UAB

Universitat Autònoma de Barcelona

Further approaches on sperm cryopreservation protocol towards the establishment of the sperm bank from endangered native Catalan ram breeds (Xisqueta and Aranesa)

Uchebuchi Ike Osuagwuh

Doctoral Thesis

Facultat de Veterinària

Departament de Medicina i Cirurgia Animal

Universitat Autònoma de Barcelona

2017

Dra. María Jesús Palomo Peiró, Profesora Titular del Departament de Medicina i Cirurgia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona

Certifica:

Que la tesis titulada "**Further approaches on sperm cryopreservation protocol towards the establishment of the sperm bank from endangered native Catalona ram breeds (Xisqueta and Aranesa)**", presentada por **Uchebuchi Ike Osuagwuh** para optar al grado de Doctor en Veterinaria se ha realizado bajo mi direccion y, considerándola acabada, autorizo su presentación para que sea juzgada por la comisión correspondiente.

Bellaterra (Cerdanyola del Vallés), Septiembre de 2017.

Dra. María Jesús Palomo Peiró   **Sr. Uchebuchi Ike Osuagwuh**

Directora   Candidato

TABLE OF CONTENT

SUMMARY

The new interest towards the characterization and conservation of *Aranesa* and *Xisqueta* ram breeds was constituted by ex situ programs using assisted reproductive techniques (ARTs) such as semen cryopreservation and the development of sperm banks. Therefore, the most commonly used application in the development of sperm banks are semen collection and freezing, and this requires the knowledge of the reproductive physiology for these species. Besides semen collection and freezing, its utilization towards artificial insemination (AI) as well as the suitable assisted reproductive technologies is also a key aspect in order to prevent the disappearance of these breeds and maintain their genetic diversity. However, there are still concerns due to issues regarding frozen-thawed sperm quality. With the present technological advances made in recent years on sperm evaluating techniques such as the use of computer-assisted sperm analysis (CASA) and flow cytometry, a wealth of information on their sperm characteristics may be achieved. Furthermore, as the application of refrigerated and frozen-thawed semen technology increases, more information on sperm quality and advances are required. To this regard, the general aim of this thesis was to provide some basic information on *Aranesa* and *Xisqueta* sperm processes for cryopreservation and to determine the efficacy of some strategies towards improving post-thawed sperm quality prior to testing its usefulness for further application.

In this doctoral thesis, we evaluated the seasonal effect and the melatonin implantation on scrotal circumference, time of semen emission and characteristics of fresh ram ejaculates collected by electroejaculation. In addition, the sperm qualitative characteristics stored at 5^0C and held for 24h in a cryopreservation media from the various treated males during non-breeding was determined, and directly compared with same parameters from a breeding season. The goal was to provide some useful information on holding spermatozoa below body temperature to various time periods prior to cryopreservation or as an alternative to frozen-thawed semen for AI. In this study, semen was collected (n=8) from ten rams (*Aranesa* and *Xisqueta* breeds) via electro-ejaculation during the non-breeding season; five males were implanted with Melovine®, while the other five rams remained untreated. Thereafter, semen was collected from six rams (n=8) during the breeding season. Results from this study showed no difference in any sperm parameter between melatonin treated and breeding season groups but both groups differed significantly (P < 0.05) when compared with the untreated/non-breeding semen donor group except on scrotal circumference. Also, despite all parameters being negatively influenced by the chilled liquid storage time, melatonin treated group showed better sperm quality parameters when compared to other groups. This

study demonstrates that melatonin implantation during the non-breeding season improved almost all qualitative sperm parameters studied during chilled liquid semen storage.

This thesis also addresses the efficacy of collection methods electroejaculation (EE) vs. artificial vagina (AV)) on fresh and post-thawed sperm quality of *Aranesa* ram sperm towards the establishment of a sperm bank. The aim being that for the successful sperm cryobank establishment towards conservation, information on semen recovery methods on pre- and post-thawed sperm quality is essential. This may lead to rapid constitution of the sperm bank especially from a large number of untrained males within a short period of time. Results in this study on fresh semen showed no significant difference between methods except for volume, concentration, total sperm count and functional sperm membrane integrity. Motility parameter was significantly higher in EE samples than those obtained via AV in pre-freeze samples but none was observed after thawing. Post-thawed samples evaluated by flow cytometry showed that semen sample obtained by EE had lower percentage of viable cells with damaged acrosome and active mitochondria (cryo-capacitation) and total acrosome damage than those via AV. Results herein supports the inclusion of EE method as a viable alternative and quicker method for semen collection towards the ongoing conservation program and establishment of *Aranesa* sperm cryobank.

Furthermore, in this thesis, we evaluated the sperm motility and qualitative characteristics of ram spermatozoa cryopreserved with or without seminal plasma under a controlled condition and subjected to a 4 h post-thawing thermal evaluation test. The aim being that exposing these frozen-thawed sperm to thermal resistance test will provide some knowledge on their survivability in the female genital tract towards fertilizing the ovum. This was achieved by collecting semen from 5 males (5 years old) via artificial vagina, and split into two aliquots. One aliquot was diluted (1:5) in Tris-citric acid-glucose (TCG) solution and washed twice by centrifugation at $600 \times g$ for 10 min, while the other aliquot was kept unwashed. Thereafter, washed and unwashed sperm were extended in a TCG-based powdered egg yolk media and frozen. Frozen–sperm were thawed and incubated at 37°C for 4 h. Results from this work showed that unwashed samples generally had better results than washed samples as regards to sperm motility characteristics irrespective of incubation. Also, post-thawing incubation time had a significant effect on acrosome integrity and mitochondria functionality irrespective of sperm treatment. This study demonstrates that the presence of seminal plasma prior to cryopreservation was beneficial in maintaining post thawed sperm motility, and as such, may be useful for ex situ ram sperm cryopreservation towards its use for artificial insemination.

The addition of seminal plasma to frozen-thawed sperm has been suggested as an alternative measure to improving its sperm quality. Therefore, we evaluated the effect of removing seminal plasma and freezing media by washing via centrifugation and subsequently supplementing with autologous whole seminal plasma (20%) collected by EE from a non-breeding and breeding season or by AV from a breeding season on frozen-thawed sperm quality. Furthermore, the effect of incubating frozen-thawed sperm (washed and unwashed) with the various seminal plasma sources for up to 90 min on sperm qualitative parameters was evaluated. The aim being that supplementing these sperm samples with different seminal plasma source following a thermal resistance test at 37°C will provide some information as regards their quality and survivability in the female genital tract. Results herein on sperm kinematics immediately after thawing showed that the addition of seminal plasma regardless its source on cyropreserved sperm (washed and unwashed) generally had a positive effect on sperm motility parameters. Also, after 90 min of incubation period, there was a decrease in sperm motility, irrespective of sperm treatments. Furthermore, no difference was observed amongst treatment groups with seminal plasma on all sperm function parameters evaluated by flow cytometry immediately after thawing except on total acrosome integrity and mitochondria functionality of washed sperm incubated with only PBS. This study demonstrates that supplementing frozen-thawed sperm with seminal plasma irrespective of its source maintained sperm functions and motility characteristics during a post-thawing incubation period. Furthermore, washing by centrifugation or removal of freezing component after thawing was not necessary.

We can therefore conclude from the results obtained in this thesis that melatonin implants on male during a non-breeding season provided a beneficial effect on ram sperm during chilled liquid storage, the inclusion of EE as method for semen collection towards the establishment of a sperm bank is recommendable, and finally, the presence of seminal plasma prior to cryopreservation or its addition to post-thawed sperm was important in maintaining sperm motility and functions.

CHAPTER I

Introduction

Aranesa and *Xisqueta* are native Catalan breeds of sheep located in marginal areas across the north of Catalonia, Spain. In the case of *Aranesa* sheep breed, they are characterized by their meat and lamb production, and are highly adapted to the harsh conditions of traditional grazing system in mountains and valleys during winter and summer periods (Esteban, 2003). The geographical distribution of this species is in the Valley of Aran and to the north of the province of Lleida, bordering the French border. In 2003, the Agriculture, Livestock and Environment Services of the Aran Valley General Council reported that there were 64 sheep farmers with a total of 2,569 *Aranesa* sheep, of which 1,489 were considered of pure race following the racial standard described by Parés (2008). As regards the *Xisqueta* sheep, they are located mainly in the Leridan regions of Pallars Jussà, Pallars Sobirà, Alta Ribagorça and Ribargoza. They are also scattered around in the regions of Monegros and Sobrarbe as well as in regions that makes up the Plane of Lleida. They are characterized by their meat and lamb production. Nonetheless, Avellanet et al. (2005) reported that the number of breeding stock is between 12,000 and 15,000 individuals.

However, a recent survey has indicated a sharp decline of these breeds due to the low generational changes in farming and was listed as endangered species with the brink of extinction (Avellanet et al., 2005). Therefore, a new interest towards the characterization and conservation of these breeds was constituted by ex situ programs using assisted reproductive techniques (ARTs) such as semen cryopreservation and development of sperm bank with its ultimate use for artificial insemination (AI), which may eventually help to conserve these breeds (Garcia, 2014). Despite the availability of literatures on the characteristics of semen and factors affecting the reproductive abilities from several breeds of rams (Boland et al., 1985; Wiemer et al., 1987; Osinowo et al., 1988), not much is known about these Catalonian breeds. Nonetheless, to efficiently apply ART, it is important to understand the basic reproductive physiology of these rams. However, ram semen preservation still remains a critical factor either as frozen-thawed or in liquid storage for AI (Allai et al., 2015), or chilled extended semen prior to freezing.

Moreover, a recent investigation by our group demonstrated the optimization of cryopreservation protocols for these breeds by studying the substitution of several components used in sperm freezing as well as the beneficial importance of melatonin implants (Garcia, 2014). Despite the aforementioned, more work is required to provide

prognostic information in identifying sperm kinematics, morphological or physiological behaviours thus indicating the state of the sperm at any given time in other to correlate its quality and fertility (Gadea et al., 2005; Rodriguez-Martinez, 2006; Silva and Gadella, 2006).

With the present technological advances made in recent years on sperm evaluating techniques such as the use of computer-assisted sperm analysis (CASA) and flow cytometry, a wealth of information on their sperm characteristics may be achieved. Furthermore, as the application of refrigerated and frozen-thawed semen technology increases, more information on sperm quality and advances is required. Therefore, the failure towards these investigations may result to loss of superior genetics, and if conception with AI could be improved through strategies that enhance semen post-thaw quality, a greater chance for maintaining superior genetics may be achieved. Several studies have focused on exploring different approaches to improve semen quality or conception rate of domestic, wild and endangered animals. This includes holding spermatozoa below body temperature to various time periods prior to cryopreservation (Fiser and Fairfull, 1984; Purdy, 2006); refrigerated extended semen as an alternative sperm storage to frozen semen (Evans and Maxwell, 1987; Rodriguez-Gil, 2006); reversing seasonality effects on reproductive performances using subcutaneous melatonin implants to give a desirable effect (Abecia et al., 2007); understanding the benefits and limitation of various semen collection methods in domestic species or breeds in creating a sperm bank as well as its effect on post-thawed semen quality (Nikolovski et al. 2013); sperm washing by centrifugation to remove seminal plasma prior to freezing and its effect on the post-thaw sperm quality (Moore et al., 2005; Fraser et al., 2007; El-Bahrawy, 2010; Naing et al., 2011; Natali, 2011; Jiménez-Rabadán et al., 2012); supplementing post-thawed sperm with seminal plasma and its effect during a post thawing incubation period on sperm quality (Maxwell et al., 2007; Rovegno et al., 2013; Rebolledo et al., 2007; Rickard et al., 2014); evaluation and maintenance of frozen-thawed sperm quality over an extended period of time at 37ºC (thermo-resistance test) as an indication towards sperm survivability in achieving high fertility rates (Alvarez et al., 2000; Bag et al., 2004; Joshi et al., 2005). All these approaches with some variations while using an established cryopreservation protocol in our laboratory constitute the main components of this thesis.

Chapter II

OBJECTIVES

The main goal of this thesis has been the creation of a sperm cryobank for *Aranesa* and *Xisqueta* ram breeds using an established cryopreservation protocol with the aim of understanding and improving sperm survival and functionality either as refrigerated or frozen-thawed. Therefore, the following specific objectives were suggested:

1. To determine the influence of melatonin implantation during a non-breeding season on scrotal circumference, time of semen emission and characteristics of fresh ram ejaculates, and further, to evaluate their sperm qualitative characteristics held for 24h at 5^0C of liquid storage in a cryopreservation media with direct comparison to a breeding season.

2. To evaluate the potential effect of two semen collection methods (electroejaculation vs. artificial vagina) on sperm production and quality of fresh and frozen-thawed *Aranesa* ram spermatozoa for the rapid constitution of a sperm bank.

3. To determine the resilience of washed (seminal plasma removal by centrifugation) and unwashed frozen-thawed sperm samples during a 4-h incubation period. This was aimed at providing some knowledge as regards sperm processes before cryopreservation prior to testing its usefulness for further application.

4. To evaluate the effect of adding whole seminal plasma (20%) collected via electroejaculation (breeding and non-breeding season) or via artificial vagina (breeding season) on post-thawed washed and unwashed ram sperm quality. Furthermore, to determine the resilience of these frozen-thawed sperm treatments during a 90 minutes post-thawing incubation period at 37ºC (thermo-resistance test).

Chapter III

LITERATURE REVIEW

3.1. FACTORS INFLUENCING SPERM PRODUCTION AND QUALITY ON RAMS

In sexually matured male ruminants, sperm cells are produced in the seminiferous tubules of the testis through a process called spermatogenesis and stored in the epididymis where they undergo maturation (Hafez and Hafez, 2000). This process is regulated by hormones called gonadotropins which are released into the bloodstream by the pituitary gland (Evans & Maxwell, 1987). The hormones responsible for maintaining and regulating spermatogenesis are the follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH has a critical role in regulating spermiogenesis, by regulating the differentiation and transformation of germ cells into spermatozoa thus controlling the fertilizing ability of the sperm (Moudgal & Sairam, 1998). On the other hand, LH acts on the Leydig cells of the testis to stimulate the production of testosterone which in turn acts on the seminiferous tubules to promote spermatogenesis (Evans & Maxwell, 1987). Nonetheless, the processes involved in sperm development and production reaches full maturation in about 50 days in the ram (Hafez and Hafez, 2000).

In creating a sperm bank for its use towards artificial insemination (AI) which is increasing becoming utilized, opportunities for substantial increase in the production and use of frozen semen abounds. Therefore, to supply this demands, the AI industry must optimize the factors affecting sperm production and semen quality.

3.1.1. Donor Age

In sheep, as in most other species, the quality and quantity of semen is strongly influenced by age. The semen volume, motility and sperm concentration increased with advancing age, while the percentage of dead and morphologically abnormal spermatozoa decreased (Mandiki et al., (1998). Furthermore, a similar report has also been demonstrated in bulls in which semen production and quality was improved in older males when compared with younger ones (Brito et al., 2002). Also, Shannon and Vishwanath, (1995) and Garner et al., (1996) reported that sperm morphology, concentration, motility and volume of the ejaculate improved with an increase in the age of the bulls. These findings seem to indicate that the genital system of the ram undergoes maturational changes during this period (Osinowo et al., 1988; Toe et al., 2000). For example, a recent study by Lymberopoulos et al., (2010)

demonstrated that matured rams (4-5 years) had significantly lower values of sperm hyperactivated motility, higher percentages of live non-capacitated sperm and cells with intact plasma membrane, functional mitochondrial as well as greater lambing rate than younger males (1-2years).

3.1.2. Size of testis

The testicular size of the testis has been regarded as a trait of choice in male (Matos and Thomas, 1992). Furthermore, it has been demonstrated that genetic correlation exists between scrotal circumference and semen volume as well as sperm motility (Rege et al., 2000). Langford (1987) reported that there was an increase in sperm output from males with an increased scrotal circumference. Therefore, scrotal circumference has been suggested as a good indicator towards libido and sperm producing ability as well as the attainment of puberty (Toe et al., 2000).

3.1.3. Method of collection

In small ruminants, semen collection via artificial vagina (AV) or electroejaculation (EE) are commonly used methods. Nevertheless, the methods of semen collection have been reported to affect sperm quality both in fresh ejaculates (Greyling and Gobbelaar 1983) as well as on post-thawed sperm (Salamon and Maxwell 1995; Watson 1995). For example, Marco-Jimenez *et al.* (2005) reported that the semen volume and concentration in the Guirra ram was higher when collected by the AV method than the EE technique. In another study, ejaculates via EE had larger seminal volume than those obtained via AV (Jiménez-Rabadán et al. 2016), suggesting that the larger volume is as a result of increased seminal plasma (SP) from the accessory sex glands due to electric stimulation (Marco-Jiménez et al. 2008). Nonetheless, controversies still abounds on sperm quality as regards method of semen collection.

3.1.4. Breed

The breed of males has been reported to significantly affect the semen characteristics (Fernandez et al.*,* 1993). For example, Corriedale rams was reported to have a lower semen

production and sperm concentration with higher incidence of sperm abnormalities than rams of other breeds like Polwarth, Merilin and Merino breeds of ram (Fernandez et al., 1993). Furthermore, a similar study by Langford et al. (1998) reported that Canadian rams produced more sperm per ejaculate (7.0 billion) than Finnish Landrace (3.3 billion).

3.1.5. Season and environment

Although, semen production in ram continues all year round (Chemineau et al., 1992), the most important environmental factors influencing sperm production and quality are season/photoperiod and temperature. Seasonal effects on semen quality are caused by several factors, such as ambient temperature or humidity, day length, and available feed quality. Seasonal variations in total protein content of the seminal plasma were found in rams, being higher in autumn than in summer and winter (Gundogan, 2006). Furthermore, the exposure of males to hot temperature has been reported to produce high abnormal morphological semen profile (Marai et al., 2007).

Also, libido, semen production and fertilizing ability are significantly reduced in heat stressed rams (Daader et al., 1985). This is suggested to be as a result of elevated temperature on sperm quality due to the damage sustained during the late stages of spermatogenesis (Howart, 1969). On the other hand, photoperiod has been demonstrated to have a significant effect on the growth of testes and LH pulses, highest being in the short photoperiod seasons such as autumn and winter and lowest in spring and summer (Lindsay et al., 1984; Malejane et al., 2014). The gonadotropins and gonadal hormones act in concert with prolactin to determine the function of the reproductive axis; this interaction potentially affects the function of the gonads, accessory sex glands, secondary sex characteristics and the expression of sexual and aggressive behaviour during sexual cycle in seasonal breeders (Lincoln, 1996).

