. Mass motility and linear progressive motility were determined subjectively using phase contrast microscope in a warm stage at 37°C. In some instances, ejaculates from males were mixed together (pooled) following no differences in semen characteristics.

4.5. Collection of seminal plasma

Briefly, an aliquot of pooled fresh semen was centrifuged at 10,000 g for 10 min at 5°C. The supernatant was collected and centrifuged again at 10,000 g for 10 min at 5°C to remove the remaining sperm and cell debris. Afterwards, the clear supernatant (SP) was recovered in an aliquot of 1.5mL tubes and freeze dried in liquid nitrogen before being stored at -80°C.

4.6. Freezing and thawing protocol

Fresh ejaculates were washed twice by dilution (1:5) in TCG and centrifuged at 600×g for 10 min. Thereafter, the supernatant was carefully removed and the sediment was diluted (1: 4) with the above basic extender. All extended samples equilibrated for 4 hours at 5°C (pre-freezing). Thereafter, extended semen was packed into 0.25ml plastic straws (IMV Technologies, L'Aigle, Cedex, France) at a final concentration of 400 x 10^6 sperm/mL. All straws were kept in liquid nitrogen vapour (5cm above the liquid nitrogen level) for 10 minutes before plunged into liquid nitrogen for cryopreservation. Thawing was carried out by

immersion in a water bath at 37 °C for 30 s. The straws were cleaned, and the content was poured into a dry 1.5mL tube and kept in a water bath (37 °C) until analysis.

4.7. Single Layer Centrifugation (SLC) procedure

Thawed sperm sample was diluted (1:4) with PBS to a final concentration of 100 x 10^6 sperm/mL. Afterwards, a single layer centrifugation (SLC) procedure was performed for all thawed and diluted samples. Prior to the procedure, the colloid Bovipure® (Nidacon, Mölndal, Sweden) was brought to room temperature and 80% Bovipure® solution was made according to the manufacturer's instruction by diluting with Bovidilute® (Nidacon, Mölndal, Sweden), then mixed thoroughly and kept in a warm water bath (37°C). Thereafter, 1 mL of the diluted colloid solution was pipetted into a centrifuge tube and an aliquot of the extended semen (1 mL of sperm diluted in PBS containing 100 x 10^6 sperm/mL) was layered on top carefully in a slanting position. After centrifugation at 300 x g for 25 mins, the supernatant was removed using a pipette. The pellet was diluted with 1 mL of Boviwash® (Nidacon, Mölndal, Sweden) and centrifuged again at 300 x g for 5mins. The supernatant was discarded and the sperm pellet was transferred to a clean tube and diluted with a modified PBS.

4.8. Incubation of washed sperm samples

Immediately after SLC/washing, all sperm samples from the various treatments/groups were incubated for 3h in three different media: (a) Modified PBS or control media (b) In-vitro fertilization commercial media (BO-IVF media, IVF Biosciences, UK) and (c) BO-IVF media + Seminal Plasma (20%). All seminal plasma samples were collected during the breeding season (autumn) from the same males used in this study. Prior to use, the SP was thawed in a warm water bath at 37°C. For each treatment/group, sperm samples were diluted in the various incubating media at a concentration of 40x10^6sperm/ml and placed in an incubator (5% CO_2) at 38.5°C for 3hours.

4.9. Analysis of sperm viability

The flow cytometry analysis was performed using the BD FACSCanto flow cytometer (BD Biosciences, CA) and samples were analysed using BD FACSDiva software (BD Biosciences, CA). Plasma and acrosome membrane integrity as well as mitochondrial

function of all sperm samples were evaluated by flow cytometry, using a quadruple-staining, technique as described by Tabarez et al. (2017). The following fluorescent probes were used for this study: LIVE/DEAD® sperm viability kit (SYBR-14 and Propidium Iodide (PI); L-7011, Invitrogen S.A.) for plasma membrane integrity, Mitotracker deep red (M22426, Invitrogen S.A.) for the detection of mitochondrial activity, and finally PE-PNA (GTX01509, Antibody Bcn, and S.L.) for acrosome integrity.

The analysis was performed using a final concentration of 1 nM of SYBR-14 (diluted in DMSO), 1.5 µM of PI, 2.5 µg/mL PE-PNA (1 mg/mL of stock solution in a buffer composed of 3.0 M ammonium sulphate, 50 mM sodium phosphate and 0.05% sodium acid, pH 7.0 containing 1 mM [Ca2+] and [Mn2+] ions) and 1.5 nM of Mitotracker deep red (diluted in DMSO) with 1 mL of diluted semen in PBS to a final sperm concentration of 1×10^6 /mL. Sperm samples were thoroughly mixed and incubated at 37 °C for 10 min in the dark to enable proper staining. Prior to analysis, sperm samples were re-mixed and sperm suspensions were subsequently run through a flow cytometer. Fluorescent probes SYBR-14, PE-PNA and PI were excited in the flow cytometer using a 488 nm blue solid-state laser while the Mitotracker deep red was excited using a 633 nm He/Ne excitation laser. Dead cells were positive for PI, thus producing a red fluorescent signal which was detected using the 679LP filter detector (detects photons emitted at more than 670 nm wavelength). SYBR-14 positive or fluorescent green signal live cells were detected using the detector with filter 530/30BP (detects photons emitted in the range 515-545 nm wavelength). Cells with acrosome damage were positively stained by PE-PNA emitting fluorescent orange signal which was detected using the 585/42BP filter detector (detects photons emitted in the range 564-650 nm). Sperm mitochondria function was detected with Mitotracker deep red using a 660/20BP filter detector.

After evaluation, the following sperm population were taken into consideration: Viability (total SYBR14+/PI- sperm cells), viable cells with intact acrosome and functional mitochondria (SYBR14+/PI-/PE-PNA-/Mitotracker+), viable cells with damaged acrosome and functional mitochondria (SYBR14+/PI-/PE-PNA+/ Mitotracker+), viable cells with intact acrosome and non-functional mitochondria (SYBR14+/PI-/ PE-PNA-/Mitotracker-), viable cells with damaged acrosome and non-functional mitochondria (SYBR14+/PI-/PE-PNA+/ Mitotracker-). In addition, the total acrosome damage was assessed for sperm samples.

4.10. Analyses of DNA integrity: Sperm Chromatin Structural Assay (SCSA)

All sperm samples were evaluated for DNA integrity using the technique described by Evenson et al. (2002). Sperm samples were diluted to a final concentration of 1-2 x 10^6 sperm/mL using TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 Mm EDTA, pH 7.4). The samples were freeze-dried in liquid nitrogen vapour and stored at -80°C until ready for evaluation. Prior to analysis, frozen samples in 1.5 mL micro centrifuge tubes were thawed for 2 minutes at 37°C until the ice had melted. The reference samples were kept on ice for few minutes before staining. Acid–induced denaturation of DNA *in situ* was achieved by adding 200 µL of thawed sperm sample to 400 µL of acid detergent (0.1% (v/v) Triton X-100, 0.15 M NaCl, 0.08 M HCl, pH 1.2). At exactly 30 seconds, 1.20 mL of Acridine Orange (AO) staining solution (0.037 M citric acid, 0.126 M Na_2HPO_4, 0.0011M sodium EDTA, 0.15 M NaCl, pH 6.0, 4°C), containing 6 µg/mL of electrophoretically purified AO (Polysciences,Inc, Warrington, Pa) was added. Stained sperm samples were immediately kept on ice for 2 minutes and subsequently analysed by flow cytometer using the BD FACS Canto flow cytometer (BD Biosciences, CA). The flow cytometer was adjusted using standard sperm samples. In general, about 5,000 events were measured. The percentage DNA fragmentation index (DFI) was calculated as the ratio of denatured single stranded DNA (red colour) to the total cells acquired (red + green colour). The High DNA stainability (HDS) index was also evaluated, defined as the population with an elevated green value, outside the main population recorded.

4.11. Analyses of sperm motion parameters

Sperm motion parameters were evaluated immediately after thawing using the computer-assisted sperm analysis (CASA) system, ISAS® (PROISER S.L., Valencia, Spain). Sperm samples were diluted (1:10) in PBS, and 10 µL drop of sperm suspension was placed on a preheated slide on a heat stage and covered with a 24 x 24 mm coverslip. Sperm motion parameters were assessed at 38 °C at x 200 using a phase contrast microscope (Olympus BH-2, Japan). For each sample, more than three fields per drop were analysed and a minimum of 200 sperm cells evaluated. The total motile sperm cells (TM), progressively motile sperm cells (PM), curvilinear velocity (VCL, µm/s), linear velocity (VSL, µm/s), mean velocity (VAP, µm/s), linearity coefficient (LIN=[VSL/VCL]x 100, %), straightness coefficient

(STR= [VSL/VAP]x 100, %), amplitude of lateral head displacement (ALH, µm) and beat-cross frequency (BCF, Hz) were all evaluated. The settings used for the sperm image analyses were as follows; number of images (25/s), optical (Ph-), scale (20xOlympus), particle area (> 3 a <70 micras^2), slow sperm (10-45 micras/s), average sperm (45-75 micras/s), rapid sperm (> 75 micras/s), progressive (80% STR). All samples and reagents were maintained at 37°C.

4.12. Statistical analyses

Statistical analyses were performed using the SAS statistical package (Version 9.4, 2015, SAS Institute Inc., Cary, NC, USA). A non-hierarchical multivariate cluster analysis was performed using the k-means model based on Euclidean distances calculated from kinematic parameters to identify motile sperm subpopulations within sperm samples, using the FASTCLUS procedure and the number of subpopulations was established using the elbow method previously described by Bravo et al. (2011). The frequency distribution of the motile sperm subpopulations was performed for each replicate using the FREQ procedure. Then a correction was made for the proportion of each subpopulation based on the total motility (TM) obtained from each sperm sample (SP = [SP/100] ×TM), since the subpopulations resulting from the analysis correspond only to motile spermatozoa. In this way, a new subpopulation was obtained that precisely grouped the static spermatozoa (SP4 =100% ─ TM). An ANOVA was applied to compare the parameters of each sperm subpopulation, using the GLM procedure and the differences between means were analysed with the Tukey test ($P < 0.05$).

A one-way non-parametric procedure was applied to perform the Kruskal-Wallis test in order to compare different factors under study (season, age, melatonin and treatment). With regards to the significant difference, pairwise comparisons were made by adjusting the P value (<0.05) with the Bonferroni method. Results were expressed in means ± standard error of the mean (SEM) and mean ± S.D.

Chapter V

RESULTS

Study 1

Single layer centrifugation using Bovipure® colloid as sperm selection method on cryopreserved goat sperm samples after thawing.

Abstract

This study was designed to evaluate the effectiveness of the sperm selection method by means of single layer centrifugation (SLC) through Bovipure® colloid on the quality of cryopreserved buck sperm considering different factors such as male age, season of semen collection and melatonin treatment of the donors. Therefore, two ejaculates/male were collected (n = 6 days) via artificial vagina from 8 males during 2 consecutive years in the breeding and non-breeding periods, at 12 and 24 months old in autumn and at 18 and 30 months old in spring. Prior to semen collection in the non-breeding period, males were randomly divided into two groups: one group was implanted with melatonin, while the other was not. All samples were pooled, centrifuged twice and diluted in an extender containing 15% powdered egg yolk and 5% glycerol before freezing. After thawing, sperm samples were analysed immediately as the Non-SLC group while the remaining samples were diluted in PBS and then SLC procedure was carried out (SLC group). Sperm motility was evaluated by CASA, while plasma and acrosome membrane integrity, mitochondria activity and DNA fragmentation were analysed by flow cytometer. No major significant differences were observed between the unselected and the SLC sperm samples with regards to total and progressive motility, viability and sperm DNA integrity. Also, no huge differences were observed on motile sperm subpopulation distribution before and after SLC amongst the different sperm samples. However, slightly higher progressive motility and viability were observed in autumn samples just after thawing, although these differences were not reflected after SLC. In regards to High DNA Stainability, higher values were observed in autumn samples, especially after SLC was performed. In conclusion, SLC through Bovipure® colloid after thawing did not improve cryopreserved buck sperm quality regardless of the male age, season and melatonin implant.

Introduction

A suitable protocol after cryopreservation, assuring the quality of thawed sperm are the fundamentals for assisted reproduction techniques (ART) implementation, otherwise the succes can not be fully guaranteed (Leboeuf et al., 2000). Sperm cryopreservation induces various damages, reducing their post-thaw surviving and fertilizing ability (Purdy, 2006; Watson, 2000). Furthermore, the damages encountered during thawing, handling and processing cannot be ignored, as minor injuries may lead to serious deleterious effect at the end of the process (Tuli et al., 1991).

Different approaches have been proposed to improve the quality of thawed sperm for optimal productivity by selecting spermatozoa free of debris/cryoprotectants, good morphology and motility (Morton et al., 2006). Selection methods such as filtration, migration and colloid centrifugation has been demonstrated for selecting efficient spermatozoa. The density gradient centrifugation (DGC) has been suggested as a potential means of improving semen quality after the detrimental effect of cryopreservation (Muratori et al., 2019). However, in recent times, a more flexible technique known as the single layer centrifugation (SLC) using only one layer of specie-specific colloid formulation has been proven to select spermatozoa with good quality, less complicated and shorter preparation time (Morrell & Rodriguez-Martinez, 2009). The SLC technique involves the centrifugation of spermatozoa through a column of glycidoxypropyltrimethoxyl silane-coated silica in a species-specific formulation, whereby, motile sperm with a good morphology, chromatin integrity and intact membranes are selected from non-motile/moribund sperms, cryoprotectants and underlying debris (Morrell et al., 2009). Improvement in post-thawed semen quality by SLC using Androcoll-B® colloid has been reported in goats (Jiménez-Rabadán et al., 2012), however there are no reports with regards to the influence of SLC method with Bovipure® colloid on the quality of frozen-thawed buck semen.

Most breeds of goats present another limiting factor of sexual activity in relation to season, with some reports showing better semen quality during the autumn and winter period (Elsheikh & Elhammali, 2015). In this regards, male goats have been reported to be seasonal breeders due to their reproductive activities being influenced by photoperiod, with better breeding performances seen during the autumn, considered as the breeding season (Delgadillo et al., 2004). Nevertheless, this adverse seasonality effects on semen production may be reversed or manipulated by using subcutaneous melatonin implants especially during a non-breeding season by artificially mimicking day length effect (Zarazaga et al., 2010). Furthermore, semen quality can also be influenced by donor age, indicating that sexual maturation and semen quality increases as male gets older due to the increase in testicular size (Moru et al., 2016). However, some reports have shown no differences in semen quality between yearlings and mature bucks (Islam et al., 2008; Kridli et al., 2005).

Therefore, the aim of this study was to investigate the efficiency of Single Layer Centrifugation through Bovipure® colloid in order to improve the quality of thawed buck sperm, taking into consideration different factors such as donor age, season of semen collection and melatonin treatment of males.

Materials and Methods

The experiment was carried out over 2 consecutive years. Two ejaculates/male/day were collected via an artificial vagina twice a week from 8 males of *Cabra Blanca de Rasquera* breed in autumn (breeding season; n=6 days of semen collection) at 12 months old. Semen collection was repeated from the same males in the following year, now 24 months old. Similarly, in spring (non-breeding season), ejaculates were also collected from the 8 males via an artificial vagina. To this regard, and prior to semen collection in spring (n=6), males were equally and randomly divided into two groups (4/group): one group was implanted with a slow releasing implant containing 18mg of melatonin (Melovine®, CEVA-Animal Health, U.K) which was placed at the base of the ear for 60 days prior to semen collection, while the other group had no implants. All procedures in spring were carried out when males were 18 and 30 months old. All fresh ejaculates collected were immediately pooled to eliminate individual differences, washed in TCG solution by centrifuging twice (600 x g, 10 min), and diluted in the extender (15% powdered egg yolk and 5% of glycerol, final concentration). Thereafter, sperm concentration was adjusted to 400 x 10^6 sperm/mL and equilibrated for 4 h at 5°C for 10 mins before freezing in liquid nitrogen vapours.

Sperm thawing and Single Layer Centrifugation (SLC)

After cryopreservation, two straws per replicate (n = 6) from the various treatments, breeding season (12 vs. 24 months), non-breeding season (18 vs. 30 months) with and without melatonin were sorted and thawed by immersion in a water bath at 37 °C for 30 seconds and the content was poured into a dry eppendorf tube and kept in a water bath, maintained at the same temperature. Thereafter, each thawed sperm sample was diluted (1:4) with PBS to a concentration of 100 x 10^6 sperm/mL. Afterwards, a single layer centrifugation (SLC) procedure was performed for all thawed and diluted samples as has been described in the general section of Materials & Methods. Thereafter, sperm samples were evaluated before SLC (non-selected samples) and after SLC.

Sperm viability analyses

Plasma and acrosome membrane integrity as well as mitochondrial function of all groups, Non-SLC and SLC sperm samples were evaluated by flow cytometry, using a quadruple-staining technique Tabarez et al. (2017) as described in the general Materials & Methods section. After evaluation, the following sperm population were taken into consideration: Viability (total SYBR14+/PI- sperm cells), viable cells with intact acrosome and functional

mitochondria (SYBR14+/PI-/PE-PNA-/Mitotracker+), viable cells with damaged acrosome and functional mitochondria (SYBR14+/PI-/PE-PNA+/ Mitotracker+), viable cells with intact acrosome and non-functional mitochondria (SYBR14+/PI-/ PE-PNA-/Mitotracker-), viable cells with damaged acrosome and non-functional mitochondria (SYBR14+/PI-/PE-PNA+/ Mitotracker-). In addition, the total acrosome damage was assessed for all non-SLC and SLC sperm samples.

Analyses of DNA integrity

All sperm samples from the breeding season (12 vs 24 months old), non-breeding season (18 vs 30 months) from males with and without melatonin were evaluated for DNA integrity using Sperm Chromatin Structural Assay (SCSA) described by Evenson et al. (2002) as it was mentioned in previous section. The percentage DNA fragmentation index (DFI) was calculated as the ratio of denatured single stranded DNA (red colour) to the total cells acquired (red + green colour). The High DNA stainability (HDS) index was also evaluated, defined as the population with an elevated green value, outside the main population recorded.

Analyses of sperm motion parameters

Sperm motion parameters were evaluated immediately after thawing (Non-SLC) and after SLC for all samples collected in the breeding season (12 vs 24 months old) and non-breeding season (18 vs 30 months old) from males with and without melatonin implants. The non-SLC and SLC samples were assessed for motion parameters using the computer-assisted sperm analysis (CASA) system, ISAS® (PROISER S.L., Valencia, Spain). The total motile sperm cells (TM), progressively motile sperm cells (PM), curvilinear velocity (VCL, µm/s), linear velocity (VSL, µm/s), mean velocity (VAP, µm/s), linearity coefficient (LIN), straightness coefficient (STR), amplitude of lateral head displacement (ALH, µm) and beat-cross frequency (BCF, Hz) were all evaluated.