However, reports on the significant effects of season on semen production differs (Fuerst-Waltl et al., 2006). For example, Stalhåmmer et al. (1989) observed the highest sperm concentration and total number of spermatozoa during summer, Mathevon et al. (1998) found higher values during winter and spring, while Brito et al. (2002) failed to detect any effect of season on semen production. Nevertheless, with the above-mentioned knowledge, sperm production can be manipulated to give a desirable effect, especially in an out of breeding

season with subcutaneous melatonin implants (Abecia et al., 2007), thereby reversing seasonality effects on reproduction. Thus, melatonin implants have been reported to increase scrotal size, improve semen quality and enhance reproductive performances outside breeding periods (Palacin et al., 2008; Casao et al., 2008). In this regard, seasonality of spermatogenesis may not be a limiting factor for an all year semen collection in the ram. However, the influence of both seasonality and melatonin treatment on reproductive parameters has strong genetic component and variation between individual rams, breeds and or donor age (Mandiki et al., 1998; Karagiannidis et al., 2000; Garcia, 2014). Therefore, a better knowledge on the influence of season on semen production is essential towards creating a sperm bank.

3.2. SEMEN CRYOBANK AND SPERM CRYOPRESERVATION FACTORS

In 2007, the Food and Agricultural Organization (FAO) initiated the preservation of animal genetic resources towards preventing the loss of animal genetic variability by prioritizing *in situ* and *ex situ* preservation of genetic materials of endangered breeds. Therefore, the creation of a sperm bank (*ex situ*) is recognized as a measure of conservation. This allows for the storage of semen or other forms of genetic materials such as oocytes, embryos, etc. for a prolonged period of time thereby ensuring the genetic diversity of species (Holt and Pickard, 1999; Roldan et al., 2006). Nonetheless, the National Institute of Agricultural Research (INIA) promoted the creation of a sperm bank for these native Catalan breeds through early genetic selection of breeders towards cryopreservation (Garcia, 2014). This was done to curb losses of their genetic variability as well as preventing their possibilities of going extinct. However, in creating a sperm cryobank, the use of an effective semen collection, freezing and evaluation technique is an integral aspect towards an effective conservation program. Therefore, it is of great paramount importance in the understanding of some basic reproductive physiology and semen characteristics of these species or breeds, which needs cryopreserving before its application with the assisted reproductive techniques.

Sperm cryopreservation has been a useful tool for the storage and restoration of valuable genetic resources, and in combination with assisted reproductive techniques, conservation of endangered breeds may be practical (Maxwell and Watson, 1996; Holt and Pickard, 1999; Watson, 2000). This involves various stages of any cryopreservation protocol such as collection and extension of semen, addition of cryoprotectant, cooling rate to above 0 ºC

(equilibration time), freezing and thawing (Salamon and Maxwell, 2000). Despite the similarities in most sperm cryopreservation protocols used in small ruminants (Shipley et al., 2007; Parkinson, 2009), 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). However, cryopreservation adversely impairs ram semen quality by altering sperm function (Ledesma et al., 2016). This may be due to spermatozoa sensitivity to extreme temperature changes during cooling, freezing and thawing, invariably leading to a reduction in motility, viability, mitochondria membrane integrity, increase in reactive oxygen species production and chromatin damage (Bailey et al., 2003; Said et al., 2010). These alterations may affect the post thawing fertilizing capabilities as a result of reduced survivability of the frozen thawed spermatozoa (Salamon and Maxwell, 1995; Ledesma et al., 2016). During the last 50 years, numerous techniques have been developed and perfected in order to obtain good fertility through the application of frozen seminal doses (Watson, 1995). Although great advances in freezing bull semen and utilization have been successful, results from other species such as ovine have been inconsistent and extremely low (O'Meara et al., 2008; Barbas and Mascarenhas, 2009). Nevertheless, there are many factors affecting sperm cryopreservation which influences the outcome of the process or fertility results (Purdy, 2006). These factors include species and individual, sperm collection technique, seminal plasma, season, extender, cooling rate, equilibration time, freezing and thawing rate, among others.

3.2.1. Specie and individual male factor

In small ruminants, there is evidence of qualitative differences between spermatozoa resulting to differences in their susceptibility towards freezing and thawing (Curry, 2000). This response of spermatozoa to freezing and thawing processes may vary between individuals within the same species due to differences in their composition of cell membranes (Salamon and Maxwell, 2000). It has also been demonstrated that sperm membrane lipid composition influences the susceptibility of sperm cells to damage during cryopreservation (Holt, 2000a). For example, ram sperm are more resistant to cold shock damage in comparison with boar sperm (Holt, 2000b). Furthermore, inter-male and inter-ejaculates has been shown to vary in sperm freezability during cryopreservation (Soler et al., 2003; Ramon et al., 2013). To this regards, ejaculates are usually referred to as good freezers (GFE) or bad freezers (PFE) due to the variation between ejaculates in accordance to their resilience to

withstand freeze-thawing process (Yeste, 2016). This has been reported in some species such as in boar (Yeste et al., 2013), goats (Dorado et al., 2010) and stallions (Yeste et al., 2015). Furthermore, Yeste, (2016) reported that the individual response is not only related to cryopreservation but to other semen processing procedure such as liquid storage. Nonetheless, the post-thawing survival and motility of GFE has been demonstrated to be more resistant on their chromatin structure than PFE (Casas et al., 2009). Although the differential mode of action involved between GFE and PFE is not known, it has been suggested that the freezabilty differences between them may be related to their genetic differences, however, inconsistency still exist between species (Vilagran et al., 2015). However, the addition of seminal plasma from GPE to PFE has been reported to increase its freezability thereby improving motility and viability of PFE frozen-thawed sperm (Vilagran et al., 2015). Therefore, for the successful application of cryopreservation towards conservation, information on their sperm physiology and appropriate freezing methodology is essential in other to prevent cellular damage during cryopreservation processes (Purdy, 2006).

3.2.2. Semen collection method

In creating a sperm cryobank, the use of an effective semen collection and evaluation technique is an integral aspect to achieving this goal. In small ruminants, semen collection via artificial vagina (AV) or electroejaculation (EE) are commonly used methods. Although, AV is the preferred method (Leboeuf et al., 2000), semen collection from large numbers of untrained rams makes sperm bank creation difficult as this technique requires previous training periods (Wulster-Radcliffe et al., 2001). Alternatively, EE could be a useful procedure for semen collection especially from untrained males (Jiménez-Rabadán et al., 2012). This has been successfully used in collecting semen from wild ruminants (Asher et al., 1990; Garde et al., 2003; Santiago-Moreno et al., 2009). Nevertheless, differences in collection methods have been reported to affect sperm quality both in fresh ejaculates (Greyling and Gobbelaar 1983) and post-thawed sperm (Salamon and Maxwell 1995; Watson 1995).

3.2.3. Seminal plasma effect

Many studies have tested the effect of seminal plasma on sperm quality during cryopreservation with variable results. While there are reports demonstrating the positive effects such as improved sperm resistance to cold or thermal shock, increase sperm motility

and its ability to migrate through the cervical mucus (Rebolledo et al., 2007; Rickard et al., 2014), others have demonstrated detrimental (Maxwell et al., 2007; Rovegno et al., 2013) or no effect (Morrier et al., 2003). These inconsistencies may be due to the complexity and variance of seminal plasma amongst species (Mandiki et al., 1998), donor age (Muiño-Blanco et al., 2008), seasons (Leahy et al., 2010) and or method of collection (Ledesma et al., 2014). Nonetheless, seminal plasma is a complex mixture of secretion originating from the male accessory organs (Gundogan, 2006). It is synthesized and secreted by the testes and accessory sex glands and plays a significant role in the maintenance of sperm motility and freezability (Turner et al., 2006). Furthermore, it contains proteins, enzymes, lipids, electrolytes, sugars and various other factors, which may play significant roles in the metabolic regulation of sperm cells (Maxwell et al., 1999). Many studies have shown that the low content of seminal plasma proteins is associated with poor semen quality (White et al., 1987; Ashworth et al., 1994). Also, Taha et al., (2000) reported that the reduction in sperm concentration and motility are associated with a decrease in seminal plasma lipid content. Seminal plasma has also been reported to maintain sperm motility and viability in many species (Maxwell et al., 1996) and increase the sperm resistance to cold shock injury (Barrios et al., 2000), by providing specific components that stabilize the membrane of the frozen-thawed sperm cells (Maxwell & Watson, 1996). In addition, Maxwell et al. (1999) reported that the penetration of sperm cells through the cervical mucus was improved and fertility after cervical insemination of ewes significantly increased when frozen-thawed sperm supplemented with 20% seminal plasma was used. Nonetheless, other studies on seminal plasma have also demonstrated detrimental effect (Maxwell et al., 2007; Rovegno et al., 2013) or no effect (Morrier et al., 2003). Therefore, in order to prevent this adverse effect, some works have suggested that the removal of seminal plasma from the ejaculates by centrifugation increased post thawed sperm motility and functions (Kozdrowski et al., 2007; Garcia, 2014). Despite the abovementioned suggestion, other studies have shown no effect (Cabrera et al., 2005) or detrimental effect (Barrios et al., 2000; 2005) of seminal plasma removal on post thawed sperm quality.

3.2.4. Season

Although, semen production in small ruminants continues all year round (Chemineau et al., 1992), season and photoperiod has been an important factor towards the success of sperm collection and quality (Shackell et al., 1977). Several positive changes have been associated

with season such as increase in testicular weight, volume of ejaculates, libido and fertility, and these has been associated during the period of autumn and winter considered as breeding season (Shackell et al., 1977; Mandiki et al., 1998; D'Alessandro and Martemucci, 2003). Furthermore, the effect of season has been demonstrated to affect the response of spermatozoa to the freeze-thawing process in several domestic animals (Fiser and Fairful, 1983; Janett et al., 2003; D'Alessandro and Martemucci, 2003; Medrano et al., 2010; Coloma et al., 2011). Also, the improvement of fertility of frozen-thawed sperm from rams has been associated with the changes in seminal plasma obtained during the breeding season (Smith et al., 1999). In addition, many works have demonstrated the beneficial effect on sperm quality by supplementing sperm with seminal plasma obtained during the breeding season (Dominguez et al., 2008; Leahy et al., 2010). However, the interaction between season and seminal plasma to frozen thawed sperm has yielded variable results (El-Rajj Ghaoui et al., 2007).

3.2.5. Freezing extender and cryoprotectant

Semen extenders (or diluents) were developed in order to provide an acceptable buffering capacity, osmolality, various sources of energy, to minimize bacterial growth and provide protection due to decreases in temperature (Vishwanath and Shannon, 1997; Purdy, 2006). The composition of an extender in which semen is diluted before freezing is a major factor that influences the success of cryopreservation (De Leeuw et al., 1993; Woelders et al., 1997). However, a cryopreservation media includes a non-penetrating cryoprotectant, a penetrating cryoprotectant, a buffer, one or more sugars, salts and antibiotics (Evans and Maxwell, 1987). The need to control ice crystal formation during freezing is of great importance (Mazur, 1980) and several penetrating cryoprotentants have been tested with glycerol being the most commonly used (Holt, 2000b). Its protective effect is its ability to reduce thermal stress thereby preventing fractures in the frozen media by reducing the total ice volume expansion during water crystallization (Gao et al., 1995). However, glycerol also has a negative effect on spermatozoa (Hammerstedt et al., 1990; Holt, 2000b) and this depends on the extender composition, method of glycerol addition, cooling and freezing rates (Salamon and Maxwell, 1995; Salamon and Maxwell, 2000). Nonetheless, other penetrating cryoprotectants such as sucrose, raffinose, trehalose and lactose, polymers such as polyvinyl pyrrolidone (PVP) and the amphipathic compounds such as glycine betaine, glutamine and proline have been identified as potentially cryoprotective (Lahnsteiner et al., 1996; Holt,

2000a; Sztein et al., 2001, Sànchez et al., 2011). The most common non-penetrating cryoprotectant used in semen cryopreservation is egg yolk due to its protecting effect on sperm acrosomal and plasmatic membranes (Salamon and Maxwell, 2000). Egg yolk helps in protecting sperm cells against cold-shock which is a lipid-phase transition effect (Drobnis et al., 1993). However, with current need for disease control and deviation from animal sources for cryoprotective media, research is ongoing to find alternatives to egg yolk (Holt, 2000b). A recent investigation has demonstrated the success of cryopreserving semen in a Tris (hydroxymethyl-aminoethane)-citric acid-glucose (TCG) based media containing 5% glycerol and 15% (v/v) powder egg yolk (PEY) from *Aranesa* and *Xisqueta* rams (Garcia, 2014). Such a test was designed to study the use PEY as an alternative cyroprotectant to fresh egg yolk in order to reduce the potential risk of microbiological contamination due to concerns regarding bacterial or viral transmission between extenders containing fresh egg yolk. Moreover, the pasteurization process during the production of PEY helps to destroy microorganisms and give a higher homogenous composition makes it safe to use (Thibier and Guerin, 2000).

3.2.6. Refrigeration

When ram semen is diluted in a freezing extender, it is cooled to 4-5ºC (refrigeration/equilibration time). This period is the interval in which the sperm cell remains in contact with glycerol before freezing thereby allowing cryoprotectant to penetrate the cells and aiding equilibrium between intra and extracellular medium (Evans and Maxwell, 1987b). This equilibration time varies between species. This decrease in temperature produces a reduction in metabolic activity of cells as well as maintaining spermatozoa functionality. However, changes in sperm membrane that alter its functionality are produced during this period (Amann, 1999). The different rates (slow and fast) have been studied in several species with variable results (Fernández-Santos et al., 2006; Salazar et al., 2011; Memon et al., 2013). Some authors have demonstrated the benefits on holding spermatozoa below body temperature to various time periods prior to cryopreservation or for artificial insemination in various species: stallion (Backman et al., 2004), boar (Guthrie and Welch, 2005), bull (Foote and Kaproth, 2002) and ram (Fiser and Batra, 1984; Purdy, 2006). This is due to the fact that chilled liquid stored semen seems to be an alternative to frozen-thawed semen due to its less marked detrimental effects on sperm physiology and less expensive to maintain (Evans and Maxwell, 1987; Rodriguez-Gil, 2006). Therefore, with the importance and increase in chilled

semen utilization, several techniques have been demonstrated regarding short-term or long-term chilled liquid sperm storage or preservation (Moradi et al., 2013; Mata-Campuzano et al., 2015). However, prolonged chilled liquid storage of sperm can be detrimental due to cold shock leading to sperm structural damages, and factors such as season (Salamon and Maxwell, 1995; D'Alessandro and Martemucci, 2003) and type of extender (Kasimanickam et al., 2011) have been incriminated to affect ram sperm during such storage.

3.2.7. Freezing and thawing rates

Freezing and thawing rates have been shown to influence post-thaw survival of sperm cells (Mazur, 1985). This is as a result of ice formation and dissolution due to freezing and heating rates (Watson, 1995). However, most cell types do not survive freezing in the absence of a cryoprotective agent. The incorporation of these substances into the cell suspension helps to increase the percentage of post-thaw sperm survival. The freezing of sperm samples is done in straws or pellet form. Freezing of sperm in pellets is rapid and inexpensive but it is usually problematic because the samples can not be labelled (Evans and Maxwell, 1987a). On the other hand, freezing in straws are expensive and cumbersome than the pellet form, however, accurate labelling of samples can be achieved.

As regards thawing, adverse effects also occur in the sperm cell (Watson, 1995). Traditionally, straws are thawed by placing them in a 37 ℃ water bath for 12-30 seconds (Deka and Rao, 1987), although, other authors have demonstrated good sperm quality after thawing at 70-75 ℃ for 7-10 seconds (Tuli et al., 1991). However, thawing at 37 ℃ is more commonly done and the risk of overheating which can result in tremendous sperm mortalities is avoided (Evans and Maxwell, 1987a). Therefore, post-thaw sperm quality during this process requires a successful freezing and thawing stage. Two damaging mechanisms have been reported during the freeze-thawing process. First, if the sperm are frozen very quickly and there is slow heating, a phenomenon called re-crystallization occurs by virtue of the formation of micro-crystals inside the cell which tend to cluster and form larger crystals with consequent lethal consequences (Mazur, 1985). And, secondly, slowly frozen cells in the presence of a penetrating cryoprotectant may be injured by osmotic stress if thawed too quickly due to the fact that the cryoprotectant can not leave the cell fast enough to maintain the osmotic equilibrium with the swelling due to entry of water (Watson, 1990). The optimum defrosting speed, therefore, depends on the cooling pattern followed, but should represent an intermediate situation between the two previous ends, which minimizes damages

caused by inappropriate transport speeds of solutes and water through the membranes, and by the clustering of intracellular ice micro-crystals (Hammerstedt et al., 1990). Molecular destabilizations have also been observed in the plasma membrane because of change in temperature thus contributing to the appearance of lesions during this phase (Hammerstedt et al., 1990). Holt et al., (1992), observed the appearance of alterations in the plasma membrane that manifested only during the thawing stage, and suggested a causal mechanism based on the phase transitions phenomenon of the lipids membrane, thus highlighting the importance of thawing in the cryopreservation cycle. The authors further suggested that membranes are initially destabilized during the freezing step, both by the effect of low temperatures and by exposure to high salt concentrations leading to post-freeze degeneration.

In addition, spermatozoa and contaminating bacteria during storage produces metabolites that can reduce the pH of the diluents (Holt and North, 1994). The internal pH of the spermatozoon is directly related to the pH of the diluents as well as motility; so if the pH of the diluents goes down, motility and sperm metabolism also decreases (Gadea, 2003). The main substance responsible for the decrease in pH is the lactic acid produced by the glycolytic metabolism of sperm cells which also is used as an indicator of seminal quality (Rigau et al., 1996). As previously mentioned, each step of the cryopreservation process reduces the viability and increases functionality failures of surviving spermatozoa (Parinaud et al., 1997; Chatterjee and Gagnon, 2001; Kankofer et al., 2005) thus diminishing its fertilizing potential. This damage may also be due to oxidative stress (Alvarez and Storey, 1992; O'Flaherty et al., 1997; Chartterjee and Gagnon, 2001), and has been demonstrated in horse (Baumber et al., 2000, 2003), ram (Peris et al., 2007), bull (Bilodeau et al., 2002; Nair et al., 2006) and cat (Thuwanut et al., 2008). It has been suggested that one of the causes leading to oxidative stress is the production of reactive oxygen species (ROS) which is generated from the metabolism of oxygen (Aitken et al., 1993). Also, other chemically reactive molecules formed during this process includes both radicals (superoxide (O_2-) and hydroxyl ($\bullet OH$) and a non-radical, hydrogen peroxide (H_2O_2) (Ferreira and Matsubara, 1997). It has been shown that the main targets of ROS are the lipids contained in the cellular membrane thereby inducing membrane lipid peroxidation which modifies the membrane permeability and fluidity (Awda et al., 2009). Therefore, an increase in ROS levels has been associated to cause sperm DNA fragmentation and low motility (Mahfouz et al., 2010). Nevertheless, low quantities of ROS have been demonstrated to also play an important role in sperm capacitation, hyperactivity of sperm motility, acrosomal reaction in bulls (O'Flaherty

et al., 2003), boars (Awda et al., 2009) and rams (Periset al., 2007). Furthermore, the imbalance between the production of ROS and the scavenging capacity of antioxidants due to the excessive by-products generated by oxygen metabolism eventually lead to sperm death (Guerra et al., 2004). Although, ram sperm have been shown to be affected by the cryopreservation process, it is not known whether these alterations occur simultaneously or at different stages of the freezing and thawing process (Salamon and Maxwell, 1995).

3.3. SEMEN ANALYSIS

Semen quality and its relationship to fertility are of major concern in animal production. The accurate evaluation of semen quality is therefore of utmost importance. The conventional laboratory test for standard semen evaluation is usually the use of light microscopy to estimate sperm survival and the percentage of motile sperm (Rowe et al., 1993). Nonetheless, different methods have been demonstrated throughout the last decades, but only few methods have been adopted for practical or research purposes (Gadea, 2005). Most of these studies have used light microscopic evaluations of classical sperm parameters, including sperm concentration, motility, morphology and viability. Although useful, these tests are not completely reliable because of the lack of objectivity as well as human bias (Graham et al., 1980). More objectivity and repeatability in the assessment of sperm motility and functions can be achieved with the aid of the computer assisted aperm analysis (CASA) system (David et al., 2007) and flow cytometry (Axner and Linde Forsberg, 2007) thus providing a wealth of information towards the study of sperm characteristics. To this regard, more information on seminal quality both for fresh and frozen-thawed semen may be determined to speculate the performance of the semen used for artificial inseminations (Rodriguez-Martinez, 2005).

3.3.1. Evaluation of Sperm Motility

Sperm motility has been considered as one of the most important characteristics in connection with fertilizing ability (Rodriguez-Martinez, 2006; Awad, 2011), and its evaluation is regarded as the simplest method to evaluate the quality of a semen sample. This assessment involves the subjective microscopic estimation of the sperm motility by examining a uniform drop of semen on a warm slide with a coverslip, under a phase contrast microscope, fitted to a warm stage at 37°C (Hafez and Hafez 2000). Preliminary subjective

evaluations of sperm motility are mass motility and individual motility. Mass motility assessment requires a high sperm concentration in other to observe the swirls or movement, and this is usually the preliminary and practical level during semen analysis of ruminants as regards to sperm motility (Mehta et al., 1972). As regards the individual motility, percentage of motile sperm as well as individual progressive movement can be estimated via observation with an optical microscope from diluted seminal drop placed on a warm slide and covered with a cover slip (Mehta et al., 1972). However, despite the usefulness of this technique, sperm motility evaluations are not completely reliable due to subjective human bias (Graham et al., 1980). Therefore, the computer assisted sperm analysis (CASA) has been introduced as a routine method to improve the accuracy and repeatability of sperm quality data thereby avoiding errors resulting from the subjective evaluation of different technicians as well as to reduce the time spent during semen evaluation (Jane et al., 1996). Several CASA systems are available commercially, and may differ in their mode of functioning as well as their ability to detect and measure the motility of sperm in different species (Quintero-Moreno et al., 2003). However, the programs designed for this equipment includes a series of descriptive parameters of the sperm movement that are common to all CASA systems (Boyers et al., 1989; Farell et al., 1995). The CASA system is able to determine a series of semen variables, including the number of moving sperm, curvilinear velocity (VCL), linear velocity (VSL), linear coefficient (LIN), straightness coefficient (STR), beat-cross frequency (BCF), etc. The kinematic parameters obtained from the CASA system are thus useful for research purposes (Quintero-Moreno et al., 2003; Holt et al., 2007).

They are described as follows (Holt et al., 2007):
- **Total motility (%)**: percentage of sperm with a total velocity greater than 10μm/s.
- **Progressive motility (%)**: percentage of motile sperm that show straightness greater than 80%.
- **Curvilinear velocity** (VCL) (μm/s): distance travelled by the spermatozoa along its actual path as a function of time.
- **Average path velocity** (VAP) (μm/s): distance travelled by the spermatozoa along its average path as a function of time.
- **Straight-line velocity** (VSL) (μm/s): velocity over the straight-line distance between the beginning and the end of a sperm track.

- **Linearity** (LIN) (%): straight-line distance divided by the incremental deviations along the actual path. It is also the percentage relationship between the curvilinear and straight-line velocity.
- **Beat-cross frequency** (BCF) (Hz.): the frequency with which the undulatory movement of the sperm head crosses the average path in function of time.
- **Straightness** (STR) (%): the percentage relationship between the curvilinear and the average path velocity.
- **Amplitude of lateral head displacement** (ALH) ((μm/s): shift that makes the head in its curvilinear trajectory of the one part and the other part of the trajectory average or linear.

Despite the advantage of the CASA system, there are also problems associated with its use. One major problem with CASA is the high cost of the instrument, which suggests that its use is only in sophisticated laboratories that perform a high number of routine semen evaluations (Verstegen et al., 2002). Also, the instrument settings are relatively subjective and different CASA instruments use different mathematical algorithms (Rabinovitch, 2006). Furthermore, there are also problems encountered as regard the accurate counting of high and low sperm concentrations (Rabinovitch, 2006). This is due to the fact that the kinetic parameters are regulated by the characteristics and composition of the diluents in which sperm is diluted (Rijsselaere et al., 2003). Also, the measurements obtained following sperm counting and statistical counting error may arise depending on the technician who performed the analysis. Therefore, to avoid subjectivity in sperm analysis, the use of CASA requires extensive training and cross validation as regards the technician competencies (Verstegen et al., 2002).

3.3.2. Sperm analysis through flow cytometry

Flow cytometry is now a recognized methodology within animal spermatology, and has moved from being a research tool to become routine in the assessment of animal semen destined to breeding (Hossain et al., 2011). This technique is a process in which fluorescently labeled cells travel individually at high speed through a flow cell, where they are illuminated by one or more lasers. This causes light scattering and fluorescence excitation of markers located on specific parts of the sperm which are dectected by the photodetectors and sent to a

computer program. The computer program presents the information in the form of relative fluorescent intensity units, which are typically displayed as either scatter plots or histograms (Martinez-Pastor et al., 2010). The scatter plots and histograms can be analyzed and various sperm populations can be separated to produce information regarding fluorescence intensity, percentage of sperm population with certain fluorescent characteristics within a total sample (Christensen et al., 2004). In addition to the speed, repeatability, and accuracy, flow cytometry allows close examination of numerous sperm characteristics, including sperm viability/membrane integrity (Garner et al., 1994), mitochondrial function and membrane potential (Garner et al., 1997) chromatin structure (Bochenek et al., 2001), and acrosomal status (Thomas et al., 1997; Nagy et al., 2003).

3.3.3. Analysis of the sperm plasma membrane integrity

The sperm membrane is directly or indirectly related with many sperm functions, warranting the capability of the cell to maintain homeostasis and depict motility as well as the capacity to interact with the environment, including the lining epithelium of the female genital tract or the oocyte–cumulus cell complex (Rodriguez-Martinez, 2003). Therefore, it is regarded as one of the structures most seriously affected by the freezing-thawing process and its analysis is of great importance either for research or commercial purpose (Nagy et al., 2003, 2004). Evaluation of sperm plasma membrane can be done either by conventional staining or by using fluorescents. Conventional stains include eosin/nigrosin, blue tripan, quick green/eosin, eosin/aniline blue or naphthol/erythrosine yellow, and their mode of action is to penétrate ruptured cells thereby changing the color (Rodríguez-Martínez, 2005). Nonetheless, this methodology is usualy compromised due to complications such as the appearance of partially dyed sperms that hinders the interpretation of their status (Watson, 1990). On the other hand, fluorocromes are capable of determining with greater objectivity of the state of membrane integrity. The first fluorescent probes used in the evaluation plasma membrane integrity were fluorescein diacetate (Matyus et al., 1984) and carboxyfluorescein diacetate (Garner et al., 1986). These dyes are non-fluorescent, but converted to fluorescent derivatives by intracellular esterase enzymes which are trapped by the intact plasma membrane thus producing a fluorescing cell. The most commonly used combination of dye for membrane integrity SYBR-14/propidium iodide (PI). In this combined stain, the nuclei of viable spermatozoa fluoresce green, while those with eroded plasmalemma are red, including a

moribund cell subpopulation (green–red). Propidium iodide is normally used as the viability probe of choice as this supravital stain rapidly penetrates non-viable spermatozoa when their plasma membrane is disrupted (Gillan et al., 2005). In addition, it is able to simultaneously evaluate sperm cell viability together with some other attributes, for example; in combination with fluorescently labeled plant lectins for simultaneous assessment of plasma membrane and acrosome integrity (Flajshans et al., 2004). This technique has been demonstrated in many species such as in boar, ram, human (Garner et al., 1995).

Another technique used in the evaluation of plasma membrane via its functionality is known as the hypo-osmotic swelling test (HOST). When sperm cells are exposed to hypo-osmotic conditions, water enters the spermatozoon in an attempt to reach osmotic equilibrium. This inflow of water will increase sperm volume and the plasma membrane will bulge giving minimum surface to volume ratio. The ability of the sperm tail to swell or curl in the presence of a hypo-osmotic solution is a sign that transport of water across the membrane occurs normally, i.e. is a sign of membrane integrity and normal functional activity (Jeyendran et al., 1984). The values obtained in this test are correlated with other sperm quality parameters such as motility, viability or morphology (Jeyendran et al., 1984).

3.3.4. Analysis of mitochondrial functionality

Sperm mitochondria are located in the mid-piece and generate a major part of the ATP required for sperm metabolism, membrane function and motility as well as anaerobic glycolysis in the cytoplasm (Aitken et al., 2004). Furthermore, they are the coordinators of apoptosis mechanisms in a number of cell systems (Hu et al., 2007; Rasola et al., 2007) and are also involved in sperm maturation (Aitken et al., 2004), as well as protection against damage induced by cryopreservation (Peña et al., 2009). Nonetheless, the fluorescent probes mainly used to detect damage to the mitochondria that may occur after ejaculation or as a result of cryopreservation are JC-1 and MitoTracker (Peña and Rodriguez, 2006). The JC-1 probe is transported into the interior of functioning mitochondria and detects the potential of the inner mitochondrial membrane. This emits a green fluorescence when it exists as a monomer but when the concentration of JC-1 inside the active mitochondria increases, the stain forms aggregates which fluoresce orange (Graham, 2001). On the other hand, the MitoTracker probe is absorbed only by active mitochondria and specifically binds to

functional mitochondria membrane lipids (Fraser et al., 2001). Also, the MitoTraker can be incorporated into different protocols of double and triple staining (Sutovsky et al., 1999).

3.3.5. Analysis of acrosomal integrity

The acrosome is a membrane-enclosed structure covering the anterior part of the sperm nucleus and contains hydrolyzing enzymes which is a basic feature of the sperm head of all mammals (Yanagimachi, 1994). Therefore, acrosomal integrity is regarded as a prerequisite for fertilization and essential for sperm penetration of the zona pellucida (Rodrıguez-Martınez, 2006). The most commonly used probes are plant lectins labeled with a fluorescent agent, most commonly PSA or peanut agglutinin (PNA). PSA is a lectin from the pea plant that binds to mannose and galactose moieties of the acrosomal matrix. As PSA can not penetrate an intact acrosomal membrane, only acrosome reacted or damaged spermatozoa will stain (Cross et al., 1986). On the other hand, PNA is believed to display less non-specific binding to other areas of the spermatozoon, and this makes it a choice for most workers (Graham, 2001). Also, it is the lectin of choice when evaluating sperm extended in egg yolk-containing media due to the fact that PSA has a nonspecific binding affinity to egg yolk (Thomas et al., 1997) as well as several nonspecific binding sites on the sperm cell surface (Graham, 2001). Nonetheless, spermatozoa with reacted, damaged, or abnormally formed acrosomes acquire fluorescence after PNA labeling, while intact, normal acrosomes have no fluorescence (Nagy et al., 2003). However, a quadruple staining technique (SYBR-14/ PNA/ PI/ MitoTracker) procedure has been demonstrated and was found out to be an efficient method for evaluating acrosomal integrity together with other sperm characteristics (Tabarez et al., 2017). This combination labels every DNA containing event and the colors of the fluoroprobes fit to the standard filter set of most commercial flow cytometry. This quadruple stain produces the appearance of eight sperm populations: 1) viable cells with intact acrosome and active mitochondria (SYBR14+/ IP-/PE-PNA-/Mitotracker deep red +); 2) viable cells with damaged acrosome and active mitochondria (SYBR14+/IP-/PE-PNA+/Mitotracker +); 3) viable cells with intact acrosome and inactive mitochondria (SYBR14+/IP-/ PE-PNA-/Mitotracker-); 4) viable cells with damaged acrosome and inactive mitochondria (SYBR14+/IP-/PE-PNA+/Mitotracker-); 5) dead cells with intact acrosome and active mitochondria (SYBR14-/IP +/PE-PNA /Mitotracker +); 6) dead cells with damaged acrosome and active mitochondria (SYBR14-/IP+/PE-PNA+/Mitotracker +); 7) dead cells

with intact acrosome and inactive mitochondrial (SYBR14-/IP +/PE-PNA-/Mitotracker-) and 8) dead cells with damaged acrosome and inactive mitochondria (SYBR14-/IP+/PE-PNA+/Mitotracker-).

3.3.6. Oxidative stress analysis

The influence of oxidative stress on sperm adversely affects sperm survival by impairing critical functions such as motility, membrane integrity and ultimately their fertilizing capabilities (Aitken et al., 2014). This is due to increased cellular damage triggered by oxygen and oxygen-derived free radicals known as reactive oxygen species (ROS) (Agarwal et al., 2014). Among the ROS compounds, one is an unpaired electron known peroxides (H_2O_2) which is an oxidizing agent generated as a by-product from the metabolism of oxygen and is a highly reactive molecule (Henkel et al., 2011). However, sperm cells are equipped with antioxidant defence mechanisms that modulate the effect ROS, thus protecting the cells from oxidative damage (Henkel et al., 2011) but when production of ROS exceeds the antioxidant capacity of spermatozoa, the cells will be unable to restore the damage induced by the oxidative stress due to the lack of strong cytoplasmic-enzyme repair systems (Saleh et al., 2002). Therefore, oxidative stress results from the imbalance between ROS production and the intracellular/extracellular antioxidants system used by sperm (Saleh and Agarwal, 2002).

Nonetheless, evaluation of ROS can be estimated by reagents that accumulate intracellularly and become fluorescent upon oxidation. Dichlorofluorescin diacetate (DFCH-DA) has been used to measure intracellular H_2O_2 using the flow cytometry. Evaluation of intracellular ROS using the flow cytometry has been reported in boar (Guthrie and Welch, 2006), human (Mahfouz et al., 2008) and dog (Kim et al., 2010). The mechanism of action of H_2DCFDA consists in penetrating into the cell through its plasma membrane. 2′,7′-dichlorodihydrofluorescein diacetate is a stable cell-permeable nonfluorescent probe that is highly intracellularly de-esterified and becomes fluorescent 2′,7′-dichlorofluorescein (DCF) on oxidation (Guthrie and Welch, 2006). The simultaneous differentiation of viable from non-viable spermatozoa is performed by co-staining the spermatozoa either with PI. This allows for the removal of the non-viable population from the analysis via the cytometry.

3.3.7. Analysis of free-cysteine residues in sperm nucleoproteins

Disulfide bonds are bonds found between proteins as well as between other macromolecules like nucleic acids. These bonds are links between two cysteine amino acids and their thiol groups and are known to stabilise the tertiary and quaternary structure of proteins (Brewer et al., 2003). Therefore, these bonds are the main structures responsible for holding proteins in their respective conformations and, consequently, to maintain the specific protein folding and stability. Furthermore, these bonds represent a bridge between nucleoproteins and DNA, and any alterations to protein-DNA linking may compromise certain processes such as DNA replication (Brewer et al., 2003; Dorigo et al., 2004; Balhorn R., 2007).

Nonetheless, a good stability is considered useful in maintaining sperm with a compact chromatin core thus increasing their hydro dynamicity, motility and the possibility to fertilize the oocyte (Balhorn R., 2007). It is well known, however, that mechanisms such as osmotic oxidative stress induced by osmotic disturbances are able to destroy disulfide bonds in a whole series of cellular structures (Cumming et al., 2004; Yang et al., 2007). This is important, due to the fact that osmotic and oxidative stress are important factors causing alterations in sperm cells such as changes in structural cellular membranes, mitochondria volume and shape as well as rupture of the peri-mitochondrial and head actin network (Hinshaw et al., 1986; Silvia and Gadella, 2006; Correa et al., 2007). Hence, the evaluation of the amount of free cysteine levels in head and tail sperm through a spectrophotometric method may be of great importance. Nonetheless, studies have demonstrated that oxidative stress caused by osmotic disturbances induced changes between nucleoproteins and DNA bonding by affecting the strength of disulfide bonds, ultimately destabilising nucleoprotein integrity (Yang et al., 2007; Flores et al., 2011). Also, changes in the number of intact disulfide bonds have been linked to some processes such as cryoresistance as well as the achievement of feasible *in vitro* capacitation (Yeste et al., 2013). To this regard, it is hypothesized that the variations in free cysteine levels will be an indicative of various changes in the sperm nucleoprotein structure and chromatin structure stability.