Statistical analyses

Statistical analyses were performed using the SAS statistical package (Version 9.4, 2015, SAS Institute Inc., Cary, NC, USA). The different analyses were performed as they have been previously described in the Material & Methods section.

Results

No significant effect of the SLC method used was observed in sperm viability. Only a significant decrease in total acrosome damage after SLC was found in the non-treated male spring samples (Table 1.1). Total sperm viability and the proportion of viable cells with intact acrosome and active mitochondria were higher in autumn samples, although not always differed significantly. Nevertheless, after SLC no differences were found amongst ages, seasons or melatonin treated groups. Total acrosome damage was higher ($P<0.05$) in autumn samples, especially after SLC. Regards to viable sperm with damaged acrosome and active mitochondrial, few significant differences were observed, showing very low percentages (Table 1.1).

Table 1.1: Effect of single layer centrifugation after cryopreservation on buck sperm viability analyzed by the flow cytometer.

Sperm Parameter (%)	Treatment	Autumn		Spring		Spring + Melatonin	
		12 months	24 months	18 months	30 months	18 months	30 months
Viability	NSLC	42.5 ± 6.1^{a}	44.3 ± 3.0^{a}	39.4 ± 4.0^{ab}	22.9 ± 2.7^{b}	33.9 ± 3.3^{ab}	27.0 ± 5.2^{ab}
	SLC	37.2 ± 3.7	39.7 ± 3.0	33.0 ± 5.1	29.5 ± 5.7	34.1 ± 5.5	30.9 ± 4.7
Intact acrosome, active mitochondrial	NSLC	42.4 ± 6.1^{a}	44.2 ± 3.0^{a}	39.2 ± 3.9^{ab}	22.1 ± 3.0^{b}	33.1 ± 3.4^{ab}	26.6 ± 5.2^{ab}
	SLC	37.0 ± 3.8	39.7 ± 3.0	32.6 ± 5.2	29.4 ± 5.7	33.9 ± 5.4	30.7 ± 4.7
Damaged acrosome, active mitochondrial	NSLC	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0^{1}	0.1 ± 0.0	0.0 ± 0.0
	SLC	0.0 ± 0.0^{b}	0.1 ± 0.0^{ab}	0.0 ± 0.0^{b}	$0.2 \pm 0.1^{a,2}$	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
Acrosome damage	NSLC	31.0 ± 4.6^{a}	29.4 ± 4.7^{ab}	12.9 ± 3.6^{bc1}	$11.4 \pm 1.2^{c,1}$	20.8 ± 2.8^{abc}	14.7 ± 7.0^{bc}
	SLC	25.0 ± 4.7^{ab}	35.6 ± 3.2^{a}	6.0 ± 0.8^{c2}	$6.5 \pm 0.9^{c,2}$	14.2 ± 3.0^{bc}	8.5 ± 2.2^{c}

$^{a-c}$ Different letters represent significant differences ($P<0.05$) between seasons and ages within each treatment. ^{1,2} Different numbers represent significant differences ($P<0.05$) between samples before and after SLC within the parameters at each season and age. NSLC: Non- selected; SLC: Single layer centrifugation. Data are shown as means ± S.E.M.

No differences were observed after SLC in the proportion of sperm with High DNA Stainability (HDS), only a significant increase was found in samples from younger treated males. However, after SLC the DNA fragmentation index (DFI) was significantly lower in spring samples from older treated males. Furthermore, no significant effect was observed in

the DFI with regards to age, season or melatonin treatment after thawing as well after SLC. Related to HDS percentages, higher values were observed in autumn samples, although not always significantly (Table 1.2).

Table 1.2: Effect single layer centrifugation after cryopreservation on buck sperm DNA integrity

Sperm parameter (%)	Treatment	Autumn		Spring		Spring + Melatonin	
		12 months	24 months	18 months	30 months	18 months	30 months
HDS	NSLC	2.7 ± 0.6^{ab}	3.2 ± 0.9^{a}	1.5 ± 0.4^{ab}	1.2 ± 0.6^{ab}	$0.6 \pm 0.1^{b.1}$	1.6 ± 0.7^{ab}
	SLC	4.7 ± 0.9^{a}	4.2 ± 0.7^{a}	2.4 ± 0.6^{ab}	1.1 ± 0.5^{b}	1.8 ± 0.4^{ab2}	2.2 ± 0.8^{ab}
DFI	NSLC	9.5 ± 1.7	9.8 ± 1.5	10.0 ± 2.6	8.5 ± 1.5	9.6 ± 1.8	11.1 ± 2.1^{1}
	SLC	5.9 ± 0.6	6.8 ± 1.1	7.1 ± 1.8	12.9 ± 5.2	5.8 ± 0.5	5.6 ± 1.1^{2}

$^{a-c}$ Different letters represent significant differences ($P<0.05$) between between seasons and ages within each treatment. ^{1,2} Different numbers represent significant differences ($P<0.05$) between samples before and after SLC within the parameters at each season and age. NSLC: Non- selected; SLC: Single layer centrifugation; HDS: High DNA Stainability; DFI: DNA Fragmentation index. Data are shown as means ± S.E.M.

After thawing, Progressive motility in autumn samples were higher, but not always significantly. Nevertheless, after SLC no differences were found on PM amongst experimental groups. As well, no SLC effect was observed in motility, only a significant reduction was seen in PM after SLC in spring samples from younger males (Table 1.3).

Table 1.3: Effect of single layer centrifugation after cryopreservation on buck sperm motility analyzed by CASA.

Sperm parameter (%)	Treatment	Autumn		Spring		Spring + Melatonin	
		12 months	24 months	18 months	30 months	18 months	30 months
Total Motility	NSLC	48.2 ± 3.9	46.3 ± 3.8	43.7 ± 3.5	43.5 ± 3.3	41.0 ± 4.6	37.9 ± 5.2
	SLC	42.7 ± 4.1	50.5 ± 2.9	34.0 ± 5.1	33.2± 3.9	36.2 ± 3.7	39.8 ± 4.8
Progressive Motility	NSLC	21.0 ± 2.3^{a}	21.4± 1.8^{a}	14.1± 0.5^{b1}	15.3±1.0^{ab}	15.2±1.5^{ab}	14.5 ±1.6^{b}
	SLC	17.2 ± 2.6	16.0 ± 2.6	10.5± 1.6^{-2}	15.5±1.2	12.9 ± 1.4	17.6 ± 2.4

^{a,b} Different letters represent significant differences (P<0.05) between seasons and ages within each sperm treatment. ^{1,2} Different numbers represent significant differences (P<0.05) between samples before and after SLC within the parameters at each season and age. NSLC: Non- selected; SLC: Single layer centrifugation. Data are shown as means ± S.E.M.

In regards to motile subpopulations, a total of 36,740 sperms were analyzed. From those, 16,658 sperm presented some type of movement and four sperm subpopulations (Sp) were identified with different kinetic patterns (P<0.05). In this study, static sperm constituted the fourth motile subpopulation (Sp4) (Table 1.4). No consistent effect of the SLC method was found in the distribution of the different subpopulations (Figure 1.). Concerning to Sp1, only a significant increase was observed in spring samples from implanted males. After SLC, the Sp2 sperm was statistically lower in all spring samples except in older treated male samples, while there was a significant increase in the Sp3 sperm in all the groups except in autumn samples from younger males and in spring samples from older males without melatonin. No differences were found in the static sperm subpopulation (Sp4) after SLC in any experimental group. Finally, related to male age, season and melatonin treatment factors, only slight differences were observed in Sp3 before SLC, which were not evident after SLC (Figure 1.).

Table 1.4: Structure and kinematics characteristics of sperm motile subpopulations in buck thawed sperm

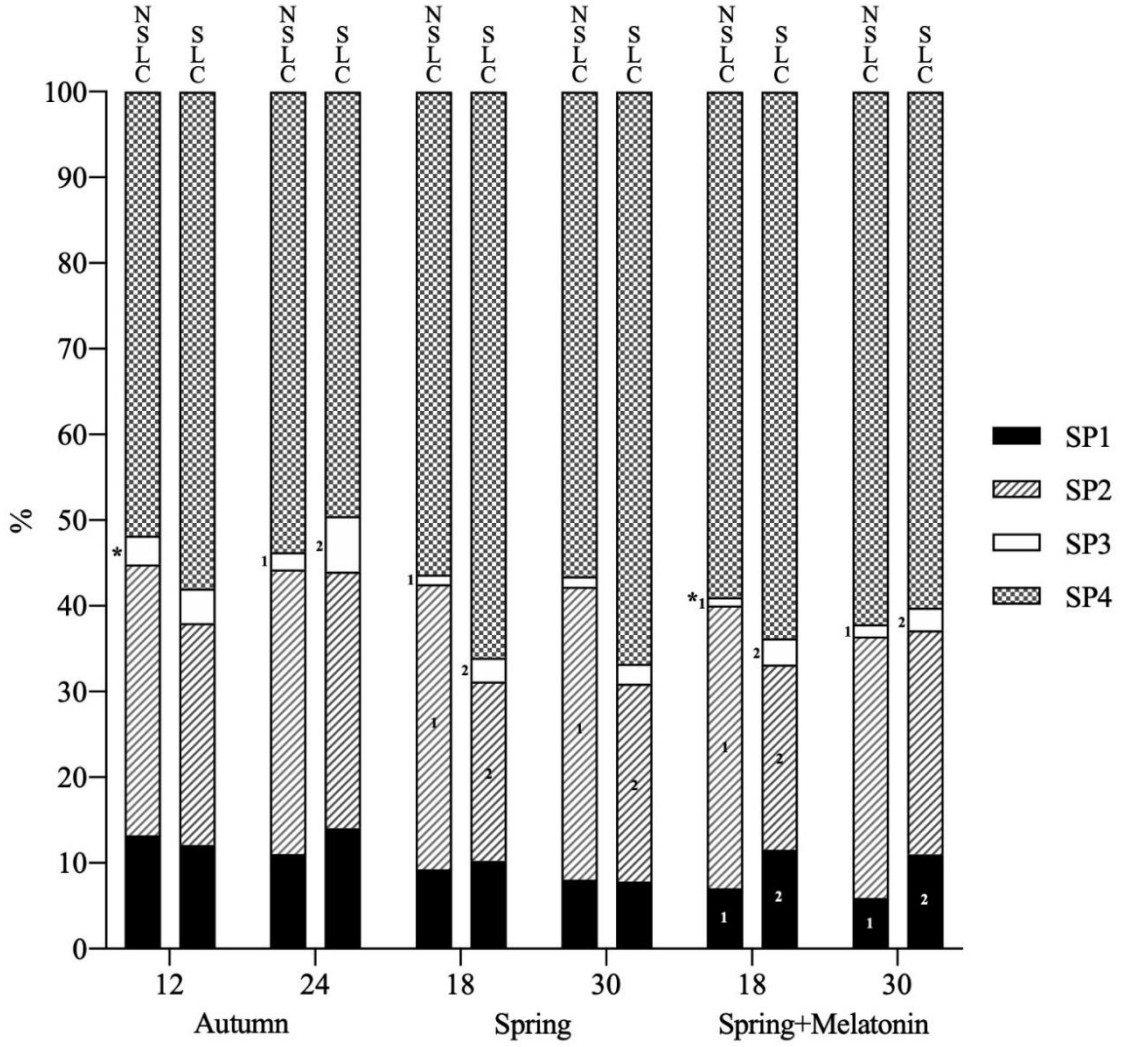
Parameter	Sp1	Sp2	Sp3	Sp4
VCL (µm/s)	116.7 ± 20.9[a]	47.4 ± 22.6[b]	167.3 ± 21.3[c]	N-D
VSL (µm/s)	43.0 ± 26.1[a]	18.5 ± 15.5[b]	49.3 ± 29.1[c]	N.D
VAP (µm/s)	67.1 ± 22.7[a]	28.9 ± 16.9[b]	87.7 ± 24.3[c]	N.D
LIN (%)	35.8 ± 18.9[a]	40.0 ± 23.0[b]	29.0 ± 16.1[c]	N.D
STR (%)	60.3 ± 24.0[a]	61.3 ± 25.3[a]	53.8 ± 24.4[b]	N.D
ALH (µm)	5.1 ± 0.8[a]	2.4 ± 1.0[b]	7.4 ± 1.2[c]	N.D
BCF (Hz)	7.3 ± 4.1[a]	4.7 ± 3.1[b]	7.4 ± 4.5[a]	N.D
n	4,142	11,436	1,080	20,082
%	11.27	31.13	2.94	54.66

[a-c] Different represent significant differences ($P<0.05$) between subpopulations.

n: Number of sperm. %: Percentage of sperm in each subpopulation. Sp: Subpopulation

ND: no data. Data are shown as means ± SD.

Figure 1.1: Effect of single layer centrifugation after cryopreservation on buck motile sperm subpopulation distribution.



(*) represents significant differences (P<0.05) in the same sperm subpopulation between seasons and ages within each treatment. [1,2] Different numbers represent significant differences (P <0.05) in the same sperm subpopulation before and after SLC within each season and age. NSLC: Non- selected; SLC: Single layer centrifugation. SP: Subpopulation.

Discussion

As an established fact, the biggest difficulty in the utilization of buck frozen semen is that cryopreservation leads to reduction in motility and viability mainly due to membrane damages (Ahmad et al., 2014; Watson, 2000). However, our studies did not reveal any positive effect of the SLC technique used as sperm selection method, only acrosome integrity was improved in spring samples. The lack of sperm quality improvement may be due to the susceptibility of the sperm membrane to mechanical damages encountered during centrifugation, which may be severe for thawed sperm that had undergone injury during

cryopreservation (Sharma et al., 1997). Specifically in goat sperm, centrifugation has been reported to alter plasma membrane integrity and decreasing sperm motility (Azerêdo et al., 2001). Similarly, in bovine thawed sperm, viability decreased after SLC with iodixanol (Gloria et al., 2016) as well a decrease in total motility was detected in thawed bull semen after SLC with Bovicoll® colloid (Szlendak et al., 2020) or even no effect was found on boar sperm motility (van Wienen et al., 2011). On the contrary, Jiménez-Rabadán et al. (2012) found greater motility and acrosome integrity in bucks when SLC was performed after thawing, using Androcoll-B® as the selection colloid. No reports have demonstrated the use of Bovipure® colloid for thawed buck sperm selection, therefore, we might only suggest that thawed buck sperm may have exhibited a different behaviour due to the composition of the colloid solution used. Though, some reports have demonstrated a positive effect with Bovipure® colloid after SLC was performed in thawed bull sperm (Samardzija et al., 2006; Valeanu et al., 2015).

Moreover, SLC through specie specific silica colloid (Androcoll-E™) has been demonstrated to effectively select motile and morphologically normal spermatozoa with an intact chromatin integrity (Šterbenc et al., 2019). In our study, no effect was seen on sperm DNA integrity after SLC owing to the fact that since SLC with Bovipure® colloid did not improve viability and motility, no positive effect might be expected on DNA integrity as well. Also changes in sperm DNA may have not been revealed immediately after thawing/centrifugation. Previous studies in bovine showed that DNA sperm damage was higher during incubation (Valeanu et al., 2015) which also correlates with the fact the most degradation of sperm DNA occurs during incubation as opposed to the first minutes after thawing (Crespo et al., 2013).

HDS has been described to reflect high immaturity of the sperm nucleus due to poor histone to protamine ration, which affects sperm nucleic compaction thus making it prone to DNA damage (Ward, 2017). In the present study, the slightly higher values observed in autumn after thawing agrees with higher HDS values showed also in breeding season in several species (García-Macías et al., 2006).

In our study, four sperm subpopulations were identified, including the static sperm subpopulation, which were quite stable after SLC with very few differences between experimental groups. Only the SP3 with highly active, non-progressive and vigorous sperm was significantly increased after SLC, in most of the studied groups, in agreement with Moohan et al. (1995) using density Percoll gradient centrifugation. This SP3 subpopulation

which represent about 2.94% of the total sperm may require special attention as this sperm exhibited motility characteristics which depict “hyperactivation” as described by Mortimer and Mortimer (1990). This “hyperactivated” like movement could also be due to cryopreservation process which causes an impairment in the mechanism responsible for calcium ion efflux, thus leading to high intracellular calcium (Cormier & Bailey, 2003). Nevertheless, the hyperactivity observed in our study may have been induced by previous damages encountered during cryopreservation which were exacerbated after centrifugation. In any case, no reduction after SLC of the static subpopulation was observed in any of the different samples regardless of different factors. Invariably SLC with Bovipure® colloid has been shown to be ineffective in selecting spermatozoa with specific kinematic parameters.

To our knowledge, no reports have demonstrated the effectiveness of SLC through Bovipure® colloid on frozen-thawed buck sperm quality as well as on the structure of motile sperm subpopulations, which could differ not only between species, also when sperm are subjected to various centrifugations and selection techniques (Anel-López et al., 2015). Furthermore, centrifugation for buck semen is recommended at speeds ranging from 500 to 1000 x g for 10 to 15 min (Purdy, 2006). In our study, SLC procedure was carried out with a relatively low g-force, but a longer duration which may have altered sperm survival. The use of short-term centrifugation with a relative high g-force has a positive influence on boar sperm cryo-survival (Carvajal et al., 2004), whereas in bull sperm after Percoll gradient centrifugation at high rotational force, an increase in reactive oxygen species and a decrease in motility and viability were described (Guimarães et al. 2014). Definitely, speed and duration of sperm centrifugation is still a matter of controversy.

Finally, no huge differences on buck sperm quality just after thawing were seen between samples from different male age or melatonin treatment or season. Furthermore, these factors had no clear interaction on the capacity of this method in selecting intact viable and motile cells from initial sperm samples. Only slightly higher progressive motility and viability after thawing were observed in autumn samples, although this difference was not reflected after SLC. In conclusion**,** the present study suggests that SLC through Bovipure® colloid was not effective in the selection of buck spermatozoa after cryopreservation. Further investigation may be required to determine the ideal buck thawed sperm selection method.

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Study 2

The seminal plasma effect on post-thawed buck sperm and its resilience under *in vitro* capacitation conditions

Abstract

In order to evaluate the effect of seminal plasma (SP) on buck thawed sperm under *in vitro* capacitation conditions, semen was collected via artificial vagina from 8 males during 2 consecutive years in the breeding and non-breeding periods. Prior to semen collection in the non-breeding season, males were split into two groups: one group was implanted with melatonin, while the other was not (control group). All samples were pooled, centrifuged twice and diluted in an extender containing 15% powdered egg yolk and 5% glycerol before freezing. After thawing, sperm was washed by Single Layer Centrifugation technique and incubated in three different media: (a) modified PBS or control (b) *in vitro* fertilization (IVF) commercial media and (c) IVF media + 20% Seminal Plasma (SP). Sperm motility was evaluated by CASA, while plasma and acrosome membrane integrity, mitochondria activity and DNA fragmentation were analysed by flow cytometer at 0h (after washing) and after 3h incubation under capacitation conditions. A significant reduction on total motility and viability were observed in most of the experimental groups after 3 h incubation under *in vitro* capacitation conditions regardless of the presence of SP while DNA integrity was not affected. However, acrosome damage was significantly higher in all sperm samples incubated in the presence of SP, regardless of the donor age, season or male treatment In conclusion, addition of SP failed to improve post thaw buck sperm quality irrespective of male age, season of collection and melatonin implant.