3.3.8. Thermal resistance test

The duration of sperm motility and characteristics during a post-thawing incubation period may be an indication in predicting the survivability of sperm within the female reproductive tract and undergo capacitation and fertilize the ovum (Fiser et al., 1991). Therefore, the exposure of frozen-thawed sperm to a thermal resistance test may expose damages during cryopreservation and which may not be revealed immediately after thawing (Aisen et al., 2000). Thus, thermal resistance test may be a useful technique in assessing the fertilizing ability of ram sperm prior to its use for AI.

Chapter IV

RESULTS

Study 1

Influence of melatonin implantation during a non-breeding season to semen donor rams on semen parameters held for 24h at 5ºC.

Abstract

The aim of this study was to evaluate the efficacy of melatonin implantation during a non-breeding season, and to compare with semen donor rams in a breeding season on scrotal circumference (S.C), semen emission time (S.E.T) as well as semen quantitative and qualitative parameters. Furthermore, the effect of chilled liquid storage for up to 24h on semen qualitative parameters was assessed. Therefore, semen was collected (n=8) from ten rams (*Aranesa* and *Xisqueta* breeds) via electro-ejaculation during the non-breeding season; five implanted with Melovine®, while the other five rams remained untreated. Thereafter, semen was collected from six rams (n=8) during the breeding season. Scrotal circumference was measured, semen emission time was recorded and fresh ejaculates were analysed macroscopically and microscopically. All samples were re-suspended in a Tris citric acid-glucose (TCG) based media containing 5% glycerol and 15% powdered egg yolk and chilled at 5ºC for 24h. Analysis on refrigeration time (4 and 24h) on sperm kinematic parameters was evaluated by CASA while viability and morphology where evaluated via eosin-nigrosin staining. Sperm plasma membrane functionality was done by hypoosmotic swelling test while sperm nucleoprotein integrity was done by the determination of overall levels of sperm-head disulfide bonds via spectrophotometric analysis of free-cysteine radicals' levels. The statistical analysis revealed no difference in any sperm parameter between melatonin treated and breeding season group but both groups differed significantly ($P < 0.05$) when compared to untreated/non-breeding semen donor group except on scrotal circumference. However, we observed that despite all parameters been negatively influenced by storage time, melatonin treated group showed better sperm quality parameters when compared to other groups. This study demonstrates that melatonin implantation during the non-breeding season improved almost all qualitative sperm parameters studied during chilled liquid semen storage.

Introduction

In sheep industry, artificial insemination (AI) with fresh or refrigerated extended semen has been successfully used (Karagiannidis et al., 2000), but still raises more concerns due to issues regarding inseminating frozen ram semen (Purdy et al., 2010). Therefore, liquid stored semen seems is the only alternative to frozen-thawed semen due to its less marked detrimental effects on sperm physiology and less expensive to maintain (Evans and Maxwell, 1987; Rodriguez-Gil, 2006). Benefits abound in literatures on holding spermatozoa below

body temperature to various time periods prior to cryopreservation or for AI in various species: stallion (Backman et al., 2004), boar (Guthrie and Welch, 2005), bull (Foote and Kaproth, 2002) and ram (Fiser and Batra, 1984; Purdy, 2006). Furthermore, frozen–thawed ram semen results in stronger damages to spermatozoa when compared to liquid storage (Evans and Maxwell, 1987).

Nonetheless, with the importance and increase in chilled semen utilization, several techniques have been demonstrated regarding short-term or long-term liquid sperm storage or preservation (Moradi et al., 2013; Mata-Campuzano et al., 2015). However, prolonged chilled liquid storage of sperm can be detrimental due to cold shock leading to sperm structural damages, and factors such as season (Salamon and Maxwell, 1995; D'Alessandro and Martemucci, 2003) and type of extender (Kasimanickam et al., 2011) have been incriminated to affect ram sperm during storage. In spite of this, ram semen preservation still remains a critical factor either as frozen-thawed, refrigerated (Allai et al., 2015), or chilled extended prior to cryopreservation (Backman et al., 2004; Purdy et al., 2006).

Although, semen production in ram continues all year round (Chemineau et al., 1992), libido and seasonality are important towards the success of semen collection and quality (Shackell et al., 1977). Nevertheless, the influence of seasonality on reproductive ability of ram has been demonstrated to be regulated by melatonin secretion (Malpaux et al., 1996). With this knowledge, reproductive performances can be manipulated to give a desirable effect, especially in a non-breeding season with subcutaneous melatonin implants (Abecia et al., 2007), thereby reversing seasonality effects on reproduction. Thus, melatonin implants have been reported to increase scrotal size, improve semen quality and enhance reproductive performances outside breeding periods (Palacin et al., 2008; Casao et al., 2008). In this regard, seasonality of spermatogenesis may not be a limiting factor for an all year semen collection in the ram. However, the influence of both seasonality and melatonin treatment on reproductive parameters has strong genetic component and variation between individual rams, breeds and or donor age (Mandiki et al., 1998; Karagiannidis et al., 2000; Garcia, 2014).

Also, a positive influence with melatonin implants has been reported to have enhanced sperm freezability and post-thaw sperm quality in rams (Kaya et al., 2001; Casao et al., 2008). Despite reports on the influence of melatonin implants on semen production,

freezability and post-thawed quality, to the authors' knowledge, there is no information on similar study evaluating sperm characteristics during chilled liquid storage.

Thus, with this background, the aim was to note the effect melatonin implantation on scrotal circumference, time of semen emission and characteristics of fresh ram ejaculates. Furthermore, to evaluate the sperm qualitative characteristics stored at 5^0C and held for 24h in a cryopreservation media during non-breeding, and directly compare with same parameters from a breeding season.

Material and Methods

Chemicals

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) unless otherwise stated. The powdered egg yolk was obtained from NIVE (Nunspeet Holland Eiproducten).

Preparation of extender

The basic extender used in this study was Tris (hydroxymethylaminoethane)- citric acid-glucose (TCG) solution as defined by Salamon and Maxwell (2000), consisting of Tris (0.3 M), citric acid anhydrous (94.7 mM), and D (+)-glucose (27.75 mM). This solution was adjusted to a pH of 7.25 ± 0.05 and the osmolarity to 333.0 ± 2.8 mOsm. Thereafter, glycerol (5% v/v, final concentration), powdered egg yolk (15% v/v) and antibiotics (1000 UI/mL sodium penicillin and 1,0 mg/ mL streptomycin sulfate) were added to the solution having a final pH of 7.0–7.17 and an osmolarity of 1327 ± 234 mOsm (Palomo et al., 2017).

Animals and semen collection

Ten adult males (5 *Aranesa* and 5 *Xisqueta*) aged 5 years were used in this study. All males were housed and managed at Caldes de Montbui in the Institut de Recerca i Tecnologia Agroalimentàries (IRTA, Spain) where they were provided with food and water *ad libitum*. During the non-breeding season, animals were randomly divided into two groups; each male in the first group i.e 3 *Aranesa* and 2 *Xisqueta* rams (MEL group) was treated with three slow releasing implants containing 18 mg of melatonin (Melovine®) while the second untreated group i.e 2 *Aranesa* and 3 *Xisqueta* rams (NB group) acted as control. Semen collection started 55 days after implantation in both groups and a total number of 80 ejaculates (8 ejaculates per male) were collected twice a week during this period. Furthermore, this

experimental procedure was repeated during the breeding season from the same rams but randomly selected (n=6). This was considered as the third group (B group) in this study. Semen collection technique was repeated as previously, and total number of 48 ejaculates was collected during this period (8 ejaculate per male) from the six rams (3 *Aranesa* and 3 *Xisqueta*).

Before semen collection, males were properly restrained in a standing position to reduce movement during the procedure. Scrotal circumference measurement was done by using a flexible measuring tape. The preputial hairs were shaved and the orifice was washed with clean water and dried to prevent contamination of samples. Faeces were removed and the accessory glands were massaged to pre-stimulate the male before electrical stimulation. Electro-ejaculation (EE) was accomplished using a manually controlled electro-ejaculator purchased from Minitube (Barcelona, Spain) attached to a 2.6 cm diameter rectal probe. Procedure was done with the probe facing ventrally inside the rectum with consecutive stimuli of 6-s pulses of similar voltage, and stopped for 6-8 s before the next stimuli. When the seminal discharge becomes cloudy, a graduated collection tube was placed over the penis to collect the semen. Time of semen emission (time from the onset of electrical stimulation to semen emission) was recorded using a stop watch.

Semen evaluation

Analysis of motility characteristics

Immediately after collection, fresh ejaculates were evaluated macroscopically for volume using a transparent graduated tube. Mass motility and individual motility was determined subjectively via a phase contrast microscope (Olympus BH-2, Japan). The mass motility was assessed and scored on a scale of 0-5 (0= no movement and 5= strong wave movement). The percentage of individual motility was subjectively estimated by diluting fresh semen (1:100) in a Tris-glucose-citrate (TGC) solution and viewing an average of 10 fields with a microscope. Also, sperm concentration was calculated using a neubauer chamber.

Sperm kinematics parameters were assessed using the computer-assisted sperm analysis (CASA) system, ISAS® (PROISER SL., Valencia, Spain). The liquid stored extended sperm was diluted (1:100) in TGC, and 10 µl drop of sperm suspension was placed on a warm slide and covered with a cover-slip (24x24mm). Sperm motility 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 analyzed and a minimum of 200 sperm evaluated. The

percentage of total motile sperm cells (TM), progressive motility (PM), curvilinear velocity (VCL, µm/s), linear velocity (VSL, µm/s), mean velocity (VAP, µm/s), linearity coefficient (LIN= [VSL/VCL]x100, %), straightness coefficient (STR= [VSL/VAP]x100, %), lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz) were 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 diluents and materials used in this study that came in contact with semen for analysis were maintained at about 38°C.

Assessment of sperm viability and morphology

The percentage of sperm viability was assessed using eosin-nigrosin stain as described by Evans and Maxwell, (1987). Smears were made from the mixture of 5µl of semen/TGC diluted sample (1:100) and 5µl eosin-nigrosin on a warm slide. The viability was calculated with bright-field microscopy from a total of 400 sperm cells counted from two slides (200 sperm cells per slide), viewed under a light microscope at 1000x magnification with oil immersion. Sperm with partial or complete purple coloration was considered non-viable. Sperm morphology was also determined from the same slide with a total of 400 sperm cells evaluated (Barth and Oko, 1989).

Assessment of sperm plasma membrane functionality

The functionality of sperm plasma membrane was evaluated using the hypo-osmotic swelling test (HOS test) as described by Forouzanfar et al. (2010). To determine the functionality of sperm plasma membrane, 20 µL of sperm was mixed in 180 µL of the HOS test solution (100 mOsm) and incubated at 37°C for 30 min. Thereafter, a smear was made from the mixture of 10 µL from the incubated sample and 10 µL of eosin-nigrosin, and viewing under the phase contrast microscope at 1000x magnification.

Determination of sperm nucleoprotein integrity

The determination of sperm nucleoprotein integrity due to disruption of the overall levels of sperm head disulfide bonds was done through spectrophotometric analysis of free cysteine radical levels. This technique was adopted from a published work on the determination of free cysteine radicals in boar sperm nucleoproteins (Flores et al., 2011). Briefly, aliquot of fresh ejaculates was centrifuged twice at 5,000x g for 10 min, seminal plasma was carefully

removed and the resultant pellet was submerged in liquid nitrogen before stored at -80ºC until analysis. After 24hrs of liquid storage, aliquot of the extended semen was washed with dilution (1:10) in PBS with a centrifugation of 5,000x g for 10 min at 5°C. The supernatant (seminal plasma/extender) was carefully removed and the resultant pellet was submerged in liquid nitrogen before been stored at -80ºC.

On analysis, all samples were resuspended in 400 µl of ice-cold 50mM Tris buffer (pH adjusted at 7.4) containing 150mM NaCl, 1% (v:v) Nonidet, 0.5% (w/v) sodium deoxycolate, 1mM benzamidine, 10µg/mL leupeptin, 0.5mM phenylmethylsulfonyl fluoride (PMSF) and 1mM sodium orthovanadate (Na_2VO_4). Spermatozoa were subsequently homogenised through sonication (Ikasonic U50 sonicator, Ika Labortechnick; Staufen, Germany). The homogenates were centrifuged at $850 \times g$ at 4°C for 20 min, and resultant supernatants and the upper layer of the pellet were removed before resuspended in 500µl of PBS. Thereafter, the levels of free-cysteine residues in sperm nucleoproteins were determined in the samples obtained by using the 2,2'-dithiodipyridine technique (2,2'-dipyridyl disulphide; Sigma; Saint Louis, USA) as described by Brocklehurst et al. (1979). This was determined by adding 10-µL of the resuspension (isolated sperm heads) obtained as described above to 990µL of an aqueous solution of 0.4mM 2,2'-dithiodipyridine. The mixture was incubated at 37°C for 1h. Afterwards, levels of free-cysteine residues in sperm nucleoproteins were determined through spectrophotometric analysis at a wavelength of 343nm. The results obtained were then normalised through a parallel determination of the total protein content of each sample through the Bradford technique (Bradford, 1976), after applying a commercial kit (BioRad; Hercules, CA, USA).

Statistical analysis

All statistical analyses were performed using the statistical package SPSS 20 and results were expressed as the mean ± S.E.M. Percentage data were arc sine transformed and analysed with ANOVA, and differences between means were determined using Duncan multiple tests. This was done to compare the various parameters across three semen donor groups. The independent sample t-test was used to test for sperm parameters during holding time between the two periods. The level of significance was set at ($P<0.05$).

Results

First of all, sperm donor groups were classified into 3 groups: NB (untreated/non-breeding season), MEL (melatonin treated/non-breeding season) and B (breeding season). Mean values and SEM on scrotal circumference, semen emission time and fresh semen quality of the various semen donor groups are shown in Table 1.1. The scrotal circumference form the NB group differed significantly ($P < 0.05$) compared to the other two groups. Also, the mean time (min) required for semen emission was much longer in NB group compared to the MEL and B sperm donor groups. As regards seminal volume, a significantly lower mean value was obtained in NB group than in MEL and B sperm donor group. No significant effect ($P > 0.05$) was observed in individual motility between donor groups but the mass motility differed significantly ($P < 0.05$) between groups with NB sperm donor group having the lowest mean value (Table 1.1). Mean values for sperm concentration and total number of sperm count showed that season had a significant effect, with very low values in NB compared to the other sperm donor groups. However, melatonin treated sperm donor group had the highest mean value amongst all donor groups. Analysis on sperm kinematic parameters within each holding refrigeration time (4h or 24hr) evaluated by CASA is shown in Table 1.2. Generally, kinematic parameters at 4h showed that NB sperm donor group had lower values with significant differences when compared to other sperm donor groups. However, there was no significant effect observed between MEL and B sperm donor groups on sperm kinematic parameters at 4hr. Similarly, total motility (TM) was the lowest in NB amongst the 3 groups, and with a significant difference when compared with MEL and B sperm donor groups. As regards to progressive motility (PM), there was a significant effect of melatonin treatment at 4h compared with NB but no significant difference with B donor sperm group despite having higher values (Table 1.2). After 24h of refrigeration, the sperm kinematic parameters of NB group had the lowest values compared to other sperm donor groups, and differed significantly except for VCL, VSL, VAP LIN and BCF when compared with B sperm donor values. Melatonin treatment caused a significant increase of VCL, VSL and VAP but no differences were seen in LIN, STR, ALH and BCF when compared with B sperm donor group. Furthermore, the enhancing effect of melatonin treatment was observed after 24h on total and progressive motility, and was significantly different when compared to NB and B sperm donor groups.

Table 1.1: Effect of melatonin implantation and season on scrotal circumference, semen emission time and characteristics of fresh semen

Parameters	Non-Breeding Season		Breeding Season
	Non-Melatonin	Melatonin	
Scrotal Circumference (cm)	35.23 ± 0.20^b	34.17 ± 0.47^a	33.75 ± 0.22^a
Semen Emission Time (min)	2.22 ± 0.15^b	1.62 ± 0.15^a	1.53 ± 0.23^a
Volume (mL)	0.43 ± 0.04^a	0.57 ± 0.06^b	0.68 ± 0.05^b
Mass Motility (0-5)	1.57 ± 0.15^a	2.75 ± 0.27^c	2.13 ± 0.16^b
Individual Motility (%)	68.75 ± 3.94	74.17 ± 5.12	67.78 ± 3.55
Sperm Concentration $(x10^6/mL)$	422.08 ± 94.56^a	1797.13 ± 325.93^c	961.07 ± 162.81^b
Total Sperm Account $(x10^6)$	195.41 ± 59.78^a	1098.73 ± 334.76^c	685.05 ± 133.79^b

Data are shown as mean $\pm$ S.E.M. Different superscripts (a, b, c) within the same row indicates a significant difference ($P<0.05$) between group parameters.

It is important to note that all sperm kinematic parameters decreased progressively ($P < 0.05$) between 4h and 24h in NB and B sperm donor groups. However, there was an increase for PM in MEL group ($P < 0.05$) after 24h of refrigeration (Table 1.2). Nonetheless, sperm kinematic parameters from melatonin treated sperm donor group had the highest values amongst other sperm donor groups during various liquid storage times.

The effect of season, melatonin treatment and storage time on the sperm morphology in fresh ejaculate and after 24h refrigeration in a cryopreservation media are shown in Table 1.3. Fresh ejaculates showed no significant difference ($P > 0.05$) in percentage means of sperm viability, normal sperm morphology, midpiece reflex, bent tails and coiled principal piece. However, significant differences were noticed in the total acrosome damage, proximal droplets, distal droplets, dag and detached heads amongst the various sperm donor groups (Table 1.3). After 24h refrigeration, there was no significant difference between MEL and B

sperm donor group on mean percentage of viability and normal sperm but both groups differed significantly (P < 0.05) when compared to NB sperm donor group.

The functionality of sperm plasma membrane, assessed by the HOS test, showed that the mean percentage value for NB sperm donors was significantly different (P < 0.05) when compared to MEL and B sperm donor groups both in fresh ejaculate and after 24h of refrigeration (Table 1.4). However, there was a reduction and significant difference (P < 0.05) in the mean percentage values in all groups when fresh and 24h refrigeration was compared. As regards to the sperm nucleoprotein integrity, a significant higher value (P < 0.05) of free cysteine radicals was seen in B donor group compared to NB and MEL sperm donor groups, both in fresh ejaculate and after 24h of refrigeration (Table 1.4). However, refrigeration time had no effect on any of the sperm donor group.

Discussion

In the present work, the influence of melatonin implantation to semen donors during a non-breeding season generally reversed seasonal effect and maintained sperm parameters during chilled liquid storage. This was achieved by directly comparing sperm parameters from ram sperm donors' in a non-breeding season: melatonin treated (MEL) and untreated (NB), and with breeding season (B). In this study, we observed no effects on mean scrotal circumference between melatonin treated and breeding season sperm donor rams. However, the mean scrotal circumference of NB sperm donor group was higher than other donor groups. An explanation for this discrepancy was due to the fact that two rams from NB group had significant larger testicular sizes compared to the rest of the animals used in this study. However, and on careful observation during the course of this experiment, we observed no marked changes on scrotal circumferences amongst individual donor rams. This is in agreement with similar works in rams (Sookhtehzari et al., 2008; Faigl et al., 2009) but in contrast with Palacin et al. (2008), who observed an increase in scrotal circumference in melatonin implanted rams.

Table 1.2: Effect of melatonin implantation and season evaluated by CASA on sperm motility and holding time after 4 h and 24h in a cryopreservation media at 5^0C

	Holding time (h)	
	4	24
TM (%)		
Non-breeding season	34.33 ± 5.48^a	28.95 ± 5.23^a
Melatonin/non-breeding	60.84 ± 6.75^b	57.71 ± 6.65^b
Breeding season	55.48 ± 4.12^{b2}	34.92 ± 3.98^{a1}
PM (%)		
Non-breeding season	12.64 ± 2.94^a	9.54 ± 2.38^a
Melatonin/non-breeding	22.40 ± 3.81^{b1}	25.29 ± 4.00^{b2}
Breeding season	18.08 ± 2.48^{ab2}	6.37 ± 1.67^{a1}
VCL (μm/s)		
Non-breeding season	39.67 ± 5.69^a	33.20 ± 6.03^a
Melatonin/non-breeding	63.10 ± 6.77^b	63.50 ± 7.15^b
Breeding season	65.26 ± 4.37^{b2}	47.06 ± 3.84^{a1}
VSL (μm/s)		
Non-breeding season	15.68 ± 2.72^a	12.29 ± 2.33^a
Melatonin/non-breeding	24.76 ± 3.13^b	26.66 ± 3.33^b
Breeding season	23.55 ± 1.86^{b2}	12.89 ± 1.34^{a1}
VAP (μm/s)		
Non-breeding season	21.83 ± 3.39^a	16.65 ± 3.09^a
Melatonin/non-breeding	34.59 ± 4.08^b	35.50 ± 4.29^b
Breeding season	34.36 ± 2.43^{b2}	22.70 ± 1.94^{a1}
LIN (%)		
Non-breeding season	22.84 ± 3.46^a	18.20 ± 3.32^a
Melatonin/non-breeding	31.72 ± 3.49^b	33.93 ± 3.59^b
Breeding season	33.10 ± 1.82^{b2}	25.29 ± 2.35^{ab1}
STR (%)		
Non-breeding season	40.93 ± 5.71^a	34.94 ± 6.03^a
Melatonin/non-breeding	56.47 ± 5.64^b	59.34 ± 5.96^b
Breeding season	59.59 ± 3.10^{b2}	46.47 ± 3.64^{b1}
ALH (μm/s)		
Non-breeding season	1.79 ± 0.25^a	1.55 ± 0.28^a
Melatonin/non-breeding	2.78 ± 0.29^b	2.73 ± 0.29^b
Breeding season	3.16 ± 0.22^{b2}	2.41 ± 0.18^{b1}
BCF (Hz)		
Non-breeding season	4.14 ± 0.65^a	3.61 ± 0.66^a
Melatonin/non-breeding	6.27 ± 0.70^b	7.10 ± 0.76^a
Breeding season	6.18 ± 0.39^{b2}	4.60 ± 0.37^{a1}

TM: total motility; PM: progressive motility; VCL: Curvilinear velocity; VSL: Linear velocity; VAP: Mean velocity; LIN: Linear coefficient; STR: Straightness coefficient; ALH: Lateral head displacement; BCF: Frequency of heat displacement. Values are expressed as mean ± S.E.M. Different superscripts (a, b) within the column indicate a significant effect (P < 0.05) amongst donor groups within each holding time. Different numbers (1, 2) in the row for the same parameter indicates a significant difference (P < 0.05) between holding time (4h or 24h).

Table 1.3. Effect of melatonin implantation and season on sperm morphology and plasma membrane integrity for fresh ejaculate and 24h refrigerated semen

Parameter	Holding time (h)	
	F	R24
Viability (%)		
Non-breeding season	50.00 ± 4.80^2	25.78 ± 3.29^{a1}
Melatonin/non-breeding	61.39 ± 5.94^2	49.32 ± 4.67^{b1}
Breeding season	51.93 ± 3.83^2	45.02 ± 3.56^{b1}
Normal morphology (%)		
Non-breeding season	66.71 ± 3.24^2	41.21 ± 3.48^{a1}
Melatonin/non-breeding	73.33 ± 4.04^2	58.16 ± 3.16^{b1}
Breeding season	65.57 ± 3.24^2	52.74 ± 3.06^{b1}
Acrosome damage (%)		
Non-breeding season	7.24 ± 1.07^{ab1}	18.04 ± 2.00^{b2}
Melatonin/non-breeding	8.48 ± 1.77^{b1}	12.74 ± 1.79^{b2}
Breeding season	4.71 ± 0.67^{a1}	7.86 ± 0.66^{a2}
Proximal droplet (%)		
Non-breeding season	0.49 ± 0.31^a	0.70 ± 0.34^a
Melatonin/non-breeding	0.62 ± 0.31^a	1.21 ± 0.30^a
Breeding season	2.10 ± 0.61^b	2.64 ± 0.51^b
Midpiece reflex (%)		
Non-breeding season	1.76 ± 0.63	1.39 ± 0.54^a
Melatonin/non-breeding	0.76 ± 0.28	0.42 ± 0.22^a
Breeding season	1.64 ± 0.36^1	5.98 ± 0.38^{b2}
Dag (%)		
Non-breeding season	1.11 ± 0.32^a	1.52 ± 0.58^{ab}
Melatonin/non-breeding	1.00 ± 0.31^a	0.26 ± 0.21^a
Breeding season	5.31 ± 1.81^b	5.26 ± 1.69^b
Bent tail (%)		
Non-breeding	7.86 ± 1.55^1	17.65 ± 2.06^{b2}
Melatonin/non-breeding	5.71 ± 1.87^1	24.53 ± 2.44^{c2}
Breeding season	6.74 ± 0.69^1	11.43 ± 0.70^{a2}
Distal droplet (%)		
Non-breeding season	0.57 ± 0.30^a	0.78 ± 0.21^a
Melatonin/non-breeding	1.19 ± 0.54^{ab}	0.63 ± 0.21^a
Breeding season	1.76 ± 0.31^b	2.07 ± 0.37^b
Coiled principal piece (%)		
Non-breeding season	0.24 ± 0.19	0.30 ± 0.19^a
Melatonin/non-breeding	0.43 ± 0.14	0.34 ± 0.20^a
Breeding season	0.83 ± 0.40	1.86 ± 0.52^b
Detached head (%)		
Non-breeding season	6.84 ± 1.60^{ab}	8.30 ± 2.46^b
Melatonin/non-breeding	3.24 ± 1.75^a	3.89 ± 0.96^a
Breeding season	10.60 ± 2.48^b	11.12 ± 2.39^a

F: fresh ejaculate; R24: 24h refrigerated semen. Values are expressed as mean ± S.E.M. Different superscripts (a, b, c) within the same column indicate a significant effect (P < 0.05) amongst donor group within holding time. Different numbers (1, 2) in the row for the same parameter indicates a significant difference (P < 0.05) between holding time (4h or 24h).

Table 1.4. Effect of melatonin implantation and season on sperm nucleoprotein integrity for fresh ejaculate and 24h refrigerated semen

	Holding time (h)	
	F	R24
Host (%)		
Non-breeding season	31.33 ± 2.13^{a2}	15.44 ± 3.20^{a1}
Melatonin/non-breeding	51.48 ± 4.09^{b2}	32.83 ± 3.51^{b1}
Breeding season	46.32 ± 3.56^{b2}	29.79 ± 2.06^{b1}
FCR (nmolCys/ug protein)		
Non-breeding season	0.33 ± 0.11^{a}	0.03 ± 0.02^{a}
Melatonin/non-breeding	0.46 ± 0.19^{a}	0.03 ± 0.03^{a}
Breeding season	4.34 ± 0.56^{b}	6.31 ± 0.62^{b}

F: fresh ejaculate; R24: 24h refrigerated semen; HOST: hypo-osmotic swelling test; FCR: free cysteine radical. Values are expressed as mean $\pm$ S.E.M. Different superscripts (a, b) within the same column indicate a significant effect ($P < 0.05$) amongst donor group. Different numbers (1, 2) in the row for the same parameter indicates a significant difference ($P < 0.05$) between holding time (4h or 24h).

The mean time for semen emission using EE was longer in NB donor group than other sperm donor groups. This significance may be as a result of marked seasonal variation regulated by photoperiod which negatively affects semen production especially during a non-breeding season (Hanif and William, 1991: Fitzgerald and Stellflug, 1991). The analysis on various semen parameters from fresh ejaculates varied considerably among the three sperm donor groups. However, from our results, it was evident that melatonin treatment in the non-breeding season generally improved fresh semen quality by reversing the negative effect on sperm production or quality usually observed in a non-breeding season (Casao et al., 2008; Rosa et al., 2012).

As regards to sperm morphology, our results showed that melatonin treatment displayed reproductive seasonality inversely associated with photoperiod thereby presenting results similar to short day length or breeding season. Although, melatonin administration did not exert any beneficial influence on viability, normal morphology and on some sperm defects in fresh ejaculates, it enhanced the viability and normal sperm morphology after 24h of chilled liquid storage. As far as spermatozoa defects were concerned, the most common sperm defects noticed after 24h refrigeration in all groups were acrosome damage, bent tails and dethatched sperm heads. The NB donor group was mostly affected and differed significantly when compared to other sperm donor groups after chilled liquid storage. Furthermore, no

difference on viability and normal sperm morphology after 24h refrigeration between MEL and breeding season sperm donor groups despite higher values in MEL. The general negative effect or reduction regarding chilled liquid storage to sperm parameters tested in the present work was similar to all previous observations (Purdy, 2006; D'Alessandro and Martemucci, 2003). This may be due to oxidative phosphorylation by sperm mitochondria leading to generation of reactive oxygen species (ROS) from prolonged liquid storage and temperature (Allai et al., 2015).

Results on sperm kinematics parameters by CASA showed that MEL group generally displayed better values than other sperm donor groups. The only evident significant changes in all kinematic parameters were observed in NB donor group except for BCF at 24h when compared to other sperm donor groups. Result herein does not agree with a similar work by our group at 4h of chilled liquid storage (Garcia, 2014), who hypothesise that the non-beneficial effect of the melatonin implants may be that these native breeds of *Aranesa* and *Xisqueta* seems less affected by seasons. However, we suggest that the reason for this discrepancy may be age related due to the fact that males used in the previous study were much younger (1-2years) than males in the present study (5 years), and that the beneficial effect of melatonin implants may be evident in older males than younger ones. Nevertheless, further investigation is required. This observation suggests that melatonin reversed the non-breeding season effect on sperm kinematic motility parameters.

The functionality of sperm plasma membrane was negatively affected during chilled liquid storage, showing a significant reduction of percentage values in all sperm donor groups. This is in agreement with result of Allai et al. (2015) who also reported a decline from prolonged liquid storage of sperm in a Tris egg-yolk based extender. However, no difference was observed between MEL and breeding season sperm donors after 24h of refrigeration, and both group differed significantly from NB sperm donor group which had the lowest values.

Regarding sperm nucleoprotein integrity, studies have demonstrated that oxidative stress caused by osmotic disturbances induces changes between nucleoproteins and DNA bonding by affecting the strength of disulfide bonds, ultimately destabilising nucleoprotein integrity (Yang et al., 2007; Flores et al., 2011). To the best of our knowledge, this is the first study towards the evaluation of ram sperm nucleoprotein integrity by assessing disulfide bonds rupture. This was done by determining the sperm head free cysteine radicals as a direct measure in the number of disulfide bonds in nucleoprotein-DNA structure. As indicated

above, our results showed no effect of liquid storage in all donor groups but a significant difference between seasons. Although, no studies on chilled liquid storage effect on ram sperm nucleoprotein structure has been reported, a previous work in boar has demonstrated a marked increase of sperm-head disulfide bonds rupture from oxidative stress due to freezing-thawing (Flores et al., 2011). As regards the significant difference between seasons, we hypothesise that ram sperm chromatin package in relation to nucleoprotein-DNA compactness maybe be correlated with seasonal variation but this area requires further investigation

Nevertheless, from results in this study, we suggest that the adverse effect of seasonality on sperm may have been reversed by the increased plasma melatonin levels in melatonin implanted rams during the non-breeding season (Egerszegi et al., 2014), or the increased antioxidant enzyme activities in the seminal plasma (Reiter et al., 2009; Casao et al., 2010) during storage. Our result is similar to observations made by Kaya et al., (2001), although on cryopreservation, demonstrated improved freezabilty of sperm from melatonin implanted rams. Results herein, and to our knowledge, is the first report on the effect of melatonin implantation during a non-breeding season on semen quality as regards to 24h chilled liquid storage as most similar studies have been focused on frozen-thawed sperm quality (Casao et al., 2008; Egerszegi et al., 2014).

Furthermore, another explanation for the differences on sperm quality between NB and MEL donor groups may be attributed to increase of testosterone levels observed with melatonin implantation to rams in a non-breeding season (Kokolis et al., 2000). Further confounding information demonstrates melatonin action on the pineal gland and, with stimulating effect of pineal hormone on the hypothalamus-pituitary axis, causes an increase of testosterone levels by increased GnRH and LH secretions (Misztal et al., 2002). Also, studies have shown melatonin direct action on testosterone secretion by the testis (Valenti and Giusti, 2002), and its direct involvement in androgen secretion (Frungier et al., 2005). Therefore, the essentiality of testosterone in the testis for spermatogenesis improves male fertility by regulating the latter phase of spermatogenesis, maintaining blood-testis barrier and release of mature sperm (Walker, 2009). Although the level of testosterone was not tested in the present work, we suggest that the influence of this hormone may have reversed the seasonal effect by the melatonin implants during the non-breeding season, giving similar results, and sometimes even better when compared to sperm donors in breeding season.

In conclusion, our results confirm that melatonin implantation to sperm donor rams during the non-breeding season enhanced the ability of sperm cells in almost all the parameters studied during chilled liquid semen storage. Furthermore, the mechanism by which melatonin implants acts on sperm function during liquid storage needs further investigation.

References

Abecia, J.A., Valares, J.A., Forcada, F., Palac´ın, I., Mart´ın, S., Martino, A. (2007). The effect of melatonin on the reproductive performance of three sheep breeds in Spain. Small Rumin. Res. 69, 10–16.

Allai, L., Druart, X., Contell, J., Louanjli, N., Moula, A.B., Badi, A., Essamadi, A., Nasser, B., Amiri, B. (2015). Effect of argan oil on liquid storage of ram semen in Tris orskim milk based extenders. Anim. Reprod. Sci. 160, 57–67.

Backman, T., Bruemmer, J.E., Graham, J.K., Squires, E.L. (2004). Pregnancy rates of mares inseminated with semen cooled for 18 h and then frozen. J. Anim. Sci. 82, 690–694.

Barth, A.D., Oko, R.J. (1989). Abnormal Morphology of Bovine Spermatozoa, first ed. Iowa State University Press, Ames, IA.

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248 – 54.

Brocklehurst, K., Stuchbury, T., Malthouse, J.P.G. (1979). Reactivities of neutral and cationic forms of 2,2-dipyridyl disulphide towards thiolate anions: detection of differences between the active centres of actinidin, papain and ficin by a three-protonic-state reactivity probe. Biochem. J., 183, 233–241.

Casao, A., Mendoza, N., Pérez-Pé, R., Grasa, P., Abecia, J.A., Forcada,F., Cebrián-Pérez, J.A., Mui˜no-Blanco, T. (2010). Melatonin prevents capacitation and apoptotic-like changes of ram spermatozoa and increases fertility rate. J. Pin. Res. 48, 39–46.

Casao, A., Vega, S., Palacín, I., Pérez-Pe, R., Lavi˜na, A., Quintín, F.J., Sevilla, E., Abecia, J.A., Cebrián-Pérez, J.A., Forcada, F., Mui˜no-Blanco, T. (2008). Effects of melatonin implants during non-breeding season on sperm motility and reproductive parameters in Rasa Aragonesa rams. Reprod. Domest. Anim. 45, 425–432.

Chemineau, P., Malpaux, B., Delgadillo, J.A., Guerin, Y., Pavault, J.P., Thimonier, J., Pelleiter, J. (1992). Control of sheep and goat reproduction: use of light and melatonin. Anim. Reprod. Sci. 30, 157-184.

D'Alessandro, A.G., Martemucci, G. (2003). Evaluation of seasonal variations of season freezability in Leccese ram. Anim. Reprod. Sci. 79, 93–102.

Egerszegi, I., Sarlós, P., Rátkya, J., Solti, L., Faigl, V., Kulcsár, M., Cseh, S. (2014). Effect of melatonin treatment on semen parameters and endocrine function in Black Racka rams out of the breeding season. Small Rumin. Res. 116, 192-198.

Evans, G., Maxwell, W.M.C. (1987). Salamon's Artificial Insemination of Sheep and Goats, Butterworths, Sydney, 107–141.

Faigl, V., Keresztes, M., Kulcsár, M., Nagy, S., Keresztes, Z., Amiridis, G.S., Solti, L., Huszenicza, G., Cseh, S. (2009). Testicular function and semen characteristics of awassi rams treated with melatonin out of the breeding season. Acta Veterinaria Hungarica 57, 531–540.

Fiser, P.S., Batra, T.R. (1984). Effect of equilibration time at 5 ∘C and photoperiod on survival of ram spermatozoa frozen in straws. Can. J. Anim. Sci. 64, 777–780.

Fitzgerald, J.A., Stellflug, J.N. (1991). Effect of melatonin on seasonal changesin reproduction of rams. J. Anim. Sci. 69, 264–275.