Introduction

The use of frozen-thawed caprine semen for artificial insemination (AI) remains challenging due to its low fertility rate (Dorado et al., 2007). The fertilizing capacity of cryopreserved semen has been reported to be low due to the detrimental effect of freezing resulting to ultrastructural, functional and biochemical damage (Watson, 2000). Cryopreserving media contains components widely used such as egg yolk and glycerol, regarded as cryoprotectants, which helps to minimise cryo-injuries during the freezing and thawing processes (Purdy, 2006). Despite this importance, the interaction between egg yolk and seminal plasma could be detrimental due to the egg yolk-coagulating enzymes (EYCE) found in buck seminal plasma (Roy, 1957), affecting the sperm viability and motility (Gangwar et al., 2016). Therefore, prior to freezing, the removal of the seminal plasma by centrifugation should be considered

necessary in other to minimise such deleterious effects caused by the cryopreservation procedure (Ritar & Salamon, 1982).

However, the importance of seminal plasma (SP) cannot be ignored due to its positive role in several key processes such as good source of nutrient, preventing premature sperm activation within the female reproductive tract and sperm membrane stabilization by inhibiting capacitation and fertility, among others (Rozeboom et al., 2000). Indeed, the addition of SP after thawing resulted in beneficial effects that has shown an increase on sperm motility, resistance to thermal shock, reverse damages encountered during freeze-thawing process and a general improvement of the sperm physiological parameters (Maxwell et al., 2007; Rebolledo et al., 2007). For instance, boar post-thawed sperm motility increases after 60 min of incubation with 10% of seminal plasma (Garcia et al., 2010), while improved fertility rates were observed in ewes inseminated with refrigerated ram sperm supplemented with SP (López-Pérez & Pérez-Clariget, 2012)..

On the other hand, for the maintenance of a good fertility ability of frozen semen after artificial insemination as well after *in vitro* fertilization, the longevity or intactness of sperm cell membrane and motion characteristics of thawed sperm is of utmost importance (Maurya & Tuli, 2003). Osuagwuh & Palomo (2017) reported that exposing frozen-thawed ram spermatozoa to post-thaw incubation period may reveal some sublethal damages especially in the sperm plasma and acrosomal membranes which may not be visible immediately after thawing. However, to the best of our knowledge, there is a lack of information on the effect of post-thaw incubation on buck sperm after cryopreservation.

Lastly, previous reports demonstrated that semen quality may vary depending on factors such as donor age, season and geographical location (Al-Ghalban et al., 2004), as season and melatonin treatment has been showed to influence reproductive abilities in bucks (Chemineau et al., 1992).

This study was therefore designed to assess the effect of adding seminal plasma (SP) on washed post-thawed buck sperm following a 3h incubation (thermo-resistance test) period under capacitation conditions with regards to donor age, season of collection and melatonin treatment. Our hypothesis lies on the fact that, the re-addition of seminal plasma (SP) after thawing may improve buck sperm resilience thereby increasing their survival and quality under *in vitro* capacitation conditions, thus mimicking their movement in the female genital tract.

Materials and methods

This experiment was carried out from semen samples collected (n=6 days of collection) in two consecutive years in autumn from 12 and 24 months old males, and samples were collected in two consecutive years in springs from 18 and 30 months old males with or without melatonin implants. Thereafter, single layer centrifugation (SLC) procedure was performed as described in the previous study. Then, immediately after SLC/washing, all sperm samples were incubated for 3h in three different media: (a) Modified PBS or control media (b) *In vitro* fertilization commercial media (BO-IVF media, IVF Biosciences, UK) and (c) BO-IVF media + seminal plasma (20%). All seminal plasma samples were collected during the breeding season (autumn) from the same males used in this study and processed as described in the general Material & Methods section. For each treatment/group, sperm samples were diluted in the various incubating media at a concentration of 40 $x10^6$ sperm/mL and placed in an incubator (5% CO_2) at 38.5°C for 3h.

Viability sperm analyses

All washed/SLC sperm samples from the breeding season (12 vs 24 months old), non-breeding season (18 vs 30 months) from males with and without melatonin after 3h of incubation in three different media were evaluated for plasma and acrosome membrane integrity as well as mitochondrial function using a quadruple-staining technique Tabarez et al. (2017) as described in the general Materials & Methods section. After evaluation, the following sperm population were taken into consideration: Viability (total SYBR14+/PI- sperm cells), viable cells with intact acrosome and functional mitochondria (SYBR14+/PI-/PE-PNA-/Mitotracker+), viable cells with damaged acrosome and functional mitochondria (SYBR14+/PI-/PE-PNA+/ Mitotracker+), viable cells with intact acrosome and non-functional mitochondria (SYBR14+/PI-/ PE-PNA-/Mitotracker-), viable cells with damaged acrosome and non-functional mitochondria (SYBR14+/PI-/PE-PNA+/ Mitotracker-). In addition, the total acrosome damage was assessed for all incubated sperm samples.

Analyses of DNA integrity

All washed sperm samples from the breeding season (12 vs 24 months old), non-breeding season (18 vs 30 months) from males with and without melatonin were evaluated after 3h of incubation in three different media for DNA integrity using Sperm Chromatin Structural Assay (SCSA) described by Evenson et al. (2002) as it was mentioned in the general Material & Methods section. The percentage DNA fragmentation index (DFI) was calculated as the ratio of denatured single stranded DNA (red colour) to the total cells acquired (red + green colour). The High DNA stainability (HDS) index was also evaluated, defined as the population with an elevated green value, outside the main population recorded.

Analyses of sperm motion parameters

Sperm motion parameters were evaluated immediately after 3h of incubation in the three different media for all washed samples collected in the breeding season (12 vs 24 months old) and non-breeding season (18 vs 30 months) from males with and without melatonin implants using the computer-assisted sperm analysis (CASA) system, ISAS® (PROISER S.L., Valencia, Spain), as it was described in the general Material and Methods section. The total motile sperm cells (TM), progressively motile sperm cells (PM), curvilinear velocity (VCL, μm/s), linear velocity (VSL, μm/s), mean velocity (VAP, μm/s), linearity coefficient (LIN), straightness coefficient (STR), amplitude of lateral head displacement (ALH, μm) and beat-cross frequency (BCF, Hz) were all evaluated.

Statistical analyses

Statistical analyses were performed using the SAS statistical package (Version 9.4, 2015, SAS Institute Inc., Cary, NC, USA) as described in the Material & Methods section.

Results

A significant reduction ($P<0.05$) in total viability percentages, as well as in the proportions of viable sperm with intact acrosome and active mitochondria was observed after 3h incubation in IVF media regardless the presence of SP (Table 2.1). Although not always significantly, samples collected in autumn showed higher viability under capacitation conditions

than in those collected in spring regardless of melatonin implant. The mean percentage of the total viable sperm with damaged acrosome and active mitochondria was low, though a significant increase was seen after 3h incubation in IVF media regardless of the presence of SP in autumn samples (Table 2.1). The proportions of viable sperm with inactive mitochondria (either with intact or damaged acrosome) were very low (<0.1%), hence data were not shown.

The total acrosome damage of thawed sperm after washing (T0) was statistically higher ($P<0.05$) in the period of autumn compared to those collected in spring regardless of melatonin implant and male age, while after 3h incubation, a significant increase was observed when samples were incubated in IVF media in the presence of SP with no differences in between male age, season, or melatonin treatment (Table 2.1.).

As regards to sperm DNA integrity (Table 2.2), no effect was found on High DNA Stainability (HDS) index after 3h incubation regardless of the media in any of the experimental groups, except in sperm samples from older males collected in autumn which showed a significant difference between control media and IVF media+SP. Similarly, there were no significant differences on HDS index between male age, except for samples collected during spring from older males which showed a significant reduction compared to samples collected in autumn but not in all the treatments. Furthermore, DNA fragmentation index (DFI) was not significantly affected after washing irrespective of age, season and melatonin treatment (Table 2.2). However, an increase in the DFI was recorded after 3h incubation in PBS (control media) in all the different samples, except for those collected in spring from 30 months old males. Similar increase on DFI was observed after 3h incubation in IVF, with or without SP, during spring regardless of melatonin implant, meanwhile autumn samples incubated in IVF media regardless the presence of SP did not differ from samples analysed immediately after washing.

The statistical analysis of CASA parameters showed that no significant differences were found in the total motility (TM) and progressive motility (PM) immediately after washing and after incubation in the control media between sperm samples from different male age, season or melatonin treatment. However, after 3h incubation period, TM and PM significantly were reduced in most of the experimental groups, especially when SP was added to the media (Table 2.3). Furthermore, samples collected in autumn showed a higher TM and PM when incubated in IVF media compared to those in spring regardless of male age or melatonin treatment, though no significant differences were observed amongst all the experimental groups.

Table 2.1: Effect of seminal plasma and its resilience under *in-vitro* capacitant conditions on the viability of thawed buck sperm with respect to male age (months) season, and melatonin implant analysed by flow cytometer

Sperm parameter (%)	Incubation media	Autumn		Spring		Spring +Melatonin	
		12	24	18	30	18	30
Sperm Viability	T_0	38.4 ± 3.7^{1}	39.7 ± 3.0^{1}	33.0 ± 5.1^{1}	29.8 ± 5.7^{1}	34.2 ± 5.5^{1}	30.8 ± 4.7^{1}
	Control	33.4 ± 1.7^{1}	31.6 ± 2.7^{1}	34.5 ± 4.7^{1}	21.5 ± 4.7^{1}	30.6 ± 4.3^{1}	25.6 ± 4.1^{1}
	IVF	$13.1 \pm 2.4^{ab,2}$	$15.3 \pm 2.9^{a,2}$	$10.1 \pm 2.3^{abc,2}$	$4.1 \pm 0.9^{c,2}$	$7.1 \pm 2.1^{abc,2}$	$5.5 \pm 1.9^{bc,2}$
	IVF+SP	$19.0 \pm 4.7^{a,2}$	$15.9 \pm 1.8^{a,2}$	$6.3 \pm 2.0^{ab,2}$	$2.7 \pm 0.8^{b,2}$	$4.4 \pm 1.4^{b,2}$	$2.9 \pm 0.6^{b,2}$
Intact acrosome and active mitochondrial sperm	T_0	37.0 ± 3.8^{1}	39.4 ± 3.0^{1}	32.6 ± 5.2^{1}	29.4 ± 5.7^{1}	33.9 ± 5.4^{1}	30.7 ± 4.7^{1}
	Control	32.0 ± 1.8^{1}	29.9 ± 3.2^{1}	33.7 ± 4.6^{1}	21.0 ± 4.6^{1}	30.2 ± 4.2^{1}	25.1 ± 4.0^{1}
	IVF	$11.6 \pm 2.1^{ab,2}$	$13.5 \pm 2.2^{a,2}$	$9.9 \pm 2.3^{abc,2}$	$3.5 \pm 0.7^{c,2}$	$6.7 \pm 2.1^{abc,2}$	$5.1 \pm 1.9^{bc,2}$
	IVF+SP	$18.3 \pm 4.8^{a,2}$	$14.6 \pm 2.1^{a,2}$	$5.6 \pm 2.2^{ab,2}$	$2.4 \pm 0.7^{b,2}$	$4.0 \pm 1.4^{b,2}$	$2.7 \pm 0.7^{b,2}$
Damaged acrosome and active mitochondrial sperm	T_0	$0.0 \pm 0.0^{b,2}$	$0.1 \pm 0.0^{ab,2}$	0.0 ± 0.0^{b}	$0.2 \pm 0.1^{a,12}$	$0.0 \pm 0.0^{b.2}$	$0.0 \pm 0.0^{b,2}$
	Control	0.0 ± 0.0^{2}	0.0 ± 0.0^{2}	0.0 ± 0.0	0.0 ± 0.0^{2}	0.0 ± 0.0^{2}	0.1 ± 0.1^{12}
	IVF	$0.5 \pm 0.2^{ab,1}$	$0.9 \pm 0.4^{a,1}$	0.1 ± 0.0^{b}	$0.3 \pm 0.1^{ab,1}$	$0.2 \pm 0.1^{ab,12}$	$0.4 \pm 0.2^{ab,1}$
	IVF+SP	$0.4 \pm 0.2^{ab,1}$	$1.0 \pm 0.5^{a,1}$	0.1 ± 0.0^{b}	$0.3 \pm 0.2^{ab,1}$	$0.4 \pm 0.2^{ab,1}$	$0.2 \pm 0.1^{ab,12}$
Total Acrosome Damage	T_0	$25.0 \pm 4.7^{ab,12}$	$35.6 \pm 3.2^{a,12}$	$6.0 \pm 0.8^{c,2}$	$6.5 \pm 0.9^{c,23}$	$14.2 \pm 3.0^{bc,12}$	$8.5 \pm 2.2^{c,2}$
	Control	$20.6 \pm 4.2^{a,2}$	$19.1 \pm 2.2^{a,2}$	$13.3 \pm 6.5^{ab,12}$	$5.0 \pm 0.7^{b,3}$	$8.8 \pm 2.2^{ab,2}$	$8.6 \pm 2.5^{ab,2}$
	IVF	21.1 ± 4.5^{2}	28.0 ± 6.9^{2}	14.8 ± 5.4^{12}	15.0 ± 4.9^{12}	15.3 ± 3.0^{12}	19.5 ± 7.4^{12}
	IVF+SP	58.0 ± 8.6^{1}	52.9 ± 5.0^{1}	49.3 ± 13.3^{1}	50.7 ± 12.3^{1}	52.9 ± 12.8^{1}	50.4 ± 10.7^{1}

[a-c] Different letters represent significant differences ($P<0.05$) between treatments and age,. [1-3] Different numbers represent significant differences ($P<0.05$) between samples within the parameters. T_0= Time 0, just after sperm washing by Single Layer Centrifugation (SLC); Control= modified PBS, IVF: 3h incubation in *in vitro* fertilization media; IVF+SP: 3h incubation in *in vitro* fertilization media + 20% seminal plasma. Data are shown as mean ± S.E.M.

Table 2.2: Effect of seminal plasma and its resilience under *in-vitro* capacitant conditions on the DNA integrity of thawed buck sperm with respect to male age (months) season, and melatonin implant

Sperm parameter	Incubation media	Autumn		Spring		Spring + Melatonin	
		12	24	18	30	18	30
HDS (%)	T_0	4.7 ± 0.9^{a}	$4.2 \pm 0.7^{a,12}$	2.4 ± 0.6^{ab}	1.1 ± 0.5^{b}	1.8 ± 0.4^{ab}	2.0 ± 0.8^{ab}
	Control	4.8 ± 0.9^{a}	$5.2 \pm 1.2^{a,1}$	2.7 ± 0.5^{ab}	2.0 ± 0.8^{ab}	1.7 ± 0.4^{ab}	1.2 ± 0.3^{b}
	IVF	4.4 ± 1.2^{a}	$3.7 \pm 0.8^{a,12}$	1.4 ± 0.4^{ab}	1.6 ± 0.6^{ab}	1.5 ± 0.5^{ab}	0.8 ± 0.2^{b}
	IVF+SP	3.0 ± 0.8^{a}	$1.6 \pm 0.2^{ab,2}$	1.9 ± 0.5^{ab}	1.1 ± 0.6^{b}	1.5 ± 0.3^{ab}	0.4 ± 0.1^{b}
DFI (%)	T_0	5.9 ± 0.6^{2}	6.8 ± 1.1^{2}	7.1 ± 1.8^{2}	12.9 ± 5.2	5.8 ± 0.5^{2}	5.9 ± 1.1^{2}
	Control	$31.6 \pm 3.2^{a,1}$	$29.1 \pm 5.0^{ab,1}$	$21.2 \pm 5.1^{ab,1}$	13.9 ± 1.7^{b}	$22.8 \pm 3.7^{ab,1}$	$19.7 \pm 3.0^{ab,1}$
	IVF	$6.5 \pm 1.2^{b,2}$	$6.1 \pm 1.4^{b,2}$	$17.7 \pm 3.3^{a,12}$	15.9 ± 2.5^{a}	$18.9 \pm 3.0^{a,1}$	$17.9 \pm 2.5^{a,1}$
	IVF+SP	$7.5 \pm 1.1^{b,2}$	$7.2 \pm 0.9^{b,2}$	$15.0 \pm 3.2^{ab,12}$	18.4 ± 2.5^{ab}	$20.7 \pm 3.7^{a,1}$	$18.5 \pm 3.3^{ab,1}$

[a,b] Different letters represent significant differences ($P<0.05$) between treatments and age,

[1,2] Different numbers represent significant differences ($P<0.05$) between samples within the parameters, T_0= Time 0, just after sperm washing by Single Layer Centrifugation (SLC); Control= modified PBS, IVF: 3h incubation in *in vitro* fertilization media; IVF+SP: 3h incubation in *in vitro* fertilization media + 20% seminal plasma, HDS: High DNA stainability. DFI: DNA fragmentation index. Data are shown as mean ± S.E.M.

In respect to sperm subpopulations, a total of 54,751 sperms were analyzed to establish motile subpopulations, we observed that 12,445 sperm presented a certain type of movement. By means of cluster analysis, four sperm subpopulations were obtained with different kinetic patterns ($P<0.05$) (Table 2.4). The subpopulation 1 (Sp1) sperm had moderately high velocities, LIN and ALH, although the BCF was the highest compared to the other two subpopulations, while subpopulation 2 (Sp2) presented the lowest velocities, ALH and BCF, but the highest LIN. Subpopulation 3 (Sp3) sperm presented the highest velocities and ALH, moderately high BCF and the lowest LIN compared to the others. Finally, in this study, static sperm constituted the fourth motile subpopulation (Sp4) (Table 2.4).

Significant reductions were observed in the Sp1, Sp2 and Sp3 proportions after 3h incubation in most of the experimental groups ($P<0.05$) (Table 2.5). No differences were found between ages or treatments in the Sp1 and Sp2 sperm percentages except after incubation in IVF media, where samples collected in autumn had greater values ($P<0.05$) compared to those in spring. Also, the percentage of Sp3 was significantly higher just after washing (0h) in samples collected in autumn. The Sp4 significantly increased after 3h incubation, whereas no differences were found across ages and season regardless of melatonin treatments, except in IVF media where samples collected in autumn showed lower percentages of static sperm.

Table 2.3: Effect of seminal plasma and its resilience under *in-vitro* capacitant conditions on the total and progressive motility of thawed buck sperm with respect to male age (months) season, and melatonin implant.

Sperm parameter	Incubation media	Autumn		Spring		Spring + Melatonin	
		12	24	18	30	18	30
TM (%)	T_0	42.0 ± 4.1^{1}	50.5 ± 2.9^{1}	34.0 ± 5.1^{1}	33.2 ± 3.9^{1}	36.2 ± 3.7^{1}	39.8 ± 4.8^{1}
	Control	22.4 ± 3.1^{2}	21.6 ± 3.3^{23}	18.8 ± 2.1^{12}	18.0 ± 2.1^{2}	13.8 ± 2.4^{2}	20.4 ± 5.4^{12}
	IVF	$24.1 \pm 0.8^{ab,2}$	$32.7 \pm 4.2^{a,12}$	$17.3 \pm 3.5^{abc,12}$	$15.4 \pm 1.1^{bc,2}$	$14.4 \pm 2.8^{bc,2}$	$10.0 \pm 2.9^{c,2}$
	IVF+SP	18.5 ± 8.0^{2}	16.1 ± 1.7^{3}	15.3 ± 3.3^{2}	11.0 ± 0.9^{3}	11.4 ± 3.5^{2}	8.9 ± 3.2^{2}
PM (%)	T_0	17.2 ± 2.6^{1}	16.0 ± 2.6^{1}	10.5 ± 1.6	15.5 ± 1.2^{1}	12.9 ± 1.4^{1}	17.6 ± 2.4^{1}
	Control	13.9 ± 3.0^{12}	9.5 ± 1.5^{12}	6.4 ± 1.1	8.6 ± 1.0^{2}	6.2 ± 1.4^{2}	8.5 ± 1.7^{12}
	IVF	$13.1 \pm 0.9^{a,12}$	$18.1 \pm 3.4^{a,1}$	6.6 ± 1.5^{b}	$6.3 \pm 1.3^{b,23}$	$5.6 \pm 1.2^{b,2}$	$4.0 \pm 1.6^{b,2}$
	IVF+SP	$5.9 \pm 1.9^{ab,2}$	$8.3 \pm 0.5^{a,2}$	4.6 ± 0.9^{ab}	$4.1 \pm 0.5^{ab,3}$	$4.2 \pm 1.1^{ab,2}$	$3.6 \pm 1.2^{b,2}$

[a-c] Different letters represent significant differences ($P<0.05$) between treatments and age.

[1-3] Different numbers represent significant differences ($P<0.05$) between samples within the parameters, T_0= Time 0, just after sperm washing by Single Layer Centrifugation (SLC); Control= modified PBS, IVF: 3h incubation in *in vitro* fertilization media; IVF+SP: 3h incubation in *in vitro* fertilization media + 20% seminal plasma. TM: Total Motility, PM: Progressive Motility. Data are shown as mean ± S.E.M.

Table 2.4: **Kinematic characteristics of the four sperm subpopulations in thawed buck sperm samples after washing by SLC.**

Parameter	Sp1	Sp2	Sp3	Sp4
VCL (µm/s)	109.5 ± 21.9^{b}	46.9 ± 23.4^{c}	163.2 ± 23.5^{a}	N.D
VSL (µm/s)	41.9 ± 32.1^{b}	20.6 ± 20.0^{c}	46.5 ± 32.8^{a}	N.D
VAP (µm/s)	65.9 ± 30.0^{b}	30.3 ± 20.8^{c}	88.1 ± 33.0^{a}	N.D
LIN (%)	36.4 ± 23.0^{b}	43.5 ± 26.6^{a}	28.1 ± 18.3^{c}	N.D
STR (%)	57.3 ± 25.3^{b}	63.1 ± 26.8^{a}	49.2 ± 24.2^{c}	N.D
ALH (µm)	4.9 ± 1.0^{b}	2.2 ± 1.0^{c}	7.6 ± 1.4^{a}	N.D
BCF (Hz)	6.2 ± 3.4^{a}	4.7 ± 3.2^{c}	5.7 ± 3.6^{b}	N.D
n	2815	9111	519	42306
%	5.14	16.64	0.95	77.27

$^{a\text{-}c}$ Different letters represent significant differences ($P<0.05$) between subpopulation

n: Number of sperm

%: Percentage of sperm in each subpopulation

Sp: Subpopulation, Data are presented as mean values ± SD.

N.D: No Data

Table 2.5: **Effect of seminal plasma and its resilience under *in-vitro* capacitant conditions on thawed buck sperm motile subpopulation with respect to age (months), season, and melatonin implant.**

Sperm parameter	Incubation media	Autumn		Spring		Spring + Melatonin	
		12	24	18	30	18	30
Sp1 (%)	T_0	12.5 ± 1.0^{1}	14.0 ± 2.7^{1}	7.9 ± 1.5^{1}	7.0 ± 1.3^{1}	7.1 ± 1.2^{1}	7.7 ± 2.3^{1}
	Control	6.7 ± 1.7^{2}	6.4 ± 0.8^{12}	3.5 ± 0.9^{2}	3.6 ± 0.9^{12}	2.9 ± 0.6^{2}	3.4 ± 0.9^{12}
	IVF	$4.6 \pm 1.1^{ab,2}$	$6.3 \pm 1.6^{a,12}$	$1.8 \pm 0.2^{b,2}$	$1.6 \pm 0.8^{b,23}$	$1.6 \pm 0.5^{b,2}$	$1.5 \pm 0.9^{b,2}$
	IVF+SP	0.0 ± 0.0^{3}	0.2 ± 0.2^{2}	0.0 ± 0.0^{2}	0.0 ± 0.0^{3}	0.0 ± 0.0^{3}	1.0 ± 0.6^{2}
Sp2 (%)	T_0	26.3 ± 3.4	34.1 ± 1.9^{1}	25.2 ± 4.0	25.2 ± 3.4^{1}	28.0 ± 2.8^{1}	30.5 ± 2.8^{1}
	Control	15.2 ± 1.8	14.2 ± 3.0^{2}	14.6 ± 1.2	14.2 ± 1.8^{12}	10.6 ± 2.0^{2}	16.7 ± 4.7^{12}
	IVF	18.7 ± 1.3^{ab}	$25.6 \pm 3.2^{a,1}$	15.2 ± 3.4^{ab}	$13.5 \pm 1.9^{ab,2}$	$11.8 \pm 2.1^{b,2}$	$8.5 \pm 2.0^{b,2}$
	IVF+SP	18.5 ± 8.0	13.2 ± 2.3^{2}	15.3 ± 3.3	11.0 ± 0.9^{2}	11.2 ± 3.4^{2}	7.9 ± 2.9^{2}
Sp3 (%)	T_0	$3.2 \pm 0.6^{a,1}$	2.4 ± 0.4^{ab}	0.9 ± 0.4^{b}	$0.9 \pm 0.3^{b,1}$	1.1 ± 0.5^{b}	$1.6 \pm 0.5^{ab,1}$
	Control	0.5 ± 0.2^{23}	1.1 ± 0.3	0.7 ± 0.4	0.3 ± 0.2^{12}	0.3 ± 0.2	0.3 ± 0.2^{12}
	IVF	0.9 ± 0.3^{2}	0.8 ± 0.3	0.3 ± 0.2	0.2 ± 0.2^{12}	0.9 ± 0.4	0.0 ± 0.0^{2}
	IVF+SP	$0.0 \pm 0.0^{b,3}$	2.6 ± 1.3^{a}	0.0 ± 0.0^{b}	$0.0 \pm 0.0^{b,2}$	0.2 ± 0.2^{ab}	$0.0 \pm 0.0^{b,2}$
Sp4 statics (%)	T_0	58.0 ± 4.1^{2}	49.5 ± 2.9^{3}	66.0 ± 5.1^{2}	66.8 ± 3.9^{3}	63.8 ± 3.7^{2}	60.2 ± 4.8^{2}
	Control	77.6 ± 3.1^{1}	78.4 ± 3.3^{12}	81.2 ± 2.1^{12}	82.0 ± 2.1^{2}	86.2 ± 2.4^{1}	79.6 ± 5.4^{12}
	IVF	$75.9 \pm 0.8^{bc,12}$	$67.3 \pm 4.2^{c,23}$	$82.7 \pm 3.5^{abc,1}$	$84.6 \pm 1.1^{ab,2}$	$85.7 \pm 2.8^{ab,1}$	$90.0 \pm 2.9^{a,1}$
	IVF+SP	81.6 ± 8.0^{1}	84.0 ± 1.7^{1}	84.7 ± 3.3^{1}	89.0 ± 0.9^{1}	88.6 ± 3.5^{1}	91.1 ± 3.2^{1}

[a-c] Different letters represent significant differences ($P<0.05$) between treatments and age,

[1-3] Different numbers represent significant differences ($P<0.05$) between samples within the parameters, T_0= Time 0, just after sperm washing by Single Layer Centrifugation (SLC); Control= modified PBS, IVF: 3h incubation in *in vitro* fertilization media; IVF+SP: 3h incubation in *in vitro* fertilization media + 20% seminal plasma. Sp: Subpopulation. Data are shown as mean ± S.E.M.

Discussion

The functional and kinetic characteristics of cryopreserved buck sperm during an incubation period to mimic the environment of the female reproductive tract were explored. Moreover, the evaluation of SP addition to post-thawed buck sperm and its resilience under *in vitro* capacitation conditions with respect to male age, melatonin treatment and season of semen collection was assessed, and our results showed various interactions herein.

In the present study, the incubation period in the control media had no negative effect on sperm viability. However, a decrease in the viability after incubation in IVF media regardless of the presence of SP was found, probably due to structural and functional changes in buck sperm under capacitation conditions (Holt & Van Look, 2004). In other words, the IVF media may have induced an increase in live acrosome reacted sperm which eventually dies if fertilization does not take place (Williams et al., 1991). However, with the addition of SP to the IVF media, this hypothesis may be more difficult to interpret, since SP has been reported to slow down capacitation and acrosome reaction (Maxwell & Johnson, 1999). Notably, the incidence of acrosome reaction after 3h incubation period in IVF media with or without SP was low and similar across all ages and season regardless of melatonin treatment. Therefore, we may suggest that the commercial IVF media used was not efficient enough to induce *in vitro* capacitation and subsequently acrosome reaction or, alternatively, the incubation time was insufficient to see more capacitation-like changes.

The high percentage of total acrosome damage (dead and viable sperm) seen in sperm incubated in IVF media in the presence of SP cannot be explained due to the incidence of spontaneous acrosome reaction, as earlier discussed. In this regard, a potential explanation may be that buck seminal plasma could cause acrosome damage due to the presence of a hydrolysing enzyme, phospholipase-A2 found in buck seminal plasma (Ferreira et al., 2014; Pellicer-Rubio et al., 1997). Nevertheless, our results are in agreement with a report in ram (Domínguez et al., 2008a) demonstrating that the addition of SP did not improve the sperm viability and acrosome status, although seminal plasma has been reported to reverse cold shock (Barrios et al., 2000; Nunes et al., 1982). This discrepancy between researchers may be due to the heterogeneity of seminal plasma composition which have been demonstrated across species (Druart et al., 2013).

On the other hand, the survival of sperm samples collected in autumn were slightly higher after 3h incubation under *in vitro* capacitation conditions compared to those collected in spring regardless of melatonin implants, which could be related to seasonal effects

showing higher semen quality on bucks during autumn (Coloma et al., 2011; Pérez & Mateos, 1996; Roca et al., 1992). However, total acrosome damage was surprisingly lower ($p<0.05$) in samples collected in spring just after washing and after 3 h incubation in control media. This finding could be related to physiological or structural differences of sperm membranes during the non-breeding season (Dias et al., 2017), although age and season had no identifiable effect on the acrosome integrity after incubation in IVF media.

DNA integrity has shown to be a reliable indicator of sperm quality in most animal species. In our study, age of males, season or melatonin treatment appeared not to affect the extent of DNA fragmentation after washing. However, an increase was observed after 3h incubation in control media in agreement with Crespo et al. (2013). However, thawed samples collected in autumn and incubated in IVF media where DNA integrity was maintained maybe suggested to be due to the better quality of the semen during the breeding season (Coloma et al., 2011). In this point, post-thaw incubation period may reveal some information not visible immediately after thawing which could partially explain the reason why semen produced in autumn could give better fertility rates. Despite reports on the antioxidant properties of the SP preventing the degradation of sperm DNA (Twigg et al., 1998), our results showed no beneficial effect of its presence in the incubation media on the sperm DNA status.

Although no great differences on high DNA stainability were observed between male ages regardless of season or melatonin treatment, a slight decreasing tendency was observed when the samples were collected in spring. This seasonal effect agrees with a similar report showing higher HDS values in the breeding season when compared to non-breeding season (García-Macías et al., 2006). However, HDS sperm population has been also described as immature cells (Evenson et al., 2002), expecting that these peculiarities may be more related to sperm in the non-breeding season. Nevertheless, further investigation is required since this high DNA stainability in the buck sperm may reflect an unknown aspect of sperm function. No significant effect of incubation was seen on the HDS index, although Peris et al. (2004) demonstrated that incubation of ram sperm enhanced sperm damage thus leading to high values of DFI and HDS reflected by DNA strand breaks or poor chromatin condensation.

With regards to motility, the negative effect of 3h incubation, especially in IVF media in presence of SP, regardless of age, season and melatonin treatment may be due to mitochondria ageing during cryopreservation resulting in low ATP production (Vishwanath & Shannon, 1997). While viability or the intactness of plasma membrane was not

significantly affected by 3h incubation in the control media, motility was reduced, showing that cryopreservation may have different effects on sperm functionality with respect to different post-thawing methods (Gangwar et al., 2016).

Nevertheless, our results showed that TM and PM were not affected by the incubation media as much as seen in the sperm viability. This implies that the *in vitro* capacitation conditions may have affected the sperm membrane integrity much more than the ability of the sperm to be motile (Watson, 1995). Furthermore, this deleterious effect on sperm motility was more evident in spring samples which showed lower values regardless of melatonin treatment. Again, the application of a resilience test to analyse certain aspects which may not have been detected immediately after thawing is strongly recommended.

Furthermore, no improvement on the sperm motility was observed in the presence of SP regardless of age, season or melatonin treatment. On the contrary, the addition of SP or its components to ram post-thawed semen has been reported to increases sperm motility (Domínguez et al., 2008a). As earlier mentioned, differences in the sperm physiology and SP composition between species, as well the complex composition and inconsistency of SP effect on sperm has been demonstrated (Moore et al., 2005b; Rovegno et al., 2013a; Sabatini et al., 2014). Nevertheless, similar reductions were seen in sperm incubated in IVF media alone, used as capacitation media in this study. In fact, buck sperm cells may lose their motility after incubation in an *in vitro* capacitation media once they reveal a "jerk" movement (Anand et al., 1989).

In the subpopulation structure, four different subpopulations were described in our study, including the non-motile sperm subpopulation or statics, but no significant changes on their distribution amongst ages, season or melatonin treatment were found, except after IVF incubation where Sp1 and Sp2 proportions were significantly lower in spring while the static proportion (Sp4) increased. More evident was the effect of 3h incubation causing the increase of Sp4 (statics) and the reduction of the Sp1 proportions. Also, a further decrease of Sp1 was observed in sperm incubated under capacitation conditions in the presence of SP, which may also be attributed to the detrimental effect of SP as earlier discussed. Similar effect was seen in the Sp2 and Sp3 subpopulation after 3h incubation compared to the proportions just after washing. It is worthy to note that Sp3 may require special attention as this subpopulation exhibited some sperm motility characteristic (very high VCL and ALH and very low LIN) which may depict "hyperactivation" as described by Mortimer & Mortimer (1990). Our results showed that this "hyperactivated" subpopulation was generally lower in spring and

almost inexistent when seminal plasma was added in the media, which can be related to the ability of SP to slow down capacitation, acrosome reaction and hyperactivation (Maxwell & Johnson, 1999) since these three phenomenon occurred concurrently (Mortimer et al., 1998).

In conclusion, buck seminal plasma did not improve motility and viability of thawed buck sperm under *in vitro* capacitation conditions irrespective of male age, season or melatonin treatment. The addition of SP in the incubation media increased acrosome damage while the incidence of acrosome reaction was similar when IVF media alone was used. Furthermore, DNA integrity was maintained in *in vitro* capacitation media regardless of the presence of SP during the breeding season. Finally, the role of seminal plasma is still controversial due to its complex composition in bucks, and also it is important to carry out a thermo-resistance test to reveal damages that may not be detected immediately after thawing.

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Study 3

Assessment of Individual male factor on post-thawed buck sperm quality and on the effectiveness of SLC

Abstract

The aim of this study was to evaluate potential individual male factor on thawed sperm quality as well on the effectiveness of Single layer Centrifugation (SLC). Therefore, 2 ejaculates/male/day were collected via artificial vagina from 6 male goats of the *Cabra Blanca de Rasquera* breed, approximately 2 years of age in early winter (n= 6 days of semen collection). The 2 ejaculates of each male were pooled and then the pool semen sample was treated individually, washed in TCG solution by centrifuging twice (600 x g, 10 min), and diluted in the extender containing 15% powdered egg yolk and 5% glycerol before freezing. After cryopreservation, two straws per individual from each replicate (n = 6) were thawed by immersion in a water bath at 37 °C for 30 s. After thawing, individual sperm samples were analysed immediately as the Non-SLC male samples while the remaining sperm samples were diluted in PBS and then, SLC procedure was carried out (SLC group). Sperm motility was evaluated by CASA, while plasma and acrosome membrane integrity, mitochondria activity and DNA fragmentation were analysed by flow cytometer before and after SLC. Only few significant differences were observed immediately after thawing on total sperm viability, on the percentage of sperm cells with intact acrosome and active mitochondria and on the proportion of sperm cell with damaged acrosome and active mitochondria between individual males, however this differences were absent after SLC. No differences were observed between individuals before and after SLC in regards to the motility, viability, and DNA integrity, except in the total acrosome damage in which 2 males from the 6 tested males were significantly reduced after SLC. In conclusion, there was no effect of "individual males" on buck sperm quality after SLC.

Introduction

Individual differences in the quality of spermatozoa have been established between the same specie due to their varying susceptibility to freezing and thawing (Curry, 2000). Understanding the principle that might affect the survivability of individual males is an instrumental reason why sperm from different males of the same specie survive cryopreservation differently. For instance, changes in the lipid composition of the cell membrane, temperature, and time of sperm mixture during epididymal transit has contributed greatly to differences in the cryosurvival of individual males (Holt, 2000). Also, individual

male variation have been associated with alterations in seminal plasma proteins (Moura et al., 2010).

Various reports demonstrated that certain characteristics such as sperm head morphometry, DNA status, motility and velocity (Hernández et al., 2006; Martinez-Pastor et al., 2005; Thurston et al., 2001) as well as genetics can influence individual male responses to cryopreservation. Due to these variations to withstand freezing during cryopreservation, individual males can be grouped into "good freezers" or "bad freezers" (Barbas & Mascarenhas, 2009), and this has been demonstrated in stallions (Janett et al., 2003), boar (Yeste et al., 2013) and even in goats (Dorado et al., 2010).