Flores, E., Ramio-Lluch, I., Bucci, D., Fernandez-Novell, J.M., Pena, A., Rodriguez-Gil, J.E. (2011). Freezing-thawing induces alterations in histone H1-DNA binding and the breaking of protein-DNA disulfide bonds in boar sperm. Theriogenology 76, 1450-1464.

Foote, R.H., Kaproth, M.T. (2002). Large batch freeing of bull semen: effect of time of freezing and fructose on fertility. J. Dairy Sci. 85, 453–456.

Forouzanfar, M., Sharafi, M., Hosseini, S.M., Ostdadhosseini, S., Hajian, M., Hosseini,L., Abedi, P., Nili, N., Rahmani, H.R., Nasr-Esfahani, M.H. (2010). In vitro comparison of egg yolk–based and soybean lecithin–based extenders for cryopreservation of ram semen. Theriogenology 73, 480–487.

Frungieri, M.B., Mayerhofer, A., Zitta, K., Pignataro, O.P., Calandra, R.S., Gonzalez-Calvar, S.I. (2005). Direct effect of melatonin on Syrian hamster testes: melatonin subtype 1a

receptors, inhibition of androgen production, and interaction with the local corticotropin-releasing hormone system. Endocrinology 146, 1541–1552.

Garcia, W.C.V. (2014). Optimización de los protocolos de crioconservación de semen ovino de las razas autóctonas en peligro de extinción xisqueta y aranesa. Ph.D. Thesis.

Guthrie, H.D., Welch, G.R. (2005). Impact of storage prior to cryopreservation on plasma membrane function and fertility of boar sperm. Theriogenology 63, 396–410.

Hanif, M., Williams, H.L. (1991). The effect of melatonin and light treatment on the reproductive performance of yearling sufolk rams. Bri. Vet. J., 147, 49-56.

Karagiannidis, A., Varsakeli, S., Alexopoulos, C., Amarantidis, I. (2000). Seasonal variation in semen characteristics of Chios and Friesian rams in Greece. Small Rumin. Res. 37, 125–130.

Kasimanickam, R., Kasimanickam, V., Tibary, A., Pelzer, K. (2011). Effect of semen extender on sperm parameter of ram during liquid storage at 4ºC. Small Rumin. Res. 99, 208–213.

Kaya, A., Aksoy, M., Baspinar, N., Yildiz, C., Ataman, M.B. (2001). Effect of melatonin implantation to sperm donor rams on post-thaw viability and acrosomal integrity of sperm cells in the breeding and nonbreeding season. Reprod. Domest. Anim. 36, 211–215.

Kokolis, N., Theodosiadou, E., Tsantarliotou, M., Rekkas, C., Goulas, P., Smokovitis, A. (2000). The effect of melatonin implants on blood testosterone and acrosin activity in spermatozoa of the ram. Andrologia 32, 107–114.

Malpaux, B., Viguie, C., Skinner, D.C., Thiery, J.C., Pelletier, J., Chemineau, P. (1996). Seasonal breeding in sheep: mechanism of action of melatonin. Anim. Reprod. Sci. 42, 109–117.

Mandiki, S.N.M., Derycke, G., Bister, J.L., Paquay, R. (1998). Influence of season and age on sexual maturation parameters of Texel, Suffolk and Ile-de-France rams: 1. Testicular size, semen quality and reproductive capacity. Small Rumin. Res. 28, 67–79.

Mata-Campuzano, M., Soleilhavoup, C., Tsikis, G., Martinez-Pastor, F., de Graaf, S.P., Druart, X. (2015). Motility of liquid stored ram spermatozoa is altered by dilution rate independent of seminal plasma concentration. Anim. Reprod. Sci. 162, 31–36.

Misztal, T., Romanowicz, K., Barcikowski, B. (2002). Melatonin – a modulator of the GnRH/LH axis in sheep. Reprod. Biol. 2, 267–275.

Moradi, A.R., Malekinejad, H., Farrokhi-Ardabili, F., Bernousi, I. (2013). Royal jelly improves the sperm parameters of ram semen during liquid storage and serves as an antioxidant source. Small Rumin. Res. 113, 346-352.

Palacín, I., Abecia, J.A., Forcada, F., Casao, A., Cebrian-Perez, J.A., Muino-Blanco, T., Palacios, C., Pontes, J.M. (2008). Effect of exogenous melatonin treatment on out of season ram fertility. Ital. J. Anim. Sci. 7, 199–206.

Palomo, M., García, W., Tabarez, A. (2017). Effect of seminal plasma and butylated hydroxytoluene (BHT) concentration on ram sperm freezability. Small Rumin. Res. 153, 66–70.

Purdy, P.H. (2006). The post-thaw quality of ram sperm held for 0 to 48h at $5°C$ prior to cryopreservation. Anim. Reprod. Sci. 93, 114-123.

Purdy, P.H., Moce, E., Stobart, R., Murdoch, W.J., Moss, G.E., Larson, B., Ramsey, S., Graham, J.K., Blackburn, H.D. (2010). The fertility of ram sperm held for 24h at 5^0C prior to cryopreservation. Anim. Reprod. Sci. 118, 231-235.

Reiter, R.J., Tan, D.X., Manchester, L.C., Paredes, S.D., Mayo, J.C., Sainz, R.M. (2009). Melatonin and reproduction revisited. Biol. Reprod. 81, 445–456.

Rodriguez-Gil, J.E. (2006). Mammalian sperm energy resources management and survival during conservation in refrigeration. Reprod. Domest. Anim. 41, 11–20.

Rosa, H.J.D., Silva, C.C., Bryant, M.J. (2012). The effect of melatonin treatment in rams on seasonal variation of testicular size and semen production parameters. Small Rum. Res. 102, 197–201.

Salamon, S., Maxwell, W.M.C. (1995). Frozen storage of ram semen. II. Causes of low fertility after cervical insemination and methods of improvement. Anim. Reprod. Sci. 38, 1–36.

Salamon, S., Maxwell, W.M.C. (2000). Storage of ram semen. Anim. Reprod. Sci. 62, 77–111.

Shackell, G.H., Kelly, R.W., Allison, A.J. (1977). Seasonal variation in libido of rams. N.Z Journal of Experimental Agric. 5,121-123.

Sookhtehzari, A., Vojgsni, M., Niassari Naslaji, A., Bahonar, A., Gholami, G. (2008). Influence of melatonin treatment on scrotal circumference eand semen parameters in Shall rams on out of season. J. Vet. Res. 65, 297–300.

Valenti, S., Giusti, M. (2002). Melatonin participates in the control of testosterone secretion from rat testis: an overview of our experience. Ann. N. Y. Acad. Sci. 966, 284–289.

Walker, W.H. (2009). Molecular mechanisms of testosterone action in spermatogenesis. Steroids 74, 602–607.

Yang, Y., Song, Y., Loscalzo, J. (2007). Regulation of the protein disulfide proteome by mitochondria in mammalian cells. Proc. Nat. Am. Soc. 104, 10.

Study 2

A Comparison of Semen Collection Methods (electroejaculation vs. artificial vagina) on the *Quality of Pre- and Post-thawed Aranesa* Ram Spermatozoa

Abstract

This study was conducted to evaluate the efficacy of collection methods (electroejaculation (EE) vs. artificial vagina (AV) on the quality *Aranesa* ram sperm towards the establishment of a sperm bank. A total of ten *Aranesa* rams were subjected to the different collection method: 5 males for EE and 5 for AV. Fresh semen quality was evaluated microscopically, pre-freeze and post-thawed sperm motion by CASA while plasma membrane integrity (SYBR-14/PI), acrosome integrity (PE-PNA), mitochondria activity (Mitotracker deep red) and reactive oxygen species (H_2DCFDA) on post-thawed sperm were analysed by flow cytometry. Sperm nucleoprotein integrity assessment was done by the determination of overall levels of sperm-head disulfide bonds via spectrophotometric analysis of free-cysteine radicals' levels. Fresh semen results showed no significant difference between methods except for volume, concentration, total sperm count and functional sperm membrane integrity. There was a significant difference between methods in almost all motion parameters except for LIN and STR in pre-freeze samples but none was observed after thawing. Post-thawed samples evaluated by flow cytometry showed better stability and functionality of sperm obtained via EE than by AV. Results herein supports the inclusion of EE method as a viable alternative and quick method for semen collection especially from a large numbers of untrained rams towards the ongoing conservation program and establishment of *Aranesa* sperm cryobank.

Introduction

Aranesa rams are native Catalonia breed of sheep located in marginal areas across the north of Catalonia, Spain. They are characterised by their meat and lamb production, and are highly adapted to harsh conditions of traditional grazing system even during summer (Esteban 2003). However, in 2003, this breed was listed as endangered with facing extinction by the Servicios de Agricultura, Ganadería and Medio Ambiente del Consejo General del Valle de Arán. Furthermore, a recent survey indicated a sharp decline due to low generational changes in farming thus sparking new interest towards characterization and conservation of this breed (Avellanet et al. 2005). To this regards, an ex situ program was initiated towards the creation of sperm cryobanks (Garcia 2014). This procedure is a useful tool for storage and restoration of valuable genetic resources, and in combination with ART, conservation of endangered breeds may be practical (Holt and Pickard 1999).

Therefore, in creating a sperm cryobank, the use of an effective semen collection and evaluation technique is an integral aspect to achieving this goal. In small ruminants, semen collection via artificial vagina (AV) or electroejaculation (EE) are commonly used methods. Although, AV is the preferred method (Leboeuf et al. 2000), semen collection from large numbers of untrained rams makes sperm bank creation difficult as this technique requires previous training periods (Wulster-Radcliffe et al. 2001). Alternatively, EE could be a useful procedure for semen collection especially from untrained males (Jiménez-Rabadán et al. 2012). Nevertheless, literature abounds on the benefits and limitation on various semen collection methods in some domestic species or breeds (Nikolovski et al. 2013), but to our knowledge, nothing is known about this Catalonia breed. Furthermore, differences in collection methods have been reported to affect sperm quality both in fresh ejaculates (Greyling and Gobbelaar 1983) and post-thawed sperm (Salamon and Maxwell 1995; Watson 1995). Despite the similarities in most sperm cryopreservation protocols used in small ruminants (Parkinson 2009; Shipley et al. 2007), differences may still exist within species due to breed characteristics and response of sperm to cryopreservation protocols (Jiménez-Rabadán et al. 2016). Also, evaluation to semen recovery method on rams' post-thawed sperm quality may not be directly similar due to various handling techniques or freezing protocols (Álvarez et al. 2012).

To this regard, there is no information on the most effective method of semen collection from *Aranesa* rams as regards their semen characteristics, although collection via AV has

been mainly used but on trained males (Garcia 2014). Therefore, for the successful application of ARTs towards conservation, information on semen recovery method on pre- and post-thawed sperm quality is essential. This may lead to a rapid constitution of sperm bank especially from a large number of untrained males within a short period of time towards conservation purposes for this breed. Thus, the present study aims at evaluating the potential effect of two semen collection methods (electroejaculation and artificial vagina) on sperm production and quality of fresh and frozen-thawed *Aranesa* ram spermatozoa.

Materials and Methods

Animals and location

Animal handling was performed in accordance to Spanish Animal Protection Regulation, RD 53/2013, which conforms to European Union Regulation 2010/63. Experimental animals were housed and managed at Caldes de Montbui in the *Institut de Recerca i Tecnologia Agroalimentàries* (IRTA, Spain) where they were provided with food and water *ad libitum*. The freezing protocol and different sperm analysis were conducted in the Autonomous University of Barcelona, Spain, 25 Km away of IRTA facilities.

Reagents and Media

Unless otherwise stated, all reagents were acquired from Sigma Chemical Co. (St. Louis, Mo, USA). Fluorescence probes and analysis kits (LIVE/DEAD® sperm viability kit (L-7011; SYBR-14 and PI) and Mitotracker deep red (M22426) were purchased from Invitrogen (Barcelona, Spain). All fluorochrome solutions were kept in the dark at -20^0C. The freezing extender was prepared in our laboratory using powdered egg yolk as described by Palomo et al. (2017) obtained from NIVE (Nunspeet Holland Eiproducten).

Collection and processing of fresh semen

Semen samples were collected during the breeding season from ten adult (*Aranesa*) rams (5 years old). Prior to collection, males were randomly divided into two groups: EE or AV group comprising of five males per group.

For the electroejaculation, Electrojac IV® stimulator (Minitub Iberica SL, Reus, Spain) was used with a rectal probe of 22 cm long, 2.5 cm in diameter. This procedure was accomplished by first shaving the preputial hairs and cleaning the orifice with clean water to prevent

contamination of samples. Feaces were removed, and the accessory glands were massaged to pre-stimulate the males before electrical stimulation. Afterwards, the probe was inserted facing ventrally inside the rectum and consecutive stimuli of 6-s pulses of similar voltage, and stopped for 6-8 s before the next stimuli. When the seminal discharge became cloudy, a graduated collection tube was placed over the penis to collect the ejaculate.

For collecting semen with an AV, trained rams were brought close to a ewe in the presence of a handler with an artificial vagina. Temperature of water in the lining of the AV ranged from 40 to 44°C during semen collection. Collection was done twice per day on weekly basis from each male using both methods. A total number of six replications (n=6) was carried out regardless the method, totaling 120 ejaculates (12 ejaculates per male). Immediately after collection, ejaculates were maintained in water bath at 37^0C following initial analysis.

Evaluation of ejaculates

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 (warming stage at 37°C). Mass motility was scored on a scale of 0-5 (0: no movement - 5: strong wave movement). Percentage of linear progressive motility was subjectively estimated by diluting fresh ejaculate (1:10) in Tris-glucose-citrate (TGC) solution and assessing 10 fields with a microscope. Fresh sperm viability and morphology was estimated by eosin-nigrosin staining as described by Evans and Maxwell (1987). Sperm plasma membrane functionality was evaluated by hypo-osmotic swelling test (HOST solution: 0.519g fructose, 0.282g sodium citrate diluted in 50ml of milli Q water, 100 mOsm) as described by Forouzanfar et al. (2010). A total number of 400 spermatozoa were counted under the microscope at ×1000 magnification with oil immersion from every staining.

Cryopreservation of semen

The basic extender used in this study was Tris (hydroxymethyl-aminoethane)-citric acid-glucose (TCG) solution as demonstrated by Salamon and Maxwell (2000), consisting of Tris (0.3M), citric acid anhydrous (94.7 mM), and D(+)-glucose (27.75 mM). This solution was adjusted to a pH of 7.25 ± 0.05 and osmolarity 333 ± 2.80 mOsm. Thereafter, glycerol (5% v/v, final concentration) and antibiotics (1000 UI/mL sodium penicillin and 1mg/mL streptomycin sulfate) were added to the solution having a final pH 7.0–7.17 and 1327 ± 234

mOsm. Powdered (PEY) egg yolk was then added to a final concentration of 15% as described by Palomo et al. (2017). First and second ejaculates from each male were mixed together following no differences in semen characteristics. Semen samples were extended in a TCG-based media containing a final concentration of 5% glycerol and 15% powdered egg yolk (PEY). All extended samples were held for equilibration 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 400×10^6 sperm/ml. All straws were kept in liquid nitrogen vapor (5cm above the nitrogen level) for 10 minutes before plunged into liquid nitrogen.

Sperm motion assessment by CASA

Pre-freezing (after 4h cooling) and post-thawed sperm motion parameters were assessed using the computer-assisted sperm analysis (CASA) system ISAS® (PROISER SL., Valencia, Spain). For frozen sperms, two straws from every replicate were thawed by immersion in a water bath at 37 °C for 30 s and the content poured in a dry tube kept at the same temperature for analysis. Analysis by CASA was done by diluting sperm samples (1:10) in PBS, and placing 5µl of sperm suspension on a slide, and covered with a cover-slip. Sperm motion was assessed at 37°C at x200 magnification using a phase contrast microscope (Olympus BH-2, Japan). For each sample, more than three fields per drop were analyzed and a minimum of 200 spermatozoa evaluated.

The percentage of total motility (TM), progressive motility (PM), curvilinear velocity (VCL, µm/s), linear velocity (VSL, µm/s), mean velocity (VAP, µm/s), linearity coefficient (LIN= [VSL/VCL]x100, %), straightness coefficient (STR= [VSL/VAP]x100, %), lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz) were 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).

Flow cytometry analysis

Flow cytometry was performed using the BD FACSCanto platform (BD Biosciences, USA), and data were analyzed by BD FACS DIVA software (BD Biosciences, USA). Plasma

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

This analysis was done 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 sulfate, 50 mM sodium phosphate and 0.05% sodium azide, 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 sperm diluted in PBS to a final concentration of 1×10^6/mL. Samples were mixed and incubated at 37 °C for 10 min and then remixed just before analysis. Stained sperm suspensions were subsequently ran through the 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.

After evaluation, sperm populations taken into consideration for this study were sperm viability (total alive sperm cells), viable cells with intact acrosome and active mitochondria (SYBR14+/ IP-/PE-PNA-/Mitotracker +), viable cells with damaged acrosome and inactive mitochondria (SYBR14$^+$/IP$^-$/PE-PNA$^+$/Mitotracker $^-$), viable cells with damaged acrosome and active mitochondria (SYBR14+/IP-/PE-PNA+/Mitotracker +) and viable cells with damage acrosome and inactive mitochondria (SYBR14$^+$/IP$^-$/PE-PNA$^+$/Mitotracker$^-$). Also, the total acrosome damage was recorded.

Assessment reactive oxygen species (ROS) production)

Reactive oxygen species (ROS) production of all samples were determined using fluorescent probes: 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA). This was done following a procedure modified from Guthrie and Welch (2006), and a simultaneous differentiation of viable from non-viable spermatozoa was performed by co-staining the spermatozoa with PI. Spermatozoa were stained with H$_2$DCFDA at a final concentration of 200 μM and incubated at 37 °C for 10 min in the dark. Thereafter, 1μl of PI at a final concentration of 10 μM was added to the incubation until 60min following analysis. H$_2$DCFDA is a stable cell-permeable non-fluorescent probe that is intracellularly de-

esterified and becomes a highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation (Guthrie & Welch 2006). This DCF fluorescence was collected through FL-1, whereas PI fluorescence was detected through FL-3. The index of ROS production was expressed as the measurement of the geometric mean of green intensity fluorescence unit (GMFI; geometric mean in FL1). Data are expressed as means ± SEM of fluorescence intensity of viable spermatozoa with high intracellular H_2O_2 levels (high DCF^+ fluorescence).