On the other hand, Single layer centrifugation (SLC) through specie specific colloids has been reported to select spermatozoa with a good quality, morphology, chromatin, and membrane integrity (Morrell & Rodriguez-Martinez, 2009). However, it has been reported that individual variations in sperm quality were decreased after this sperm selection method (Morrell et al. 2009). To the best of our knowledge, there is lack of information on the potential effect of individual male characteristics and the effectiveness of single layer centrifugation (SLC) on thawed buck sperm, therefore the aim of this study was to assess the effect of "individual male factor" and the efficiency of SLC on post thaw buck sperm quality.

Materials and methods

For this experiment, 6 male goats of the *Blanca de Rasquera* breed, of 2 years old were used. Two ejaculates/male/day were collected via artificial vagina from 6 male goats in early winter (n= 6 days of collection). The 2 ejaculates of each male were pooled and then the pool semen samples were treated individually, washed in TGC solution by centrifuging twice (600 x g, 10 min), and diluted in the extender containing 15% powdered egg yolk and 5% of glycerol (final concentration). Thereafter, sperm concentration was adjusted to 400 x 10^6 sperm/mL and equilibrated for 4 h at 5°C before freezing in liquid nitrogen vapours at 5cm over nitrogen level for 10mins and finally submerged into the liquid nitrogen.

After cryopreservation, two straws per individual from each replicate (n = 6) were thawed by immersion in a water bath at 37 °C for 30 s. Afterwards straws were cleaned, and the content was poured into a dry tube and kept in a water bath, maintained at the same temperature. Subsequently, each thawed individual pooled sperm sample was diluted (1:4) with normal PBS to a concentration of 100 x 10^6sperm/mL and analysed before and after

SLC. The single layer centrifugation (SLC) procedure was performed as described in the general Material & Methods section.

Viability sperm analyses

Plasma and acrosome membrane integrity as well as mitochondrial function of all groups, Non-SLC and SLC individual sperm samples were evaluated by flow cytometry, using a quadruple-staining technique (Tabarez et al., 2017) as described in the general Materials and Methods section. After evaluation, the following sperm population were taken into consideration: Viability (total SYBR14+/PI- sperm cells), viable cells with intact acrosome and functional mitochondria (SYBR14+/PI-/PE-PNA-/Mitotracker+), viable cells with damaged acrosome and functional mitochondria (SYBR14+/PI-/PE-PNA+/ Mitotracker+), viable cells with intact acrosome and non-functional mitochondria (SYBR14+/PI-/ PE-PNA-/Mitotracker-), viable cells with damaged acrosome and non-functional mitochondria (SYBR14+/PI-/PE-PNA+/ Mitotracker-). In addition, the total acrosome damage was assessed for all non-SLC and SLC individual sperm samples.

Analyses of DNA integrity

All individual sperm samples were evaluated before and after SLC for DNA integrity using Sperm Chromatin Structural Assay (SCSA) described by Evenson et al. (2002) as it was mentioned in previous chapter. The percentage DNA fragmentation index (DFI) was calculated as the ratio of denatured single stranded DNA (red colour) to the total cells acquired (red + green colour). The High DNA stainability (HDS) index was also evaluated, defined as the population with an elevated green value, outside the main population recorded.

Analyses of sperm motion parameters

Sperm motion parameters were evaluated immediately after thawing (Non-SLC) and after SLC for all individual samples, using the computer-assisted sperm analysis (CASA) system, ISAS® (PROISER S.L., Valencia, Spain). The total motile sperm cells (TM), progressively motile sperm cells (PM), curvilinear velocity (VCL, μm/s), linear velocity (VSL, μm/s), mean velocity (VAP, μm/s), linearity coefficient (LIN), straightness coefficient (STR), amplitude of lateral head displacement (ALH, μm) and beat-cross frequency (BCF, Hz) were all evaluated.

Statistical analyses

Statistical analyses were performed using the SAS statistical package (Version 9.4, 2015, SAS Institute Inc., Cary, NC, USA). The different analyses were performed as they have been previously described in the Material & Methods section.

Results

In regards to total sperm viability and the proportion of viable sperm with intact acrosome and active mitochondria analysed immediately after thawing, two males (65 and 67) showed statistically higher values compared to only one of the tested males (61), while the rest of the males (62, 63 and 66) did not show any significant difference amongst individuals. However, after SLC, there were no significant differences between males. In relation to the efficiency of the SLC method used, only one of the males (66) showed an increase in the percentage total sperm viability and sperm with intact acrosome and active mitochondria compared to the values obtained before SLC (Table.3.1). In relation to the other viable sperm categories obtained after thawing through quadruple staining technique, all the values were extremely low, although slight differences were found in the percentages of viable sperm with damaged acrosome and active mitochondria (potential acrosome reaction), the highest mean values did not exceed of 0.2 % (Table 3.1).

Similar values were obtained in the percentage of viable sperm with intact acrosome and inactive mitochondria, where the highest mean value was 2. 0 % (data not shown). In any case, no differences were seen after SLC amongst males and no huge effect of this washing method was observed in any of the tested males. Furthermore, no significant difference was observed amongst individual males in the percentage total acrosome damage after thawing or after SLC, however, in sperm samples from 2 males (63 and 66) from the six tested males, the acrosome integrity increased statistically after SLC was performed (Table 3.1).

Table 3.1: Effect of individual males on buck sperm viability and acrosome integrity after thawing and after SLC.

Sperm parameter (%)	Incubation media	61	62	63	65	66	67
Viability	NSLC	15.0 ± 3.4[b]	23.6 ± 0.7[ab]	25.0 ± 3.6[ab]	33.1 ± 4.2[a]	24.0 ±1.4[ab.2]	34.7 ± 2.9[a]
	SLC	18.4 ± 4.3	29.8 ± 4.3	23.6 ± 1.5	34.4 ± 4.2	30.7 ± 1.8[1]	31.8 ± 5.6
Intact acrosome and active mitochondrial	NSLC	13.2 ± 3.7[b]	22.2 ± 0.8[ab]	23.0 ± 4.1[ab]	32.9 ± 4.2[a]	23.6 ±1.4[ab.2]	34.5 ± 2.9[a]
	SLC	16.8 ± 4.9	29.2 ± 4.3	22.7 ± 1.8	33.5 ± 4.5	30.4 ± 1.8[1]	31.5 ± 5.5
Damage acrosome and active mitochondrial	NSLC	0.0 ± 0.0[b]	0.2 ± 0.1[a]	0.0 ± 0.0[b]	0.2 ± 0.0[a]	0.1 ± 0.0[ab]	0.2 ± 0.0[a]
	SLC	0.1 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.0
Acrosome damage	NSLC	21.8 ± 3.0	18.2 ± 2.7	13.0 ± 1.1[1]	17.0 ± 1.9	16.6 ± 1.5[1]	12.3 ± 2.4
	SLC	14.6 ± 2.1	16.8 ± 3.1	8.9 ± 1.2[2]	12.1 ± 2.2	8.3 ± 0.7[2]	9.1 ± 1.6

[a,b] Different letters represent significant differences (P<0.05) between males

[1,2] Different letters represent significant differences (P<0.05) between samples within the parameters, NSLC: Non-selected, SLC- Selected. Data are shown as mean ± S.E.M.

Related to SCSA results, there were no significant differences in the high DNA Stainability (HDS) and DNA fragmentation index (DFI) amongst individuals just after thawing as well after SLC (Table 3.2), only individual sperm samples from one male (62) showed lower DFI ($p<0.05$) after SLC was performed. Likewise, no significant differences were found between donors with regards to the total and progressive motility (Table 3.3). Furthermore, no effect of the male was found on the effectiveness of SLC.

Table 3.2: **Effect of individual males on buck sperm chromatin integrity after thawing and after SLC.**

Sperm parameter	Incubation media	61	62	63	65	66	67
HDS (%)	NSLC	3.4 ± 0.4	3.9 ± 0.7	3.1 ± 0.8	5.6 ± 1.2	2.8 ± 0.6	2.9 ± 0.8
	SLC	3.1 ± 0.7	2.7 ± 1.1	3.1 ± 0.6	5.4 ± 1.2	2.1 ± 0.4	3.0 ± 1.0
DFI (%)	NSLC	12.1 ± 1.6	13.4 ± 1.7[1]	12.3 ± 0.9	13.5 ± 1.3	11.9 ± 2.2	12.0 ± 2.3
	SLC	9.8 ± 1.6	8.4 ± 1.4[2]	9.9 ± 0.9	11.5 ± 1.4	11.8 ± 1.9	15.0 ± 2.7

[a,b] Different letters represent significant differences ($P<0.05$) between males,

[1,2] Different letters represent significant differences ($P<0.05$) between samples within the parameters, NSLC: Non-selected, SLC- Selected, HDS: High DNA Stainability, DFI: DNA Fragmentation Index. Data are shown as mean ± S.E.M.

By the CASA system, a total of 37,565 sperm tracks were analysed and four subpopulations were established. From them, 12,006 sperms presented motile movements hence can be characterised into the following: subpopulation 1 (Sp1) comprises of medium velocities, low LIN, and medium ALH, and BCF, while subpopulation 2 (Sp2) presented the lowest velocities, highest LIN, lowest ALH, and BCF. The subpopulation 3 (Sp3) presented the highest velocities, moderate LIN, highest ALH, and BCF, and lastly, the static sperm (Sp4) which constituted the fourth motile subpopulation (Table 3.4).

No significant differences were found between individual male donors with regards to the distribution of motile subpopulations just after thawing, and after SLC. Furthermore, no effect of the males was found on the effectiveness of SLC between individual male samples. (Figure 3.1.)

Table 3.3:.Effect of individual male factor on thawed buck sperm total and progressive motility after thawing and after SLC

Sperm parameter	Incubation media	61	62	63	65	66	67
TM (%)	NSLC	24.8 ± 3.4	25.5 ± 2.0	29.2 ± 6.0	40.9 ± 4.8	34.8 ± 3.5	32.5 ± 2.7
	SLC	24.7 ± 3.7	21.1 ± 4.6	25.1 ± 5.6	41.4 ± 8.1	47.1 ± 6.2	36.6 ± 7.2
PM (%)	NSLC	2.8 ± 0.4	3.2 ± 0.7	2.5 ± 0.6	4.2 ± 1.0	4.5 ± 0.7	5.9 ± 1.0
	SLC	4.5 ± 1.5	3.0 ± 0.8	3.3 ± 0.5	4.4 ± 1.6	5.8 ± 2.2	3.8 ± 0.8

[a,b] Different letters represent significant differences (P<0.05) between males,

[1,2] Different letters represent significant differences (P<0.05) between samples within the parameters,

TM: Total motility, PM: Progressive motility, NSLC: Non-selected, SLC- Selected. Data are shown as mean ± S.E.M.

Table 3.4: Kinematic characteristics of the four sperm subpopulations identified in thawed buck sperm samples.

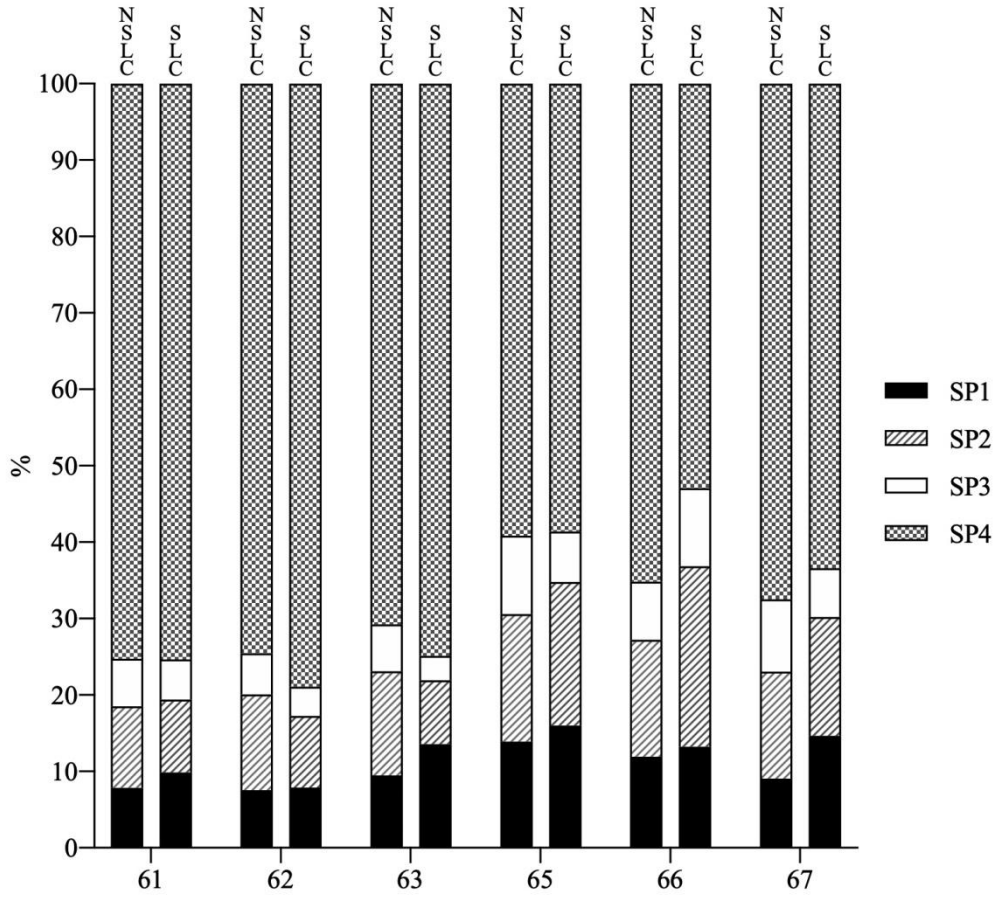
Parameter	Sp1	Sp2	Sp3	Sp4
VCL (µm/s)	54.6 ± 24.6[b]	33.1 ± 15.5[c]	115.6 ± 30.5[a]	N.D.
VSL (µm/s)	9.8 ± 7.0[c]	18.9 ± 10.0[b]	50.1 ± 23.2[a]	N.D.
VAP (µm/s)	27.1 ± 12.9[b]	24.1 ± 11.9[c]	74.1 ± 22.2[a]	N.D.
LIN (%)	18.2 ± 10.2[c]	58.1 ± 16.7[a]	45.6 ± 20.7[b]	N.D.
STR (%)	36.4 ± 19.4[c]	78.5 ± 12.5[a]	67.3 ± 20.2[b]	N.D.
ALH (µm)	3.0 ± 1.3[b]	1.9 ± 0.8[c]	5.0 ± 1.7[a]	N.D.
BCF (Hz)	4.0 ± 2.4[b]	3.3 ± 2.6[c]	6.7 ± 3.6[a]	N.D.
n	4,316	5,433	2,257	25,559
%	11.49	14.46	6.01	68.04

[a-c] Different represent significant differences (P<0.05) between subpopulations

n: Number of sperm,%: Percentage of sperm in each subpopulation, Sp: Subpopulation

N.D: No data. Data are shown as mean ± S.D.

Figure 3.1: Effect of individual male factor on thawed buck sperm motile subpopulation after thawing and after SLC.



NSLC: Non- selected; SLC: Single layer centrifugation. SP: Subpopulation.

Discussion

Highlights of this study showed that male 65 and 67 showed better quality after thawing compared to 61. As earlier stated, variations that exist in spermatozoa after cryopreservation may be due to the chemical composition of the sperm membrane which may affect its permeability, and osmotic tolerance (Graham 1996). Previous works conducted few years back by our group (Tabarez, 2014) found differences between the same individual males after thawing, classifying these individual differences into 3 categories: good, moderate, and bad freezers. However, in our recent study, similar results were found except that these individual differences were not expressed fully. A possible explanation may be due to the period of storage of these sperm samples, since prolonged time of storage of spermatozoa has been reported to alter semen characteristics as a result of the natural ageing process of the spermatozoa (Johnson et al., 2000), which could have decreased the

differences between males seen after a period of time in liquid nitrogen. Similarly, Ducha et al. (2012) found that prolonged storage may affect the motility of individual male spermatozoa after cryopreservation.

On the other hand, in this study, even though individual differences existed after thawing with regards to the viability and mitochondria function, the differences between males were absent when SLC was performed. Furthermore, there was no significant improvement of sperm viability after SLC with Bovipure® colloid except in one of the males, while acrosome integrity was better after SLC in 2 of the 6 tested males. Therefore, beside the potential differences on sperm freezability between males, the effectiveness of the method of sperm selection could be influenced by the individual since the behaviour of the sperm after SLC was different depending on the sperm donor. Also some reports found a significantly bigger improvement of sperm quality after SLC in sperm samples with a low freezability, while those with medium or high freezability showed similar increase in their sperm quality after SLC (Ortiz et al., 2015).

Specific DNA gene sequences responsible for the integrity of the DNA have been established in which their spermatozoa can be classified as good or bad quality (Thurston et al., 2001). In our study, no effect was observed in the DNA status amongst individual male sperm samples after thawing and even after SLC was carried out. This finding could suggest that the males did not exhibit considerable genetic differences in the DNA strands of the sperm to highlight individual differences, although the number of tested males was not sufficient enough to express them. Similarly, Nongbua et al. (2017) did not find any difference in the DNA integrity between different male samples after thawing and after SLC in frozen-thawed bull sperm. Furthermore, a heat shock protein gene, HSP70 in Boer goat sperm (Nikbin et al., 2014) and HSP90 in pig sperm (Casas et al., 2009) have been suspected to affect their ability to withstand freezing since these proteins provide thermo tolerance to cell thereby protecting them against apoptosis during injury and other oxidative stressful conditions (Beere & Green, 2001).

SLC through Androcoll® colloid has been described to selected frozen thawed spermatozoa with better motility in several species (Jiménez-Rabadán., 2012; Macías García et al., 2009; Thys et al., 2009). Therefore, we hypothesized that the effectiveness of SLC with Bovipure® to select frozen thawed buck spermatozoa with good motile abilities could also depend on the individual male. Nonetheless, on the basis of our observation, no significant differences were observed between individual males before and after washing method,

although mean values of total motility of some males were almost twice the mean values of other males, especially after SLC. Contrary to our results, a significant improvement was found in the motility between individual males in stallions spermatozoa after SLC and DGC was performed (Morrell et al., 2009). Again, perhaps the lack of significant difference may be due to the insufficient number of males and observations. However, in relation to progressive motility, all the males showed similar, but quite low values, suggesting the prejudicial effect of the period of storage of these sperm samples as mentioned previously. Furthermore, no differences on the sperm subpopulation distribution were observed before and after washing (SLC) being that the proportion of each motile subpopulation was almost homogeneous between individual males, apart from the sP4 or static sperm which was the greater subpopulation.

In conclusion, our study did not show a fully manifested individual male factor in buck sperm quality after cryopreservation, although some differences on sperm viability were observed between males. Finally, SLC method through Bovipure® colloid may not be efficient in improving thawed buck sperm quality regardless of the donor, although different sperm behaviour were seen depending on the male.

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Study 4

Assessment of Individual male factor on post-thawed buck sperm quality after incubation with seminal plasma under *in vitro* capacitation conditions.

Abstract

The aim of this study was to evaluate the effect of individual male factor on thawed cryopreserved buck sperm quality after 3h incubation with seminal plasma (SP) under *in vitro* capacitation conditions. Semen was collected via artificial vagina from 6 male goats of the *Blanca de Rasquera* breed, 2 years old approximately. Ejaculates were treated individually, washed in TCG solution by centrifuging twice (600 x g, 10 min), and diluted in the extender containing 15% powdered egg yolk and 5% glycerol before freezing. After cryopreservation, two straws per individual from each replicate (n = 6) were thawed and washed through single layer centrifugation method (SLC). Thereafter, washed sperm samples were incubated for 3h in the following media: (a) modified PBS or control media (b) *in vitro* fertilization commercial media (BO-IVF media, IVF Biosciences, UK) (c) BO-IVF media + Seminal Plasma (SP, 20%) and (d) modified PBS + SP (20%). Sperm motility was evaluated by CASA, while plasma and acrosome membrane integrity, mitochondria activity and DNA fragmentation were analysed by flow cytometer. No significant differences were observed in the sperm motility, viability and percentage of sperm with intact acrosome and active mitochondria amongst the different individual males after washing. However after 3h incubation, some individual differences were found between males and amongst incubation media, especially when sperm were incubated in the presence of SP, showing significant lower values on viability as well as sperm motility parameters. In regards to the DNA integrity, no differences were found between males after washing in the DNA Fragmentation Index (DFI) and High DNA Stainability (HDS). Nonetheless after 3h incubation, the DFI showed some significant differences amongst males especially in IVF media. Lastly, in terms of motility and motile sperm subpopulation, no differences were found after washing and after 3h incubation between males.

In conclusion, incubation of buck spermatozoa with seminal plasma under *in-vitro* capacitation condition did not improve buck sperm motility, viability, and membrane status irrespective of the male donor.

Introduction

The variations that exist between males can also influence differences in the survival of spermatozoa in the female reproductive tract (Shannon, 1978). Some ejaculates may contain subpopulations of spermatozoa that vary in fertilizing capacity, and these characteristics are peculiar to each male (Gomes et al., 2020). However, certain attributes such as the morphology and physiology of spermatozoa during spermatogenesis can influence the fertilizing capacity of each male, and of course this can be linked to the differences in the freezability of individual males (Ramm et al., 2014). Also the lipid composition of the sperm membrane influences the susceptibility of sperm cells to damage during cryopreservation (Holt, 2000). Due to these differences, semen donors can be grouped into "good "or "bad" freezers based on the post thaw quality and these males or their samples can be excluded during artificial insemination (A.I) programs. Furthermore, even though the differential mode of action involved between good and bad freezing male is not known, Vilagran et al. (2015) suggested that individual freezabilities may also be related to genetic differences. For instance, a heat shock protein gene, HSP70 in Boer goat sperm (Nikbin et al., 2014) and HSP90 in pig sperm (Casas et al., 2009) have been suspected to affect their ability to withstand freezing.

In bull spermatozoa, reports have demonstrated that individual differences exist during *in vitro* capacitation conditions and this variations have been used to predict fertility during A.I (Blottner et al., 1990). On the other hand, seminal plasma (SP) has been reported to play an important role in improving sperm viability, sperm resistance to cold shock, and improving the fertilizing ability of spermatozoa in the female reproductive tract (Maxwell et al., 2007). It has been also shown to be beneficial in the motility, acrosome and capacitation status in "good freezers" (Al-Essawe et al., 2018), and in the chromatin status, plasma and acrosome membrane in "bad freezers" (Aurich et al., 1996; Okazaki et al., 2009).

We hypothesised that incubating thawed buck sperm samples from individual males in a capacitation media could show potential differences in the sperm behaviour analysing certain aspects which may not have been detected immediately after thawing and which can predict differences on fertility between buck thawed sperm. Furthermore, the addition of SP in the incubation media could also improve post thaw sperm quality showing a different effect depending on the male as well. To the author's knowledge, no reports on the effect of individual males on buck sperm quality after *in vitro* incubation with SP under capacitation condition have been studied. Therefore, the aim of this study was to determine if individual

male variability reflects on their response to *in vitro* incubation with SP under capacitation conditions.

Materials and methods

This experiment was carried out from individual sperm samples collected from 6 male goats of the *Cabra Blanca de Rasquera* breed of 2 years old (n=6) in early winter. Two ejaculates/male/day (n=6 day of collection) were collected, pooled by male and treated individually. Then, the cryopreservation procedure of the individual male samples was performed as described in the Material and Methods section. After thawing, two straws per individual from each replicate (n = 6) were washed through single layer centrifugation method (SLC) as described previously and immediately after SLC/washing, all individual sperm samples were incubated for 3h in four different media: (a) Modified PBS or control media (b) Modified PBS or control media+ seminal plasma (20%), (c) *In vitro* fertilization commercial media (BO-IVF media, IVF Biosciences, UK) and (d) BO-IVF media + seminal plasma (20%). All seminal plasma samples were collected during the breeding season (autumn) from the same males used in this study and processed as described in the general Material & Methods section. For each treatment/group, sperm samples were diluted in the various incubating media at a concentration of 40 $x10^6$ sperm/mL and placed in an incubator (5% CO_2) at 38.5°C for 3h.

Viability sperm analyses

All washed/SLC sperm samples from the 6 individual males after 3h of incubation in four different media were evaluated for plasma and acrosome membrane integrity as well as mitochondrial function using a quadruple-staining technique (Tabarez et al., 2017) as described in the general Materials and Methods section. After evaluation, the following sperm population were taken into consideration: Viability (total SYBR14+/PI- sperm cells), viable cells with intact acrosome and functional mitochondria (SYBR14+/PI-/PE-PNA-/Mitotracker+), viable cells with damaged acrosome and functional mitochondria (SYBR14+/PI-/PE-PNA+/ Mitotracker+), viable cells with intact acrosome and non-functional mitochondria (SYBR14+/PI-/ PE-PNA-/Mitotracker-), viable cells with damaged acrosome and non-functional mitochondria (SYBR14+/PI-/PE-PNA+/ Mitotracker-). In addition, the total acrosome damage was assessed for all incubated sperm samples.

Analyses of DNA integrity

All washed/SLC sperm samples from the different males were evaluated after 3h of incubation in four different media for DNA integrity using Sperm Chromatin Structural Assay (SCSA) described by Evenson et al. (2002) as it was mentioned in the general Material & Methods section. The percentage DNA fragmentation index (DFI) was calculated as the ratio of denatured single stranded DNA (red colour) to the total cells acquired (red + green colour). The High DNA stainability (HDS) index was also evaluated, defined as the population with an elevated green value, outside the main population recorded.

Analyses of sperm motion parameters

Sperm motion parameters were evaluated immediately after 3h of incubation in the four different media for all washed samples collected from 6 different males using the computer-assisted sperm analysis (CASA) system, ISAS® (PROISER S.L., Valencia, Spain), as it was described in the general material and methods section. The total motile sperm cells (TM), progressively motile sperm cells (PM), curvilinear velocity (VCL, μm/s), linear velocity (VSL, μm/s), mean velocity (VAP, μm/s), linearity coefficient (LIN), straightness coefficient (STR), amplitude of lateral head displacement (ALH, μm) and beat-cross frequency (BCF, Hz) were all evaluated.

Statistical analyses

Statistical analyses were performed using the SAS statistical package (Version 9.4, 2015, SAS Institute Inc., Cary, NC, USA). The different analyses were performed as they have been previously described in the material & methods section.

Results

No significant differences were observed in the sperm viability and percentage sperm with intact acrosome and active mitochondria amongst the different individual males after washing (T0) (Table 4.1) as shown in the previous study. Similarly, no differences were observed on viability between individuals, after 3hrs incubation in the control media except male 67 which was significantly higher compared to male 61 in the control media. However, incubation in control media significantly reduced viability percentages on 2 of the six males (male 63 and 66), while the same time of incubation in capacitation media (IVF media) reduced significantly the sperm viability regardless of the male donor, showing no significant

differences amongst them and lower values compared to control media in all the males. Overall, 3h incubation in the presence of SP either in the control or capacitation media significantly reduced the sperm viability of all the males compared to initial values (T0) and to the values obtained from both media without SP. Nevertheless, sperm viability on IVF media + SP was significantly higher than in control media + SP, in all the males except males 61 and 63 (Table 4.1).

In relation to the percentage of viable sperm with intact acrosome and active mitochondria, no differences were seen between males in T0 and in any of the control media with or without SP. However, after 3h incubation in IVF media, male 67 presented significantly higher value compared to male 61, while in IVF media + SP this sperm parameter was significantly higher in male 65 compared to the values showed by males 61, 62 and 63. Viable spermatozoa with damaged acrosome and active mitochondria values were low but showing significant differences between males 61 and 63 only when the sperm were incubated in IVF media. Related to the effect of different incubation media on this parameter, some significant differences were observed but without following a clear pattern. Individual males did not show any significant difference in the percentage acrosome damage after 3hrs incubation in any of the tested media, however, there was increased acrosome damage when sperm were incubated with SP compared to initial values (T0), especially in the presence of IVF media, although not always significantly (Table 4.1.).

No significant differences were observed in the high DNA stainability (HDS) between individual males, and after 3h incubation in the different media, only one male (61) showed significant higher percentages in initial values (T0) compared to the percentage obtained after 3h incubation in IVF + SP. Similarly, the DNA fragmentation index did not show significant differences between individual males, only after 3hrs incubation in IVF media, individual males 61, 62, and 63 were statistically higher compared to male 66 (Table 4.2). Related to the effect of different incubation media on the DNA fragmentation, only one male (63) showed significant difference between the values observed after 3 h in the control media compared to IVF + SP media.

The results for CASA analysis showed that no significant differences between individual males were observed in relation to the total motility (TM), and progressive motility (PM). However, an important reduction was observed in the total motility (TM) when the sperm were incubated for 3 h regardless of the media and the male compared to T0, although this reduction was not always significant.

No significant differences were observed between sperm samples incubated in the different media regardless of the male, although the lower values of TM were observed in the presence of SP, except for male 63.

Table 4.1: Effect of individual males on buck sperm viability and mitochondria function after 3hrs incubation period in capacitation conditions with or without seminal plasma.

Sperm parameter	Incubation media	61	62	63	65	66	67
Viability (%)	T0	18.4 ± 4.3^{1}	29.8 ± 4.3^{1}	23.6 ± 1.5^{1}	34.4 ± 4.2^{1}	30.7 ± 1.8^{1}	31.8 ± 5.6^{1}
	Control	$14.0 \pm 4.0^{b.1}$	$22.4 \pm 2.8^{ab.1}$	$15.6 \pm 1.3^{ab.2}$	$27.2 \pm 3.4^{ab.1}$	$23.8 \pm 0.8^{ab.2}$	$29.0 \pm 4.7^{a.1}$
	Control+SP	$0.2 \pm 0.1^{b.3}$	$0.2 \pm 0.1^{b.4}$	$0.3 \pm 0.1^{ab.4}$	$0.5 \pm 0.1^{a.4}$	0.4 ± 0.1^{ab5}	0.2 ± 0.1^{b4}
	IVF	3.2 ± 0.5^{2}	5.9 ± 1.8^{2}	5.1 ± 0.8^{3}	10.4 ± 4.5^{2}	7.3 ± 1.2^{3}	8.8 ± 2.8^{2}
	IVF+SP	$0.8 \pm 0.2^{c.3}$	$1.0 \pm 0.2^{bc.3}$	$0.9 \pm 0.3^{c.4}$	$2.9 \pm 0.3^{a.3}$	2.4 ± 0.6^{ab4}	2.0 ± 0.3^{ab3}
Intact acrosome and active mitochondrial (%)	T0	16.8 ± 4.9^{1}	29.2 ± 4.3^{1}	22.7 ± 1.8^{1}	33.5 ± 4.5^{1}	30.4 ± 1.8^{1}	31.5 ± 5.5^{1}
	Control	13.1 ± 3.7^{1}	21.9 ± 2.9^{1}	15.1 ± 1.3^{2}	25.6 ± 3.7^{1}	23.1 ± 0.8^{2}	28.1 ± 4.7^{1}
	Control+SP	0.0 ± 0.0^{3}	0.0 ± 0.0^{4}	0.0 ± 0.0^{5}	0.0 ± 0.0^{3}	0.0 ± 0.0^{5}	0.1 ± 0.0^{3}
	IVF	$2.3 \pm 0.7^{b.2}$	$4.7 \pm 1.3^{ab.2}$	$3.9 \pm 0.7^{ab.3}$	$4.6 \pm 0.9^{ab.2}$	$6.7 \pm 1.1^{ab.3}$	$8.1 \pm 2.5^{a.2}$
	IVF+SP	$0.6 \pm 0.2^{b.2}$	$0.6 \pm 0.2^{b.3}$	$0.9 \pm 0.2^{b.4}$	$2.7 \pm 0.2^{a.2}$	$1.2 \pm 0.3^{ab.4}$	$1.2 \pm 0.4^{ab.3}$
Damage acrosome and active mitochondrial (%)	T0	0.1 ± 0.0^{1}	0.2 ± 0.0^{12}	0.0 ± 0.0^{2}	0.2 ± 0.1	0.1 ± 0.1^{1}	0.1 ± 0.0^{1}
	Control	0.1 ± 0.1^{1}	0.3 ± 0.2^{1}	0.1 ± 0.0^{1}	0.2 ± 0.1^{1}	0.2 ± 0.1^{1}	0.2 ± 0.0^{1}
	Control+SP	0.0 ± 0.0^{2}	0.0 ± 0.0^{2}	0.0 ± 0.0^{2}	0.0 ± 0.0^{2}	0.0 ± 0.0^{2}	0.0 ± 0.0^{2}
	IVF	$0.0 \pm 0.0^{b.2}$	$0.1 \pm 0.1^{ab.12}$	$0.3 \pm 0.1^{a.1}$	$0.2 \pm 0.1^{ab.1}$	$0.1 \pm 0.0^{ab.1}$	$0.1 \pm 0.0^{ab.1}$
	IVF+SP	0.0 ± 0.0^{2}	0.0 ± 0.0^{2}	0.0 ± 0.0^{2}	0.0 ± 0.0^{2}	0.0 ± 0.0^{2}	0.0 ± 0.0^{2}
Acrosome damage (%)	T0	14.6 ± 2.1^{2}	16.8 ± 3.1	8.9 ± 1.2	12.1 ± 2.2	8.3 ± 0.7^{3}	9.1 ± 1.6^{2}
	Control	21.8 ± 4.4^{12}	14.4 ± 1.9	12.4 ± 2.0	11.9 ± 0.9	15.0 ± 2.4^{23}	20.5 ± 8.4^{2}
	Control+SP	33.5 ± 14.7^{12}	47.5 ± 12.1	43.3 ± 16.5	35.2 ± 14.5	51.6 ± 15.8^{12}	38.6 ± 14.8^{12}
	IVF	13.6 ± 3.5^{2}	18.8 ± 5.9	12.6 ± 1.6	28.7 ± 9.0	14.4 ± 2.5^{23}	17.6 ± 3.0^{12}
	IVF+SP	60.9 ± 12.4^{1}	54.1 ± 13.4	47.8 ± 13.4	55.2 ± 10.8	52.6 ± 6.5^{1}	62.6 ± 4.3^{1}

[a-c] Different letters represent significant differences ($P<0.05$) between males.[1-5] Different letters represent significant differences ($P<0.05$) between samples within the parameters. T0: Time 0h, SP: Seminal plasma, IVF: *Invitro* fertilization media. Data are shown as mean ± S.E.M.

Table 4.2: Effect of individual male factor on the DNA integrity of buck sperm after 3hrs incubation in capacitation conditions with or without seminal plasma.

Sperm parameter	Incubation media	61	62	63	65	66	67
HDS (%)	T0	3.1 ± 0.7[1]	2.7 ± 1.1	3.1 ± 0.6	5.4 ± 1.2	2.1 ± 0.4	3.0 ± 1.0
	Control	2.3 ± 0.3[12]	2.0 ± 0.6	2.5 ± 1.1	2.8 ± 0.8	1.4 ± 0.5	2.2 ± 0.8
	Control+SP	1.3 ± 0.5[12]	1.3 ± 0.6	1.8 ± 0.4	1.9 ± 1.0	1.3 ± 0.5	1.8 ± 0.6
	IVF	1.7 ± 0.4[12]	4.8 ± 1.5	2.7 ± 0.7	5.2 ± 1.8	2.1 ± 0.5	3.0 ± 0.9
	IVF+SP	0.9 ± 0.2[2]	1.0 ± 0.5	0.9 ± 0.4	3.9 ± 1.5	0.7 ± 0.2	0.9 ± 0.3
DFI (%)	T0	9.8 ± 1.6	8.4 ± 1.4	9.9 ± 0.9[12]	11.5 ± 1.4	11.8 ± 1.9	15.0 ± 2.7
	Control	15.6 ± 1.1	13.8 ± 2.6	16.8 ± 1.9[1]	13.1 ± 2.0	10.7 ± 2.6	16.9 ± 3.0
	Control+SP	12.4 ± 0.9	13.7 ± 1.8	12.2 ± 2.0[12]	10.0 ± 0.9	8.8 ± 2.0	9.5 ± 1.4
	IVF	13.4 ± 3.2[a]	13.6 ± 2.6[a]	13.1 ± 1.2[a.12]	12.3 ± 2.3[ab]	6.6 ± 0.5[b]	8.7 ± 0.8[ab]
	IVF+SP	9.4 ± 1.3	11.9 ± 2.1	8.3 ± 1.2[2]	12.3 ± 2.9	9.4 ± 2.1	12.4 ± 1.5

[a,b] Different letters represent significant differences (P<0.05) between males,

[1,2] Different letters represent significant differences (P<0.05) between samples within the parameters,

T0: Time 0h, SP: Seminal plasma, IVF: *Invitro* fertilization media. HDS: High DNA Stainability, DFI: DNA Fragmentation index. Data are shown as mean ± S.E.M.

In regards to the progressive motility (PM), no significant differences were observed after 3h incubation in the control media compared to T0 except in male 63, while in the presence of SP there was a significant reduction in all the males. No significant differences were observed between samples incubated in IVF media and those incubated in the control media. Moreover after 3h incubation in IVF media, males 61, 63, and 66 showed a significant difference compared to 0h whereas, after incubation in IVF+SP, all the males showed a significant reduction compared to T0 (Table 4.3). In any case, all the mean values were extremely low.

With regards to the sperm motile subpopulation, a total of 76,990 sperms were analysed and four motile subpopulations were equally identified. From them, 9,670 sperms presented motile movements hence these movements can be characterised into the following: Sp1 composed of sperm with the highest velocities, moderate LIN, highest ALH, and BCF. Sp2 constituted of sperm with lowest velocity, highest LIN, lowest ALH, and BCF. While Sp3 constituted of moderate velocities, lowest LIN, moderate ALH, and BCF. Finally, the static sperm constituted the fourth motile subpopulation (Sp4) (Table 4.4.)