Determination of sperm nucleoprotein integrity

The determination of sperm nucleoprotein integrity due to disruption of the overall levels of sperm head disulfide bonds was done through spectrophotometric analysis of free cysteine radical levels. This technique was adopted from a published work on the determination of free cysteine radicals in boar sperm nucleoproteins (Flores et al. 2011). Briefly, aliquot of frozen thawed semen was washed with dilution (1:10) in PBS with a centrifugation of 5,000x g for 10 min at 5°C. The supernatant (seminal plasma/extender) was carefully removed and the resultant pellet were resuspended in 400 µl of ice-cold 50mM Tris buffer (pH adjusted at 7.4) containing 150mM NaCl, 1% (v:v) Nonidet, 0.5% (w/v) sodium deoxycolate, 1mM benzamidine, 10µg/mL leupeptin, 0.5mM phenylmethylsulfonyl fluoride (PMSF) and 1mM sodium orthovanadate (Na_2VO_4). Spermatozoa were subsequently homogenised through sonication (Ikasonic U50 sonicator, Ika Labortechnick; Staufen, Germany). The homogenates were centrifuged at $850 \times g$ at 4°C for 20 min, and resultant supernatants and the upper layer of the pellet were removed before resuspended in 500µl of PBS.

Thereafter, the levels of free-cysteine residues in sperm nucleoproteins were determined in the samples obtained by using the 2,2'-dithiodipyridine technique (2,2'-dipyridyl disulphide; Sigma; Saint Louis, USA) as described by Brocklehurst et al. (1979). This was determined by adding 10-µL of the resuspension to 990µL of an aqueous solution of 0.4mM 2,2'-dithiodipyridine. The mixture was incubated at 37°C for 1h. Afterwards, levels of free-cysteine residues in sperm nucleoproteins were determined through spectrophotometric analysis at a wavelength of 343nm. The results obtained were then normalised through a parallel determination of total protein content of each sample through the Bradford technique (Bradford, 1976), after applying a commercial kit (BioRad; Hercules, CA, USA).

Statistical analyses

Statistical analysis was performed using the statistical package SPSS 20 and results were expressed as the mean ± S.E.M. The data obtained from the analysis of all sperm parameters were subjected to tests of normality and homoscedasticity Shapiro-Wilk and Levene, respectively. Where necessary, data were arc sine transformed. Comparison between semen collection methods were analyzed using the independent sample t-test, followed by Duncan multiple test. Analyses were considered as statistically significance at $P<0.05$.

Results

Fresh ejaculate parameters

With regards to collection methods, no influence (P > 0.05) was established for both methodologies on mass motility, progressive motility, viability, normal sperm morphology and total acrosome damage. However, a significant effect (P < 0.05) was observed in the volume, sperm concentration and total sperm count, showing ejaculates via AV having the lesser volume but the highest sperm concentration (Table 2.1). Similarly, after the hypo-osmotic test analysis, the membrane functional integrity was significantly higher (P < 0.05) in sperm samples collected by AV compared to those collected by EE.

Pre-freeze and post-thawed sperm motion parameter by CASA

Means of semen parameters for pre-freeze and frozen-thawed samples collected using both methods obtained by CASA are presented in Table 2.2. Pre-freeze analysis showed that collection method had an effect on sperm motion (P < 0.05) for VCL, VSL, VAP, ALH, BCF, TM or PM variables with sperm collected by AV having the lowest values. However, no difference (P > 0.05) was observed for LIN and STR between collection methods. After thawing, significant effects (P < 0.05) were observed in some sperm motility parameters (VCL, VSL, VAP and TM) between pre-freeze and frozen-thawed samples regardless of collection method. There was no difference on PM in AV collected samples but a significant decrease (P < 0.05) was seen in the EE collected samples. As regards to all frozen-thawed samples, no difference (P > 0.05) was observed between collection methods in all sperm motility parameters studied.

Table 2.1. Effect of method of collection on fresh ejaculate characteristics

Parameters	EE	AV
Volume (ml)	1.25 ± 0.12^a	0.79 ± 0.08^b
Mass motility	3.54 ± 0.10	3.58 ± 0.15
Progressive motilty (%)	76.04 ± 1.90	74.79 ± 2.30
Host (%)	55.29 ± 3.27^a	70.50 ± 1.63^b
Viability (%)	76.38 ± 1.61	67.63 ± 5.82
Normal morphology (%)	77.50 ± 2.28	73.42 ± 2.02
Total acrosome damage (%)	6.21 ± 0.87	5.21 ± 0.69
Concentration ($x10^6$)	1722.21 ± 201.23^a	4871.88 ± 268.94^b
Total sperm count ($x10^6$)	2104.92 ± 325.87^a	3374.83 ± 306.33^b

EE: electroejaculation; AV: artificial vagina. Data are shown as mean ± S.E.M. Different superscripts (a, b) across the same row indicates significant difference between collection methods ($P < 0.05$).

Effect of collection methods on post-thawed sperm nucleoprotein integrity

Analysis of free cysteine radicals in sperm nucleoproteins at freeze-thawing showed no significant difference ($P > 0.05$) in the mean levels of cysteine residues in sperm nucleoproteins between sperm collected via EE (0.42 ± 0.14 nmol/µg protien) compared to those by AV (0.51 ± 0.21 nmol/µg protien).

Frozen-thawed sperm quality by flow cytometry

The flow cytometry analysis of thawed sperm samples showed no significant difference ($P > 0.05$) on total viable sperm cells (alive population) regardless of collection method (Table 2.3). Also, within the above population, we observed no differences in viable sperm cells with intact acrosome and active mitochondria on sperm obtained by EE compared to those via AV. However, there was a significant increase ($P < 0.05$) in the percentage of viable cells with damage acrosome and high mitochondria activity in sperm obtained via AV compared to those collected via EE. In this study, we considered this sperm population as "cryocapacitated" sperm. Also, percentage of viable cells with intact acrosome and inactive mitochondria was significantly higher in EE collected samples compared to those collected via AV

Table 2.2. Effect of collection method on pre-freeze and frozen-thawed sperm motility

Parameters	Collection method	Pre-freeze	Post-thaw
TM *(%)*	EE	67.54 ± 2.48^{a1}	28.50 ± 2.89^{b}
	AV	53.67 ± 5.00^{a2}	29.92 ± 2.55^{b}
PM *(%)*	EE	21.71 ± 1.86^{a1}	7.67 ± 1.35^{b}
	AV	9.00 ± 2.16^{2}	8.21 ± 1.18
VCL *(µm/s)*	EE	78.04 ± 3.67^{a1}	89.55 ± 6.41^{b}
	AV	55.03 ± 3.53^{a2}	79.82 ± 4.51^{b}
VSL *(µm/s)*	EE	28.32 ± 1.55^{a1}	35.95 ± 3.10^{b}
	AV	16.99 ± 1.39^{a2}	36.82 ± 3.05^{b}
VAP *(µm/s)*	EE	42.23 ± 2.05^{a1}	53.98 ± 4.04^{b}
	AV	27.59 ± 1.93^{a2}	52.88 ± 3.36^{b}
LIN *(%)*	EE	36.76 ± 1.24	39.37 ± 1.48
	AV	35.18 ± 2.04	40.80 ± 1.71
STR *(%)*	EE	64.94 ± 1.03	61.82 ± 1.72
	AV	61.65 ± 1.89	59.80 ± 1.84
ALH *(µm/s)*	EE	3.62 ± 0.13^{1}	3.72 ± 0.21
	AV	2.84 ± 0.15^{2}	3.22 ± 0.13
BCF *(Hz)*	EE	7.11 ± 0.33^{1}	6.65 ± 0.42
	AV	5.65 ± 0.36^{2}	5.49 ± 0.32

EE: electroejaculation; AV: artificial vagina; TM: total motility; PM: progressive motility; VCL: Curvilinear velocity; VSL: Linear velocity; VAP: Mean velocity; LIN: Linear coefficient; STR: Straightness coefficient; ALH: Lateral head displacement; BCF: Beat cross frequency. Data are shown as mean ± S.E.M. Different numbers (1, 2) in the column for the same parameter indicates significant difference ($P < 0.05$) between collection methods. Different superscripts (a, b) across the same row (pre-freeze and post-thawed) indicate a significant difference ($P < 0.05$).

Viable cells with damaged acrosome and inactive mitochondria detected by this technique showed very low values with no significant differences between collection methods. Furthermore, the total acrosome damage was significantly higher in AV collected sperm compared to those via EE (Table 2.3). No significant difference was observed on reactive oxygen species (ROS) production values between collection methods.

Table 2.3. Effect of collection on frozen-thawed plasma and acrosomal membrane integrity, mitochondrial activity and production of reactive oxygen species analysed by flow cytometry

Parameters	EE	AV
Viable sperm cells (%)	24.95 ± 1.41	25.39 ± 1.30
Viable, intact acrosome and active mitochondria (%)	21.65 ± 2.92	23.31 ± 0.51
Viable, damaged acrosome and active mitochondria (%)	0.10 ± 0.03[a]	0.39 ± 0.37[b]
Viable, damaged acrosome and inactive Mitochondria	0.22 ± 0.01	0.51 ± 0.21
Total Acrosome Damage (%)	31.10 ± 4.62[a]	43.50 ± 3.06[b]
ROS (GMFI)	7.35 ± 1.37	8.70 ± 1.36

EE: electroejaculation; AV: artificial vagina; ROS: reactive oxygen species; GMFI: geometric mean fluorescence intensity of viable spermatozoa with high intracellular H_2O_2 levels. Data are shown as mean $\pm$ S.E.M. Different superscripts (a, b) across the same row indicates significant difference ($P < 0.05$).

Discussion

Various works have suggested that the effect of collection methods on pre and or post-thawed sperm quality varies in small ruminants thus showing inconsistent results depending on species and or breeds (Marco-Jiménez et al. 2008). In this study, we directly compared the effect of two semen collection methods (EE vs AV) on fresh and frozen-thawed sperm quality of endangered *Aranesa* ram using an established cryopreservation protocol in our laboratory (Palomo et al. 2017). Despite the usefulness of EE, especially for untrained males, this method has been reported to alter semen characteristic and may show different responses of sperm to cryopreservation (Jiménez-Rabadán et al. 2012; Ledesma et al. 2014).

In the present study, ejaculates via EE had larger seminal volume with lesser sperm concentration than those via AV (Jiménez-Rabadán et al. 2016), suggesting that the larger volume is as a result of increased seminal plasma (SP) from the accessory sex glands due to electric stimulation (Marco-Jiménez et al. 2008). Also, total sperm count and functional integrity of sperm membrane obtained via EE were lower compared to AV. This result agrees

with Jiménez-Rabadán et al. (2016) but in contrast with Marco-Jiménez et al. (2005) who found similar values. Furthermore, there was no effect on sperm viability, morphology and total acrosome damage between the methods (Marco-Jiménez et al. 2008; Jiménez-Rabadán et al. 2012).

Regarding sperm kinematics parameters by CASA at pre-freezing, it was interesting to observe that motility parameters was significantly higher in EE samples than those obtained by AV except for LIN and STR motion parameters. This discrepancy may be due to changes in protein composition in SP obtained by EE (Marco-Jiménez et al. 2008). Further confounding this explanation may be the greater amount of energy and buffer per spermatozoa in the seminal plasma available for maintenance of metabolism during chilled liquid storage (Leahy et al. 2010; Mata-Campuzano et al. 2015). In any case, most studies are inconclusive as to the best possible sperm concentration and SP since no direct comparison has been made, especially for chilled liquid storage (Maxwell and Salamon 1993; Mata-Campuzano et al. 2015). Whether the changes in protein composition or increased amount of SP obtained via EE improved chilled stored sperm kinematics compared to those by AV, further investigation is required. At post-thaw, an effect was observed on VCL, VSL, VAP and TM motility parameters regardless of collection method. This result agrees with other findings as regards the negative effect of freeze-thawing on sperm motility (Jiménez-Rabadán et al. 2012; Ledesma et al. 2014). On the other hand, our study agrees with a similar works showing no difference between collection methods on post-thawed sperm motility parameters (Marco-Jiménez et al. 2008; Jiménez-Rabadán et al. 2016).

Flow cytometry analysis of thawed sperm samples on viability and ROS agrees with similar study reported by Álvarez et al. (2012), but contradicts Marco-Jiménez et al. (2005) who reported better values in sperm collected by EE than via AV. We suggest that this discrepancy may be due to the cryopreservation protocol used making comparison hardly conclusive (Jiménez-Rabadán et al. 2016). Nonetheless, cryopreservation process has been reported to induce changes in sperm function such as loss of membrane permeability, sperm structural damage, reduced enzyme activity, viability and motility (Thundathil et al. 1999). In this study, we observed no effect on viable cells with intact acrosome and active mitochondria as well as in damaged acrosome and inactive mitochondria after thawing, irrespective of collection method. Our results contradict similar work by Jiménez-Rabadán et al. 2016, indicating that ram sperm collected via AV were more resistance to cold shock with

better sperm quality than via EE. However, Marco-Jiménez et al. (2005) reported a higher sperm cryoresistance quality in EE obtained samples than via AV. An explanation to this may be due the complexity and variance of SP amongst species or breeds (Leahy et al. 2010). Consequently, semen sample obtained by EE had lower percentage of viable cells with damaged acrosome and active mitochondria (cryo-capacitation) and total acrosome damage than those via AV. This agrees with Marco-Jiménez et al. (2005). Our result in the present study suggests that EE frozen-thawed sperm seem to display better stability during cryopreservation. This stability may be due to SP proteins induced by electrical stimulation of accessory glands which may have helped to suppress capacitation or acrosome reaction, and to an extent, preserved the functional characteristics of spermatozoa during freezing (Barrios et al. 2000; El-Rajj Ghaoui et al. 2007). It is worthy to note the high incidence of sperm with low mitochondrial activity in sperm collected via EE. A possible explanation may be due to the electrical stimulation, resulting to the forceful out flow of spermatozoa with various qualitative characteristics unlike AV which resembles natural mating (Wulster-Radcliffe et al. 2001).

Regarding sperm nucleoprotein integrity, studies have demonstrated that oxidative stress caused by osmotic disturbances induces changes between nucleoproteins and DNA bonding by affecting the strength of disulfide bonds, ultimately destabilising nucleoprotein integrity (Yang et al., 2007; Flores et al., 2011). Furthermore, the weakening and disruption of disulfide bonds due to oxidative stress during cryopreservation and freeze-thawing may cause damages to nucleoprotein structure (Oganesyan et al., 2007). However, to the best of our knowledge, this is the first study towards evaluating ram sperm nucleoprotein integrity by assessing disulfide bonds rupture. This preliminary study was done to determine sperm head free cysteine radicals as a direct measure in the number of disrupted disulfide bonds in nucleoprotein-DNA structure between sperm from EE and AV. Our results showed no significant difference between methods on post thawed sperm. We hypothesise that ram sperm chromatin package in relation to nucleoprotein-DNA compactness between EE and AV may be similar. However, we can only speculate about the physiological significance of this structure but further investigation is needed in order to evaluate the strength of the hypothesis, as well as the consequences during the entire cryopreservation process.

In conclusion, results in our study supports the inclusion of using EE technique in the creation of *Aranesa* sperm bank as a swift methodology towards cryopreservation without the

need for training period. This will allow for a quicker conservation program for this breed with less detrimental effect on cryopreserved sperm quality. Furthermore, future work is required to aim at improving cryopreservation protocols or extenders towards the successful conservation program on semen collected by EE from *Aranesa* rams.

References

Álvarez M, Tamayo-Canul J, Martínez-Rodríguez C, López-Urue˜na E, Gomes-Alves S, Anel L, Martínez-Pastor F, De Paz P, 2012: Specificity of the extender used for freezing ram sperm depends of the spermatozoa source (ejaculate, electroejaculate or epididymis). Anim Reprod Sci **132**, 145–154.

Avellanet R, Arangune-Méndez J, Jordana J, 2005: La raza ovina Xisqueta en España: Caracterizacion estructural de las explotaciones. Boletín de información sobre Recursos Genéticos Animales. **37**, 21-30.

Barrios B, Fernández-Juan M, Mui˜no-Blanco T, Cebrián-Pérez J, 2005: Inmuno-cytochemical localization and biochemical characterization of two seminal plasma proteins that project ram spermatozoa. J Androl **26**, 539–549.

Barrios B, Perez-Pe R, Gallego M, Tato A, Osada J, Muino-Blanco T, 2000: Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol Reprod **63**, 1531–1537.

El-Rajj Ghaoui R, Thomson P, Leahy T, Evans G, Maxwell W, 2007: Autologous whole ram seminal plasma and its vesicle free fraction improve motility characteristics and membrane status but not in vivo fertility of frozen thawed spermatozoa. Reprod Domest Anim **42**, 541–549

Esteban C, 2003: Razas ganaderas españolas ovinas. Feagas, editor. MAPA.

Forouzanfar M, Sharafi M, Hosseini S, Ostdadhosseini S, Hajian M, Hosseini L, Abedi P, Nili N, Rahmani H, Nasr-Esfahani M, 2010: In vitro comparison of egg yolk–based and soybean lecithin–based extenders for cryopreservation of ram semen. Theriogenology **73**, 480–487.

García V, 2014: Optimización de los protocolos de crioconservación de semenovino de las razas autóctonas en peligro de extinción Xisqueta y Aranesa.Universidad Autónoma de Barcelona, Barcelona, Tesis doctoral.

Greyling J, Gobbelaar J, 1983: Seasonal variation in semen quality of Boer and Angora goat rams using different collection techniques. S Afr J Anim Sci **13**, 250–252.

Guthrie H, Welch G, 2005: Impact of storage prior to cryopreservation on plasma membrane function and fertility of boar sperm. Theriogenology **63**, 396–410.

Holt W, 2000: Basic aspects of frozen storage of semen. Anim Reprod Sci **62**, 3–22.

Holt W, Pickard R, 1999: Role of reproductive technologies and genetic resource banks in animal conservation. Rev Reprod **4**, 143–150.

Jeyendran R, Van der Ven H, Perez-Pelaez M, Crabo B, Zaneveld L, 1984: Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil **70**, 219–228.