Table 4.3: Effect of individual males on buck sperm total and progressive motility after 3h incubation in the control and capacitation condition with or without seminal plasma

Sperm parameter	Incubation media	61	62	63	65	66	67
TM (%)	T0	24.7 ± 3.7^{1}	21.1 ± 4.6^{1}	25.1 ± 5.6^{1}	41.4 ± 8.1^{1}	47.1 ± 6.2^{1}	36.6 ± 7.2^{1}
	Control	12.7 ± 4.0^{12}	6.2 ± 1.8^{12}	4.3 ± 1.8^{2}	11.4 ± 3.9^{12}	10.5 ± 2.4^{2}	10.1 ± 2.7^{2}
	Control+SP	6.8 ± 2.3^{2}	1.6 ± 1.3^{2}	7.7 ± 3.3^{12}	4.1 ± 2.5^{2}	5.3 ± 3.1^{2}	10.5 ± 5.5^{2}
	IVF	11.7 ± 5.0^{12}	10.5 ± 4.1^{12}	4.7 ± 2.6^{2}	8.7 ± 1.9^{12}	14.9 ± 4.9^{2}	11.4 ± 2.9^{12}
	IVF+SP	1.9 ± 0.9^{2}	1.2 ± 1.2^{2}	7.0 ± 1.7^{12}	5.5 ± 2.6^{2}	5.4 ± 1.9^{2}	7.2 ± 2.9^{2}
PM (%)	T0	4.5 ± 1.5^{1}	3.0 ± 0.8^{1}	3.3 ± 0.5^{1}	4.4 ± 1.6^{1}	5.8 ± 2.2^{1}	3.8 ± 0.8^{1}
	Control	1.4 ± 0.5^{12}	1.6 ± 1.0^{12}	0.4 ± 0.3^{2}	2.0 ± 0.8^{1}	2.3 ± 1.0^{12}	1.7 ± 1.0^{1}
	Control+SP	0.3 ± 0.3^{23}	0.0 ± 0.0^{2}	0.6 ± 0.4^{2}	0.0 ± 0.0^{2}	0.0 ± 0.0^{3}	0.2 ± 0.2^{23}
	IVF	1.3 ± 0.8^{23}	1.4 ± 0.6^{12}	0.8 ± 0.5^{2}	1.9 ± 0.7^{12}	1.7 ± 1.0^{23}	1.6 ± 0.6^{12}
	IVF+SP	0.0 ± 0.0^{3}	0.3 ± 0.2^{2}	0.9 ± 0.5^{2}	0.0 ± 0.0^{2}	0.0 ± 0.0^{3}	0.0 ± 0.0^{3}

[1-4] Different letters represent significant differences (P<0.05) between samples within the parameters,.

TM: Total motility, PM: Progressive motility, IVF: *Invitro* fertilization media, SP: Seminal plasma. T0: Time 0h. Data are shown as mean ± S.E.M.

Table 4.4: Kinematic characteristics of the four sperm subpopulations in thawed buck sperm

Parameter	Sp1	Sp2	Sp3	Sp4
VCL (µm/s)	111.3 ± 31.2^{a}	32.9 ± 15.5^{c}	54.5 ± 25^{b}	N.D.
VSL (µm/s)	54.2 ± 24.8^{a}	18.9 ± 10.0^{b}	9.3 ± 7.0^{c}	N.D.
VAP (µm/s)	77.4 ± 24.1^{a}	23.9 ± 11.8^{c}	26.2 ± 12.9^{b}	N.D.
LIN (%)	51.3 ± 22.5^{b}	58.2 ± 17.4^{a}	17.4 ± 10.2^{c}	N.D.
STR (%)	69.7 ± 19.9^{b}	78.9 ± 12.5^{a}	35.4 ± 19.3^{c}	N.D.
ALH (µm)	4.6 ± 1.9^{a}	1.8 ± 0.8^{c}	3.0 ± 1.3^{b}	N.D.
BCF (Hz)	6.2 ± 3.3^{a}	3.2 ± 2.6^{c}	3.9 ± 2.3^{b}	N.D.
n	1,566	4,474	3,630	67,320
%	2.03	5.81	4.72	87.44

[a-c] Different represent significant differences (P<0.05) between subpopulations

n: Number of sperm

%: Percentage of sperm in each subpopulation; Sp: Subpopulation. Data was expressed as mean values ± S.D.

In regards to the distribution of motile sperm subpopulation, no differences were showed between males in any time or different media tested, however some differences were seen between incubation media in the proportion of the different sperm subpopulation. In

general, after 3h of incubation irrespective of the incubation media, the proportion of Sp1, Sp2 and Sp3 were reduced compared to T0, although not always in a significant way, while Sp4 or static subpopulation were increased in all the males (Table 4.5). In relation to the specific effect of incubation media, no homogeneous pattern was found between males, except that the presence of seminal plasma in IVF media reduced the proportion of the different subpopulations significantly, mostly in the Sp1 sperm and increasing the proportion of the static subpopulation.

Table 4.5: Effect of individual males on buck sperm motile subpopulation after 3h incubation in a control and capacitation condition with or without seminal plasma

Sperm parameter	Incubation media	61	62	63	65	66	67
Sp1 (%)	T0	5.2 ± 1.4^{1}	3.9 ± 1.1^{1}	3.1 ± 1.1^{1}	6.5 ± 1.9^{1}	10.2 ± 5.2^{1}	6.3 ± 2.0^{1}
	Control	2.3 ± 0.6^{12}	1.6 ± 1.0^{12}	0.1 ± 0.1^{2}	2.1 ± 0.7^{12}	2.2 ± 1.2^{12}	1.4 ± 0.8^{23}
	Control+SP	0.0 ± 0.0^{3}	0.0 ± 0.0^{2}	0.0 ± 0.0^{2}	0.2 ± 0.2^{23}	0.0 ± 0.0^{2}	0.0 ± 0.0^{3}
	IVF	1.5 ± 1.1^{23}	1.4 ± 0.7^{12}	0.7 ± 0.5^{12}	2.1 ± 0.8^{12}	1.9 ± 1.0^{12}	2.1 ± 0.8^{12}
	IVF+SP	0.5 ± 0.4^{23}	0.0 ± 0.0^{2}	0.0 ± 0.0^{2}	0.0 ± 0.0^{3}	0.0 ± 0.0^{2}	0.0 ± 0.0^{3}
Sp2 (%)	T0	10.0 ± 1.9^{1}	8.0 ± 1.6^{1}	13.7 ± 4.3^{1}	16.2 ± 3.0^{1}	13.2 ± 3.4^{1}	14.7 ± 3.7^{1}
	Control	4.9 ± 1.9^{12}	2.8 ± 0.6^{23}	2.1 ± 1.0^{12}	4.1 ± 0.8^{12}	3.2 ± 0.9^{123}	4.3 ± 1.3^{12}
	Control+SP	1.1 ± 0.9^{2}	0.3 ± 0.3^{34}	1.6 ± 1.4^{2}	0.5 ± 0.5^{3}	0.2 ± 0.2^{3}	1.3 ± 0.7^{2}
	IVF	4.6 ± 2.0^{12}	3.1 ± 1.5^{12}	1.8 ± 1.2^{2}	1.9 ± 0.9^{23}	5.0 ± 1.3^{12}	4.5 ± 1.0^{12}
	IVF+SP	0.5 ± 0.4^{2}	0.0 ± 0.0^{4}	2.0 ± 0.6^{12}	0.7 ± 0.3^{3}	1.7 ± 0.6^{23}	1.2 ± 0.8^{2}
Sp3 (%)	T0	9.5 ± 1.6^{1}	9.2 ± 2.2^{1}	8.4 ± 3.0^{1}	18.7 ± 4.4^{1}	23.6 ± 6.0^{1}	15.6 ± 1.8^{1}
	Control	5.5 ± 1.8^{12}	1.8 ± 0.7^{12}	2.1 ± 0.8^{12}	5.2 ± 2.6^{12}	5.1 ± 0.8^{12}	4.4 ± 1.3^{2}
	Control+SP	5.6 ± 2.4^{12}	1.3 ± 1.3^{2}	6.1 ± 2.6^{12}	3.4 ± 1.8^{12}	5.0 ± 3.1^{2}	9.1 ± 5.8^{12}
	IVF	5.7 ± 2.3^{12}	6.0 ± 2.6^{12}	2.1 ± 1.0^{12}	$4.7 \pm 1.^{2}$	7.9 ± 4.8^{12}	4.7 ± 1.7^{2}
	IVF+SP	0.9 ± 0.7^{2}	1.2 ± 1.2^{2}	5.0 ± 1.4^{2}	4.7 ± 2.3^{2}	3.7 ± 2.1^{2}	6.0 ± 2.9^{12}
Sp4 statics (%)	T0	75.3 ± 3.7^{2}	78.9 ± 4.6^{2}	74.9 ± 5.6^{2}	58.6 ± 8.1^{2}	53.0 ± 6.2^{2}	63.4 ± 7.2^{2}
	Control	87.3 ± 4.0^{12}	93.8 ± 1.8^{12}	95.7 ± 1.8^{1}	88.6 ± 3.9^{12}	89.5 ± 2.4^{1}	89.9 ± 2.7^{1}
	Control+SP	93.3 ± 2.3^{1}	98.4 ± 1.3^{1}	92.4 ± 3.3^{12}	95.9 ± 2.5^{1}	94.8 ± 3.1^{1}	89.5 ± 5.5^{1}
	IVF	88.3 ± 5.0^{12}	89.5 ± 4.1^{12}	95.4 ± 2.6^{1}	91.3 ± 1.9^{12}	85.1 ± 4.9^{1}	88.6 ± 2.9^{12}
	IVF+SP	98.1 ± 0.9^{1}	98.8 ± 1.2^{1}	93.0 ± 1.7^{12}	94.6 ± 2.6^{1}	94.7 ± 1.9^{1}	92.8 ± 2.9^{1}

[1-4] Different letters represent significant differences ($P<0.05$) between samples within the parameters,.

Sp: Subpopulation, IVF: *In vitro* fertilization media, SP: Seminal plasma, T0: Time 0h.

Discussion

The main objective of the present study was to determine the potential differences in the behaviour of thawed buck sperm under *in vitro* capacitation conditions to predict differences

on freezability or even on fertility between male bucks. Also this work was focused on investigating the effect of the presence of seminal plasma during incubation under capacitation conditions on thawed buck sperm quality depending on the semen donor. As previously mentioned, the addition of SP can reverse the damage caused by the freeze-thawing process, since SP contains some factors that can influence the status of the plasma membrane, by regulating the onset of capacitation and acrosome reaction (Töpfer-Petersen et al., 2005).

However, in our study, the presence of SP had a negative effect on the sperm viability, irrespective of their individual male and the incubation media (control or under capacitation conditions). Similarly, several reports have demonstrated the negative effect of SP on sperm viability (Garcia & Graham, 1987; Moore et al., 2005; Rovegno et al., 2013). Furthermore, Šichtař et al. (2019) reported that the functionality of the plasma membrane was reduced after incubation with SP and no improvement on the sperm membrane integrity were seen in sperm from poor freezing stallions. We may suggest that, this lack of improvement may be dependent on the initial quality after thawing as seen in our previous experiment which presented no huge differences and as such, no differences were expected after incubation with SP. However contrary to our results, some reports have demonstrated that plasma and acrosome membrane in "bad freezers" were improved after incubation with SP (Aurich et al., 1996; Okazaki et al., 2009) as previously stated in the introduction. Also another possible explanation may be due the relative ratio of capacitating and decapitating proteins which may vary between individuals males, or species (Domínguez et al., 2008; O'Meara et al., 2007).

On the other hand, even though very few differences on sperm parameters were found between males under different incubation media, these differences on the sperm behaviour could have some influence on the viability of thawed sperm in the female reproductive tract (Shannon, 1978). Therefore, more investigation is required to determine a possible explanation in regards to the male factor described on cryopreserved buck sperm survival and subsequent fertility. In fact, the successful fertilization of the oocyte relies on the ability of the plasma membrane to be intact (Gadella & Luna, 2014). Due to mechanical and thermal stress encountered by the spermatozoa during thawing, the plasma and acrosomal membranes are the focal point of damage and although few differences were seen between males in the present work, they were mainly observed in the plasma, acrosome membrane status and mitochondrial activity of the thawed sperm after 3h of incubation. Also, the percentage

acrosome damage observed when samples were incubated in IVF +SP were similar to that seen when samples were pooled. We may suggest that SP negatively affected the acrosome integrity irrespective of the donor (Moore et al., 2005).

Furthermore, DNA integrity was less affected by this procedure since most of the individual males were stable after 3h incubation. Only few differences on DFI between males were observed when the sperm were incubated under capacitation conditions, which may have some relationship with potential differences on fertility (Berlinguer et al., 2009). It is worthy to note that these differences were absent in the presence of SP. Although the protective effect of SP on the DNA integrity is still unknown, we may also suggest that buck sperm DNA was less affected during the incubation process probably due to the short incubation time, which was not enough to reveal damages in the DNA as demonstrated by Crespo et al. (2013). However, other studies have demonstrated low DFI in boars after thawing and even several hours after incubation (Gosálvez et al., 2011). Also in rams, a low DFI was observed after 0, 3 and 20 h incubation in synthetic oviductal fluid that mimics the female genital tract (Peris et al., 2004).

In relation to the HDS, even though no significant differences were found, the lowest values were seen when SP was added to the incubation media. We may suggest that the decrease in the HDS-parameters after incubation with seminal plasma may reflect additional information other than sperm immaturity as described by (Evenson et al. (2002) which is yet to be identified.

The total and progressive motility in our study were clearly affected by 3h incubation in the presence of seminal plasma (SP) regardless of the incubation media and the male donor. In fact, no significant differences between individual males in terms of their motility were observed in any of the studied conditions. Although some males presented no significant differences on motility after 3h incubation in the media without SP compared to the initial values, other bucks presented a significantly decreased values, showing a potential male factor on thaw sperm motility. Furthermore, some reports revealed that there was no beneficial effect of SP on the motility of spermatozoa from poor freezing stallions (de Andrade et al., 2011; Šichtař et al., 2019). We can also suggest that this were due to the detrimental effect of seminal plasma as previously discussed.

In the present study, the tested males did not present huge differences on sperm quality to be able to categorize them into good or bad freezers, therefore the lack of individual male factor on the response of the sperm to incubation with seminal plasma could

be explained. Nevertheless, in boars, Fernández-Gago et al. (2017) observed that individual boar sperm samples differed in motility characteristics, but incubation with SP had an overall positive effect in all the males irrespective of the motility differences. These discrepancies can be explained by differences between species as well in the composition of the seminal plasma added to the incubation media (Mandiki et al., 1998).

In regards to the motile subpopulation distribution, no differences were seen between males at any time and incubation media, although no homogeneous pattern was found, except that the presence of seminal plasma in IVF media reduced significantly the proportion of the different subpopulation with some movement which were lost as seen in the sP1 sperm, especially, with the highest velocities, moderate LIN, highest ALH, and BCF and alongside increases the proportion of the static subpopulation in all the different male samples. By contrast, a previous study found out that incubating stallion spermatozoa for 30 min in the presence of SP improved the distribution of spermatozoa in the fast subpopulation (Šichtař et al., 2019). We may suggest that the negative effect observed in our study may be due to complex nature of SP proteins, and the toxic effect of buck seminal plasma (Roy, 1957).

In conclusion, incubation of buck spermatozoa with seminal plasma under *in vitro* capacitation condition did not improve buck sperm motility, membrane status, and mitochondria function regardless of the male donor, rather a negative effect was seen in the studied sperm quality parameters, except on the DNA status.

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Chapter VI

GENERAL DISCUSSION

With the establishment of a semen bank for the endangered native Catalonian breed of *Cabra Blanca de Rasquera*, some protocols were adopted towards determining and improving the post-thawed sperm quality before its application such as in artificial reproductive programmes, which are essential for its preservation success. Therefore, in providing some information towards evaluation and improvement of thawed cryopreserved sperm from *Blanca de Rasquera* buck, sperm evaluation by CASA due to its ability to provide correct and specific information on sperm kinematic parameters (Gravance & Davis, 1995) was done. Also, sperm functional characteristics were analysed by flow cytometry to provide a better prediction of their physiological status (Evenson et al., 2002; Graham, 2001). To this regard, the degree of sperm quality can be determined before its use in assisted reproductive technique. However, the ability to evaluate the potential fertilizing ability of these spermatozoa is still of concern.

Thus, this thesis was aimed at achieving sperm selection based on natural principles in an ideal physiological conditions by separating potential fertile spermatozoa from immotile, moribund or damaged ones and debris while mimicking the physiological condition within the female reproductive tract during the process of migration (Mortimer & Templeton, 1982). Therefore, several selection methods been employed the literature processed or frozen-thawed semen *in-vitro* towards improving sperm quality. In this study, we explored the Single layer centrifugation (SLC) technique through a specie-specific colloid (Bovipure®) to improve the post-thawed sperm quality from *Blanca de Rasquera* breed. However, before discussing the importance of the effectiveness of the selection technique - SLC, we first focused on the quality of sperm samples immediately after thawing in relation to donor age, season of semen collection as well as melatonin treatment.

With respect to donor age, no major significant effects were observed, although there is a trend especially during the spring period, where samples collected from older males (30 months old) with or without melatonin implant showed lower results compared to samples collected in autumn (from 12 and 24 months olds). In fact, it is difficult to establish if the difference were due to the effect of season or due to the effect of the age of the males since there was no time to compare semen from males with the same age collected in different seasons. In any case, the effect of the age on sperm quality may be difficult to explain but reports have shown inconsistencies as regards different breeds producing higher sperm quality either at a younger age or at an older age (Al-Ghalban et al., 2004).

However, we observed that usually samples collected during the period of autumn had better sperm quality after freezing-thawing than those collected during spring in the case of pooled samples or winter period in the individual cryopreservation studies. We therefore suggest that autumn may be the optimal breeding period of this breed where better sperm quality is produced (Pérez & Mateos, 1996) and consequently better thawed quality could be achieved. Furthermore, we observed that the sperm quality was superior in pooled samples during autumn and spring (study 1) after thawing compared to individual samples collected in winter (study 3). This may be due to the effect of heterospermic interaction between samples from the different males in the pooled samples (Godet et al., 1996). However contrary to our study, a report demonstrated that sperm pooling did not modify sperm parameters in the Majorera bucks (Batista-Arteaga et al., 2011). To this regard, we may suggest that the season of collection may have negatively influenced the quality of the individual males since semen collection was done during the winter period (Al-Ghalban et al., 2004; Wang et al., 2015). Another explanation may be due to the males could be exhausted after 5-6 months of extensive semen collection leading to depletion in the epididymal storage of spermatozoa as demonstrated (Thwaites, 1995), although the optimal frequency of semen collection (3-4 ejaculates a week) was maintain along the experimental process.

Previous study carried out by our team (Tabarez, 2014) found quite similar inter-male variability when sperm samples from the various donor bucks were thawed, thus grouping the individual males as good, moderate, and bad freezers. In the present study, these individual differences were not expressed immediately after washing and less evident after the resilience test in the different incubation conditions, similar to results from Berger et al. (1994). However, slight differences were observed between the results showed by our previous team (Tabarez, 2014) from the same animals and the present results. A possible explanation may be due to the prolong time of storage of these sperm samples in the liquid nitrogen which we analysed six years after, and this may have altered the sperm characteristics of some males due to natural ageing process of the spermatozoa (Johnson et al., 2000). Furthermore, the examination of sperm data may vary due to observations by different technicians, hence the differences in current results obtained when compared with those from a previous team (Graham et al., 1980), especially in regards to the progressive motility.

On the other hand, addition of melatonin implant in the non-breeding season was not beneficial in improving the semen quality. This findings were similar to studies carried out previously by our team (Tabarez, 2014), as well as in other reports in the *Blanca Andaluza*

buck breed (Gallego-Calvo et al., 2015). On the contrary, other reports recorded positive effect of melatonin on the viability of Damascus goat breed (Ramadan et al., 2009), an increase in the post thaw seminal parameters in the Sirohi bucks (Chandra Shekher et al., 2020). The discrepancy in results could be related to the amount of melatonin that was administered to the donor males prior to cryopreservation. Following the manufacturer's instructions, we inserted 2 implants of Melatonin, while in other studies, 3 implants were used or due to the effect of cryopreservation which may have reduced the post thaw quality (Gallego-Calvo et al., 2015). Also, it could be noteworthy to consider that there could be breed differences on the degree of seasonal reproductive performances as well the different latitude areas where the photoperiod can be more or less determining (Zarazaga et al., 2021).

Towards improving sperm quality after thawing as one of the main objectives of this thesis, the effectiveness of Single Layer Centrifugation (SLC) through Bovipure® colloid in order to improve the quality of cryopreserved buck sperm was determined considering various factors such as donor age, season of semen collection and melatonin treatment of males. However, the overall sperm parameters were not improved after SLC. We only found that in the total acrosome damage, lower values were observed in the spring season in the non-implanted males after SLC compared to autumn samples. However, in other studies, SLC improved the motility and acrosome integrity of post thawed *Celtibérica* buck spermatozoa (Jiménez-Rabadán et al., 2012). This sperm selection method could be used to estimate the reproductive ability of spermatozoa after natural selection *in-vivo* during active migration through the cervical mucus (Farrell et al., 1998; Vantman et al., 1988). Furthermore, some authors found some close relationships between sperm parameters assessed in selected sperm samples *in-vitro* with *in- vivo* fertility (Alvarez Garcia et al., 2010). Nevertheless, Sánchez-Partida et al., (1999) did not find any relationship between the motility characteristics of frozen thawed spermatozoa after selection *in vitro* and pregnancy rates.

The lack of improvement in our results after SLC may be attributed to the damages on the sperm encountered previously during cryopreservation which were not removed by this selection method when Bovipure colloid is used. Although no previous report has been found in buck sperm using Bovipure colloid, this colloid has shown a positive influence for selection in frozen-thawed bull semen, and also for i*n vitro* production of bovine embryos (Samardzija et al., 2006; Valeanu et al., 2015). Although Bovipure colloid was developed for bull spermatozoa, using it in bucks may have reduced its effect due to specie differences. Secondly another reason for the lack of improvement in our studied parameters may be due to

the susceptibility of buck sperm cell membrane to mechanical damages encountered during the centrifugation procedure which may have been even more severe for cryopreserved sperm (Sharma et al., 1997). Centrifugation for buck semen is recommended at speeds ranging from 500 to 1000 x g for 10 to 15 min (Purdy, 2006) whereas, in our study, SLC procedure was carried out with a relatively low g-force (300g), but a longer duration (25 and 5 mins respectively) which may have altered sperm survival. Other authors have also reported that the force of centrifugation might affect sperm motility, and membrane integrity in bulls (Verberckmoes et al., 2000) and rams (Gil et al., 2000).

The subjection of frozen-thawed sperm samples to an *in vitro* incubation under capacitation conditions were also carried out in this thesis in order to mimic the migration of spermatozoa through the female reproductive tract and thus to be able to better predict their resilience, longevity and survivability (Bag et al., 2004). In our study, this capacitation process could be achieved by treating buck sperm with IVF commercial media. To this regard, our results showed a decrease in the viability after incubation in IVF media regardless of the presence of seminal plasma (SP).Since no literature has demonstrated the negative effect of IVF media on buck sperm viability. It may be suggested that the commercial IVF media used may not be effective for buck spermatozoa.

Nevertheless, the addition of 20% seminal plasma (SP) did not improve the sperm viability afterwards in our experiments, which is in agreement with other reports demonstrating that the addition of SP did not improve the sperm motility, viability, and acrosome integrity (Domínguez et al., 2008; Graham, 1994; Moore et al., 2005). Although in rams, penetration of sperm cells through the cervical mucus and fertility after cervical insemination of ewes significantly increased when frozen-thawed sperm supplemented with 20% seminal plasma was used (Maxwell et al., 1999).

We may assume that survivability of buck spermatozoa was shortened during the incubation process due to low sperm quality as opposed to if proper selection had taken place. However, it probably seems that the decrease on sperm viability may be attributed to the IVF media as well as the negative effect of buck seminal plasma as confirmed when individual sperm samples where incubated in control media + 20% SP (study 4) showing even lower sperm viability than when IVF media was used with and without SP. As previously mentioned, buck seminal plasma has been reported to be detrimental for buck sperm cryopreservation due to the presence of a hydrolysing enzyme, phospholipase-A2 and its interaction with egg yolk extenders (Ferreira et al., 2014; Pellicer-Rubio et al., 1997). However, after thawing once the extender was removed by SLC, this negative effect of SP

was unexpected, since the importance of seminal plasma cannot be ignored due to its positive role in several key processes such as good source of nutrient, preventing premature sperm activation within the female reproductive tract and also increases sperm membrane stabilization by inhibiting capacitation and improving fertility, among others (Rozeboom et al., 2000). Special attention may be required in regards to the high acrosome damage observed when seminal plasma was added in the media which may be due to the harmful interactions of buck SP which has also been demonstrated elsewhere (Gangwar et al., 2016), and also due to the complex nature of SP proteins which may vary amongst species (Mandiki et al., 1998).

In regards to sperm motility, no significant improvement after single layer centrifugation with Bovipure colloid and during *in-vitro* incubation conditions was observed. Contrary to our studies, sperm motility parameters were improved after SLC in bulls and stallion semen (Macías García et al., 2009; Thys et al., 2009). Yet again, we may suggest that SLC through Bovipure colloid was not suitable for buck sperm selection, or further investigation may be required. Furthermore, under capacitation conditions, spermatozoa may undergo different structural and functional changes in the movement pattern towards a fast non-linear movement prior to fertilization (García-Álvarez et al., 2014; Holt & Van Look, 2004). In our study, after analysing the motile subpopulation distribution, we observed that this group of spermatozoa (characterized by fast and non-linear movement) also known as "hyperactivated" spermatozoa were higher after SLC procedure in the pooled samples, although they were almost inexistent after incubation regardless the media. A potential explanation could be that this motile subpopulation showing "hyperactivated" like movements after SLC are already moribund sperm at the verge of dying during the incubation period (Cremades et al., 2005; Flores et al., 2008).

In the present study, the DNA integrity was not affected after SLC and after *in vitro* incubation as seen in other species (Fernández-Gago et al., 2017). The % DFI thresholds that could impact fertility have been demonstrated in different species, and these values were higher compared to those found in our study (Didion et al., 2009; Evenson & Wixon, 2006). However, high DFI in our study in control media was observed. This may justify reports by Crespo et al. (2013) who reported that the degradation of the DNA may likely occurs during incubation as opposed to the first minutes after thawing. Although incubating buck spermatozoa in IVF media regardless of the presence of SP may have a protective effect on the DNA integrity (Matsura et al., 2010).

In this thesis, one of the main goals was also to evaluate the individual male freezabilities, in relation to their ability to survive under *in vitro* capacitation, since the ability of sperm to migrate successfully through the cervix is dependent on the individual sperm quality during freezing and thawing (Shannon, 1978). The freezing of sperm from valuable genetic individuals is a major factor considered in the reproductive strategies to conserve endangered breed such as the *Cabra Blanca de Rasquera* breed. Therefore, to improve the post thaw sperm quality of semen banks already created with poor freezabilities becomes a matter of major interest. Our results confirmed that even though few individual differences existed after thawing with regards to the viability and mitochondria function, these differences between males were absent after the separation technique. This was similar to several studies in which there was no major influence of individual freezability on the quality of thawed sperm after SLC was performed (Morrell et al., 2009b; Ortiz et al., 2015; Thurston et al., 2001). However, regards to the 2 males with improved acrosome integrity after SLC, we may suggest that the effectiveness of the method of sperm selection could be influenced by the individual, since the behaviour of the sperm after SLC was different depending on the sperm donor. Furthermore, Curry (2000) suggested that cryo-survival is not essentially related to the initial quality of the sperm sample such that individuals with good sperm parameters before freezing can turn out to be bad after thawing or vice versa. To confirm this suggestion, some reports found a significantly higher sperm quality after SLC in sperm samples with a low freezability, while those with medium or high freezability showed similar increase in their sperm quality after SLC (Ortiz et al., 2015).

As it has been mentioned above, in our study, the presence of seminal plasma had a negative effect on the sperm viability, irrespective of their individual male and the incubation media (control or IVF). Similarly, several reports have demonstrated the negative effect of SP on sperm viability (Garcia & Graham, 1987; Moore et al., 2005; Rovegno et al., 2013). Furthermore, Šichtař et al. (2019) reported that the functionality of the plasma membrane was reduced after incubation with SP and no improvement on the sperm membrane integrity were seen in sperm from poor freezing stallions. Since the successful fertilization of the oocyte relies on the ability of the plasma membrane to be intact (Gadella & Luna, 2014), we may suggest that, the lack of improvement seen in our study may be dependent on the initial quality after thawing as seen in our previous experiments which presented no huge differences and as such, no differences were expected after incubation with SP. On the contrary, some reports have showed that the plasma and acrosome membrane in bad freezing stallions were improved after incubation with SP (Aurich et al., 1996). In the present study,

the DNA integrity were less affected in most individual males, Only few differences on DFI between males were observed when the sperm were incubated under capacitation conditions, which may have some potential relationship with differences on fertility (Berlinguer et al., 2009).

The total and progressive motility in our study were clearly affected during incubation regardless of the male donor. However, while some males presented no significant differences in relation to the motility after 3h incubation in the media without SP compared to the initial values, other bucks presented a significantly reduced values, showing a potential male factor on thaw sperm motility. Furthermore, some reports revealed that there was no beneficial effect of SP on the motility of spermatozoa from poor freezing stallions (de Andrade et al., 2011; Šichtař et al., 2019). Contrary to our studies Fernández-Gago et al. (2017) observed that individual boar sperm samples differed in motility characteristics, and the presence of SP had an overall positive effect in all the males irrespective of their individual variability. Our results may be due to the negative effect of buck SP as explained earlier, as well as discussed (Mandiki et al., 1998; Moore et al., 2005). These discrepancies can be explained by differences between species as well in the composition of the seminal plasma added to the incubation media (Mandiki et al., 1998).

In this thesis, with a general point of view, no significant differences were observed in the distribution of the different subpopulations amongst individual male samples or even pooled samples from males with different ages collected in different seasons. In fact, only slight differences in the distribution were seen amongst different sperm treatments or incubation media, indicating that this subpopulation structure is quite stable (Ledesma et al., 2016). Sperm subpopulation 3 (sP3) composed of spermatozoa with the fast and non-linear movement (hyperactivated) spermatozoa showed some differences on the distribution along different experiments of this thesis. At this point it is worthy to note that this sP3 showed clearly higher percentages in the individual male samples compared to the values observed in the pooled samples after thawing and even after SLC was performed. This may be due to the fact that since it was established that pooled samples showed better values when compared to samples from the individual males, hence the higher percentage of SP3 sperm in the pooled samples.

In our study, the use of the multivariate cluster analysis was used to identify the four motile sperm subpopulation with the statics considered as the 4^{th} subpopulation which was deduced from the overall CASA sperm parameters during the statistical analyses. However, this subpopulation was significantly increased after thawing and under *in vitro* incubation

with seminal plasma. Evidently, the relative importance of the different subpopulations of motile spermatozoa present in buck sperm sample for predicting in vivo fertility still needs to be investigated.

Finally, many studies have demonstrated different effects on the relationship between studied sperm parameters and *in vivo* fertility using different sperm preparation techniques (Rodriguez-Martinez & Barth, 2007; Rodriguez-Martinez, 2003; Rodríguez-Martínez, 2006), while some other authors found no relationship (Garner et al., 2001; O'Meara et al., 2008; Sánchez-Partida et al., 1999).

Chapter VII

GENERAL CONCLUSION

Therefore, according with the results obtained in this doctoral thesis, the final conclusions are:

1. Single layer centrifugation method with Bovipure® did not improve the quality of *Blanca de Rasquera* buck sperm after thawing regardless of the male age, season and melatonin implant. Further investigation is required to determine the ideal colloid and method for the selection of sperm from the *Cabra Blanca de Rasquera* breed.

2. No remarkable differences were observed on the sperm quality after thawing as well after single layer centrifugation method between samples collected from males of *Cabra Blanca de Rasquera* breed at different ages in the same season.

3. The season of semen collection had slight influence on the quality of sperm after thawing, showing better sperm quality in samples collected in autumn (breeding period) compared to samples collected in spring. No improvement on the sperm thaw quality was described on samples collected from melatonin implanted males in the non-breeding season.

4. The present study did not show a fully manifested individual male factor in buck sperm quality after cryopreservation, although some differences on sperm viability were observed between males. Furthermore, SLC method through Bovipure® colloid is not efficient in improving thawed buck sperm quality regardless of the donor, besides different sperm behaviours were seen depending on the male.

5. The sperm quality after thawing was superior in pooled samples collected during autumn and spring compared to individual samples collected in winter, suggesting a positive effect of heterospermic interaction within samples from the different males.

6. The significant differences observed on sperm quality between pooled samples as well between individual samples just after thawing were not reflected after SLC.

7. The 3h incubation in control media did not affect sperm viability, although DNA integrity and motility was negatively affected, regardless of the male age, season or melatonin treatment. However, different behaviour was observed amongst males under similar conditions on sperm viability.

8. Thaw sperm viability was significantly reduced after 3h under *in vitro* capacitation conditions compared to control media, regardless of donor age, season of semen collection and melatonin treatment of males or the individual, suggesting that thawed sperm could have achieved capacitation status and the subsequent acrosome reaction, and finally the cell death. However, the low percentages of live sperm with active mitochondria and reacted acrosome found in the different incubation media regardless of the origin of the samples may be difficult to support this hypothesis.

9. In Relation to DNA Fragmentation Index, a significant reduction was seen in pooled samples from autumn after 3h under capacitation conditions when compared to control media, suggesting a protective role of the IVF media for DNA integrity only when semen samples were collected in the breeding season (autumn). However, no effect on motility and HDS percentages was observed between control and IVF media after 3h incubation regardless of the different studied factors.

10. Incubation of buck spermatozoa with seminal plasma under *in vitro* capacitation condition did not improve buck sperm motility, membrane status, and mitochondria function regardless of the male donor, rather a negative effect was seen in the studied sperm quality parameters, except on the DNA status.

11. The presence of buck seminal plasma in the incubation media increased acrosome damage while the incidence of acrosome reaction was similar when IVF media alone was used. Moreover, DNA integrity was maintained in *in vitro* capacitation media regardless of the presence of SP during the breeding season.

12. SLC method through Bovipure® colloid is not efficient in improving thawed buck sperm quality regardless of the donor, although different sperm behaviours were seen depending on the male.

13. The DNA integrity and HDS of thawed spermatozoa from *Cabra Blanca de Rasquera* bucks were less affected compared to other studied parameters after thawing and during *in vitro* incubation.

14. In relation to buck sperm freezability, the differences in the DFI between males can be used to predict the survivability of buck spermatozoa in the female reproductive tract which was carried out *in vitro* after 3h incubation under capacitation conditions.

15. The distribution of the different subpopulations amongst individual or pooled samples from males with different ages collected in different seasons was similar along the different experiments. Only slight differences were seen in the distribution the amongst different sperm treatments or incubation media, indicating that this subpopulation structure remains quite stable regardless of the studied factor, especially when the subpopulation of static sperm is included as a specific subpopulation of the sperm structure.

16. Only the sperm subpopulation 3 (sP3) composed of spermatozoa with the fast and non-linear movement (hyperactivated) sperm showed some differences on the distribution along different experiments of this thesis. This sP3 subpopulation was higher after SLC regardless of season, male age or melatonin treatment and clearly higher in the individual male samples compared to the pooled samples after thawing, after SLC was performed and even after sperm incubation in different media. Further investigation is required to determine if these differences on sP3 proportion observed in our study are indicative of the prejudicial effect of cryopreservation which can be further exacerbated by the centrifugation procedure or a consequence of the sperm hyperactivation process.

17. Buck sperm freezability could be determined better after 3h incubation under capacitation conditions mimicking the female reproductive tract than just after thawing.

18. Results in this study indicate that the sperm motility parameters evaluated by CASA, sperm functional characteristics analysed using flow cytometry and Sperm Chromatin Structural Assay may be useful for assessing the protocols and strategies in creating a sperm bank for the *Cabra de Blanca de Rasquera* breed. However, more investigation is required especially in order to improve thaw sperm quality.

Chapter VIII

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ABBREVIATION INDEX

ALH: Amplitude of Lateral Head displacement

ANOVA: Analysis of Variance

AO: Acridine Orange

ART: Assisted Reproductive Technologies

AV: Artificial Vagina

BCF: Beat Cross Frequency

BSA: Bovine Serum Albumin

CASA: Computer assisted sperm analysis

DFI: DNA Fragmentation Index

DGC: Density Gradient Centrifugation

DNA: Deoxyribonucleic Acid

EE: Electroejaculation

EYCE: Egg Yolk Coagulating Enzyme

FAO: Food and Agricultural Organization

GRB: Genetic Resource Bank

HDS: High DNA Stainability

INIA: *Instituto Nacional de Investigaciones Agroalimentaria*

IUCN: International Union for Conservation of Nature

IVF: In Vitro Fertilization

LIN: Linearity

PBS: Phosphate Buffer Saline

PI: Propidium Iodide

PM: Progressive Motility

SCSA: Sperm Chromatin Structural Assay

SD: Standard Deviation

SEM: Standard Error of the Mean

SLC: Single Layer Centrifugation

SP: Seminal Plasma

Sp: Subpopulation

STR: Straightness

TM: Total Motility

TNE: Tris-NaCl-EDTA

VCL: Curvilinear Velocity

WHO: World Health Organization