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

Jiménez-Rabadán P, Ramón M, García-Álvarez O, Maroto-Morales A, Del Olmo E, Pérez-Guzmán M, Bisbal A, Fernández-Santos M, Garde J, Soler A, 2012: Effect of semen collection method (artificial vagina vs. electroejaculation), extender and centrifugation on post-thaw sperm quality of Blanca-Celtibérica buck ejaculates. Anim Reprod Sci **132**, 88–95.

Jiménez-Rabadán P, Soler A, Ramón M, García-Álvarez O, Maroto-Morales A, Iniesta-Cuerda M, Fernández-Santos M, Montoro V, Pérez-Guzmán M, Garde J, 2016: Influence of semen collection method on sperm cryoresistance in small ruminants. Anim Reprod Sci **167**, 103–108.

Leahy T, de Graaf S, 2012: Seminal plasma and its effect on ruminant spermatozoa during processing. Reprod Domest Anim **47**, 207–213.

Leahy T, Marti J, Evans G, Maxwell W, 2010: Seasonal variation in the protective effect of seminal plasma on frozen-thawed ram spermatozoa. Anim Reprod Sci **119**, 147–153.

Leboeuf B, Restall B, Salamon S, 2000: Production and storage of goat semen for artificial insemination. Anim Reprod Sci **62**, 113–141.

Ledesma A, Manes J, Cesari A, Alberio R, Hozbor F, 2014: Electroejaculation increases low molecular weight proteins in seminal plasma modifying sperm quality in Corriedale rams. Reprod Domest Anim **49**, 324–332.

Marco-Jiménez F, Puchades S, Gadea J, Vicente J, Viudes-de-Castro M, 2005: Effect of semen collection method on pre- and post-thaw Guirra ram spermatozoa. Theriogenology **64**, 1756–1765.

Marco-Jiménez F, Vicente J, Viudes-de-Castro M, 2008: Seminal plasma composition from ejaculates collected by artificial vagina and electroejaculation in Guirra ram. Reprod Domest Anim **43**, 403–408.

Mata-Campuzano M, Soleilhavoup C, Tsikis G, Martinez-Pastor F, de Graaf S, Druart X, 2015: Motility of liquid stored ram spermatozoa is altered by dilution rate independent of seminal plasma concentration. Anim Reprod Sci **162**, 31–36.

Maxwell W, Salamon S, 1993: Liquid storage of ram semen: a review. Reprod Fertil Dev **5**, 613–638.

Nikolovski M, Atanasov B, Dovenska M, Petkov V, Dovenski T, 2013: Variations in semen quality parameters of ovchepolian pramenka rams according to the method of collection and the meteorological season. Mac Vet Rev **35**, 79-86.

Oganesyan N, Ankoudinova I, Kim S, Kimb R, 2007: Effect of osmotic stress and heat shock in recombinant protein overexpression and crystallization. Prot Expr Purif **52**, 280–285.

Palomo M, García W, Tabarez A, 2017: Effect of seminal plasma and butylated hydroxytoluene (BHT) concentration on ram sperm freezability. Small Rum Res **153**, 66–70.

Parkinson T, 2009: Veterinary Reproduction and Obstetrics, 9th ed. Saunders Elsevier, London.

Salamon S, Maxwell W, 1995: Frozen storage of ram semen I. Processing, freezing, thawing and fertility after cervical insemination. Anim Reprod Sci **37**, 185–249.

Salamon S, Maxwell W, 2000: Storage of ram semen. Anim Reprod Sci **62**, 77–111.

Shipley C, Buckrell B, Mylne M, 2007: Current Therapy Large Animal Theriogenology, 2nd ed. Saunders-Elsevier, St. Louis.

Tabarez A, García W, Palomo, M, 2017: Effect of the type of egg yolk, removal of seminal plasma and donor age on buck sperm cryopreservation. Small Rum Res **149**, 91–98.

Thundathil J, Gil J, Januskauskas A, Larsson B, Soderquist L, Mapletoft R, 1999: Relationship between the proportions of capacitated spermatozoa present in frozen–thawed bull semen and fertility with artificial insemination. Int J Androl **22**, 366–73.

Watson P, 1995: Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev **7**, 871–891.

Watson P, 2000: The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci **60**, 481–492.

Wulster-Radcliffe M, Wiliams M, Stellflug J, Lewis G, 2001: Technical note: artificial vagina vs. a vaginal collection vial for collecting semen from rams. J Anim Sci **79**, 2964-2967.

Study 3

Effect of semen washing on thawed ram sperm subjected to a four hour post-thawing thermal evaluation test

Research paper

Effect of semen washing on thawed ram spermatozoa subjected to a four hour post-thawing thermal evaluation test

CrossMark

Uchebuchi Ike Osuagwuh*, María Jesús Palomo

Department of Animal Medicine and Surgery, Universitat Autònoma de Barcelona, Spain

ARTICLE INFO

ABSTRACT

This study was conducted to evaluate sperm motility and qualitative characteristics of ram spermatozoa cryopreserved in the presence or absence of seminal plasma under controlled condition and subjected to a 4 h post-thawing thermal evaluation test. The aim being that exposing these frozen-thawed sperm to thermal resistance test will provide useful information on their survivability in the female genital tract towards fertilizing the ovum. Briefly, ejaculates from 5 males (5 years) were collected by artificial vagina, and split into two aliquots. One aliquot was diluted (1:5) in Tris-citric acid-glucose (TCG) solution and washed twice by centrifugation at $600 \times g$ for 10 min, while the other aliquot was kept unwashed. Thereafter, washed and unwashed sperm were extended in a TCG-based media containing 5% glycerol and 15% powdered egg yolk (PEY) and frozen. Frozen-thawed sperm were thawed and incubated at 37 °C for 4 h. Sperm motility characteristics was evaluated by CASA while plasma membrane integrity (SYBR-14/PI), acrosome integrity (PE-PNA), mitochondria activity (Mitotracker deep red) and reactive oxygen species (H_2DCFDA) were analysed by flow cytometry. Sperm nucleoprotein integrity assessment was done by the determination of overall levels of sperm-head disulfide bonds via spectrophotometric analysis of free-cysteine radicals' levels. Unwashed samples generally showed better results than washed samples as regards to sperm motility characteristics irrespective of incubation. The post-thawing incubation had a significant ($P < 0.05$) effect on acrosome integrity and mitochondria functionality irrespective of sperm treatment. This study demonstrates that the presence of seminal plasma prior to cryopreservation was beneficial in maintaining post thawed sperm motility, and as such, could be useful for ex situ ram sperm preservation towards its use for artificial insemination.

1. Introduction

The use of semen cryopreservation in assisted reproductive techniques (ART) has become an indispensable tool for genetic improvement towards breeding management in sheep industry (Anel et al., 2006). However, cryopreservation adversely impairs ram semen quality by altering sperm function (Ledesma et al., 2016). This may be due to spermatozoa sensitivity to extreme temperature changes during cooling, freezing and thawing, invariably leading to a reduction in motility, viability, mitochondria membrane integrity, increase in reactive oxygen species production and chromatin damage (Bailey et al., 2003; Said et al., 2010). These alterations may affect the post thawing fertilizing capabilities as a result of reduced survivability of the frozen thawed spermatozoa (Salamon and Maxwell, 1995; Ledesma et al., 2016).

Although, laparoscopic artificial insemination (AI) has yielded acceptable results (Naqvi et al., 2001), its use is usually sophisticated and cost effective, making it routinely inappropriate due to the extensive nature of the sheep industry. Nevertheless, there is still a wide variability with cryopreserved semen resulting to frequently low fertility rates (Bag et al., 2004). This is notably observed when cervical AI is performed, leaving substantive period of time for spermatozoa to migrate to the oviduct thus impairing sperm survivability (Druart et al., 2009). Therefore, maintaining the sperm quality of frozen-thawed spermatozoa for an extended period of time is of utmost importance.

However, several researchers on different species have reported that the longevity of frozen thawed spermatozoa during *in vitro* incubation is low due to the various alterations in sperm membrane during cryopreservation (Gadea et al., 2005; Domınguez-Rebolledo et al., 2009; Mata-Campuzano et al., 2012). Therefore, many attempts to improve the quality of frozen-thawed semen in various species have been reported; one such method is the removal of seminal plasma from the ejaculate by centrifugation (semen washing) prior to freezing (Peterson et al., 2007; Webb and Dean, 2009). Despite studies reporting the beneficial importance of seminal plasma prior to cryopreservation increased sperm resistance to cold shock and reducing cryocapacitation

(Barrios et al., 2005; Fernandez-Juan et al., 2006), others have also demonstrated that removing seminal plasma was necessary to increase motility, membrane integrity and fertility after freeze-thawing (Moore et al., 2005; Maxwell et al., 2007).

Nevertheless, the necessity to eliminate the seminal plasma or not in ram sperm cryopreservation is still an ongoing issue. One reason for this discrepancy may be due to the complexity and variance of seminal plasma amongst species, breeds, donor age and or seasons (Mandiki et al., 1998; Muiño-Blanco et al., 2008; Leahy et al., 2010). However, a recent work in our laboratory has demonstrated that the removal of seminal plasma (washing by centrifugation) improved the sperm kinematics and survival of frozen-thawed ram sperm (Garcia, 2014). Despite the main aim- being to establish a semen bank for these endangered native Catalonian eed of rams (*Aranesa* and *Xisqueta*) under controlled conditions, information on sperm motility and qualitative characteristics during post-thawing incubation regarding the various sperm treatments prior to freezing is of preliminary importance. Therefore, exposing these frozen-thawed sperm to a thermal resistance test may be a useful technique in assessing their probability for survival in the female genital tract and capacitation status towards fertilizing the ovum (Aisen et al., 2000).

Therefore, in this present work, we tested the resilience of frozen-thawed sperm from washed and unwashed sperm samples during a 4-h incubation period. The objective was to determine the sperm physiological changes and qualitative characteristics by assessing their kinematic parameters, viability, nucleoprotein integrity, plasma membrane integrity, acrosome membrane integrity, mitochondrial function and reactive oxygen species (ROS) production at post thaw, and after a 4-h post-thawing incubation period. We aim at providing some basic information as regards to ram sperm processes for cryopreservation prior to testing its usefulness for further application.

2. Materials and methods

2.1. Reagents and media

Unless otherwise stated, all reagents were acquired from Sigma Chemical Co. (St. Louis, Mo, USA). Fluorescence probes and analysis kits (LIVE/DEAD* sperm viability kit (L-7011; SYBR-14 and PI) and Mitotracker deep red (M22426) were purchased from Invitrogen (Barcelona, Spain). All fluorochrome solutions were kept in the dark at −20 °C. The freezing extender was prepared in our laboratory using powdered egg yolk as described by Palomo et al. (2017) obtained from NIVE (Nunspeet Holland Eiproducten). Incubation media used in this study was a modified phosphate buffer solution (PBS) (supplemented with 36 µg/mL pyruvate and 0.5 mg/mL BSA) with osmolarity of 280–300 mOsm and pH 7.3-7.4.

2.2. Animals and sample collection

Five adult male rams of *Aranesa* and *Xisqueta* breeds (aged 5 years) were used in this study. They were maintained under an intensive management system in Institute farm (IRTA, Caldes de Montbui, Barcelona, Spain) where semen collection and initial assessment was carried out. Semen was collected twice weekly with two ejaculates per collection from each male via artificial vagina with a total of six replications. Prior to collection, the preputial hairs were shaved and the orifice was washed with clean water and dried to prevent contamination of samples. All ejaculates were collected during the breeding season and maintained in water bath at 37 °C following initial analysis. Volume, mass motility, progressive motility and concentration were accessed immediately after collection through conventional methods. Only ejaculates of good quality were used and frozen (mass motility: ≥4; sperm concentration: ≥2500 × 10⁶ sperm/ml: normal sperm morphology ≥70%).

2.3. Cryopreservation of semen

The basic extender used in this study was Tris (hydroxymethyl-aminoethane)-citric acid-glucose (TCG) solution as defined by Salamon and Maxwell (2000), consisting of Tris (0.3 M), citric acid anhydrous (94.7 mM), and D(+)-glucose (27.75 mM). This solution was adjusted to a pH of 7.25 ± 0.05 and osmolarity 333 ± 2.80 mOsm. Thereafter, glycerol (5% v/v, final concentration) and antibiotics (1000 UI/mL sodium penicillin and 1 mg/mL streptomycin sulfate) were added to the solution having a final pH 7.0–7.17 and 1327 ± 234 mOsm. Powdered (PEY) egg yolk was then added to a final concentration of 15% as described by Palomo et al. (2017). The first and second ejaculates from each male were mixed together following no differences in the semen characteristics and divided into two equal samples. One sample was washed twice by dilution (1:5) in TGC by centrifugation at 600 X g for 10 min to remove the seminal plasma while the other sample was kept unwashed. Thereafter, the supernatant was carefully removed from the washed samples leaving the resulting sediment. All samples (washed and unwashed) was then diluted with the freezing extender (1:4). All extended samples were held for equilibration for 4 h at 5 °C. After cooling for 4 h (pre-freezing), the extended semen was packed into 0.25 ml plastic straws (IMV Technologies, L'Aigle, Cedex, France) at a final concentration of 400 × 10⁶ sperm/ml and sealed with polyvinyl alcohol. All straws were kept in liquid nitrogen vapor (5 cm above the nitrogen level) for 10 min before been plunged into the liquid nitrogen and stored.

2.4. Sperm thermal evaluation test

Thermal stress test was carried out to verify the longevity of thawed semen samples. Two straws per treatment (wash and unwashed) per replicate were thawed and diluted in the modified PBS to 40 × 10⁶ sperm/ml. Thereafter, samples were incubated at 37 °C in a dry bath in the dark. Evaluation on all post thawed parameters in this study was assessed immediately after thawing (0 h) and at 4 h of post-thawing incubation.

2.5. Semen evaluation

2.5.1. Motility assessment by CASA

Motion characteristics of pre-freezing (after 4 h of refrigeration) and post-thawed (0 h and 4 h of incubation) samples were assessed using the computer-assisted sperm analysis (CASA) system ISAS* (PROISER SL., Valencia, Spain). On analysis, all sperm samples were diluted (1:10) in PBS, and 5 µl drop of sperm suspension was placed on a slide and covered with a cover-slip (24 × 24 mm). Sperm motility was assessed at 37 °C at x200 magnification 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 spermatozoa evaluated.

The percentage of total motility (TM), progressive motility (PM), curvilinear velocity (VCL, µm/s), linear velocity (VSL, µm/s), mean velocity (VAP, µm/s), linearity coefficient (LIN = [VSL/VCL]x100, %), straightness coefficient (STR = [VSL/VAP]x100, %), lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz) were 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 micras²), slow sperm (10–45 micras/s), average sperm (45–75 micras/s), rapid sperm (> 75 micras/s), progressive (80% STR). All diluents and materials used for sperm analysis were maintained at 37 °C.

2.5.2. Flow cytometry analysis

Flow cytometry was performed using the BD FACSCanto platform (BD Biosciences, USA), and data were analysed by BD FACS DIVA software (BD Biosciences, USA). Plasma and acrosomal membrane integrity as well as mitochondrial function were evaluated by flow

cytometry using a quadruple-staining technique as described by Tabarez et al. (2017). The following fluorescent probes were used: LIVE/DEAD* sperm viability kit (SYBR-14 and Propidium Iodide (PI; L-7011, Invitrogen SA)) for plasma membrane integrity (viability), Phycoerythrin-Peanut Agglutinin (PE-PNA; GTX01509, Antibody, Bcn SL) for acrosome integrity and Mitotracker deep red (M22426, SA Invitrogen) for the detection of mitochondrial activity.

This analysis was done 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 sulfate, 50 mM sodium phosphate and 0.05% sodium azide, 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 sperm diluted in PBS to a final concentration of 1×10^6 sperm/ml. Samples were mixed and incubated at 37 °C for 10 min and then remixed just before analysis. Stained sperm suspensions were subsequently ran through the 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.

After evaluation, sperm populations taken into consideration for this study were sperm viability (total alive sperm cells), viable cells with intact acrosome and active mitochondria (SYBR14 +/IP-/PE-PNA-/Mitotracker +), viable cells with damaged acrosome and active mitochondria (SYBR14 +/IP-/PE-PNA+/Mitotracker +) and viable cells with intact acrosome and inactive mitochondria ($SYBR14^+/IP^-/PE-PNA^+/Mitotracker^-$). Also, the total acrosome damage was recorded.

Reactive oxygen species (ROS) production of all samples were determined using fluorescent probes: 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). This was done following a procedure modified from Guthrie and Welch (2006), and a simultaneous differentiation of viable from non-viable spermatozoa was performed by co-staining the spermatozoa with PI. Spermatozoa was stained with H_2DCFDA at a final concentration of 200 μM and incubated at 37 °C for 10 min in the dark. Thereafter, 1 μl of PI at a final concentration of 10 μM was added to the incubated mixture and the incubation continued until 60 min before analysis. H_2DCFDA is a stable cell-permeable non-fluorescent probe that is intracellularly de-esterified and becomes a highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation (Guthrie and Welch, 2006). The index of ROS production was expressed as the measurement of the geometric mean of green intensity fluorescence unit (GMFI; geometric mean in FL1). Data are expressed as means ± SEM of fluorescence intensity of viable spermatozoa with high intracellular H_2O_2 levels (high DCF^+ fluorescence).

2.5.3. Determination of sperm head free cysteine radicals

The determination of sperm nucleoprotein integrity due to disruption of the overall levels of sperm head disulfide bonds was done through spectrophotometric analysis of free cysteine radical levels. This technique was adopted from a published work on the determination of free cysteine radicals in boar sperm nucleoproteins (Flores et al., 2011). Briefly, aliquot of frozen thawed semen was washed with dilution (1:10) in PBS with a centrifugation of $5,000 \times g$ for 10 min at 5 °C. The supernatant (seminal plasma/extender) was carefully removed and the resultant pellet were resuspended in 400 μl of ice-cold 50 mM Tris

buffer (pH adjusted at 7.4) containing 150 mM NaCl, 1% (v:v) Nonidet, 0.5% (w/v) sodium deoxycolate, 1 mM benzamidine, 10 μg/mL leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium orthovanadate (Na_2VO_4). Spermatozoa were subsequently homogenised through sonication (Ikasonic U50 sonicator, Ika Labortechnick; Staufen, Germany). The homogenates were centrifuged at $850 \times g$ at 4 °C for 20 min, and resultant supernatants and the upper layer of the pellet were removed before resuspended in 500 μl of PBS.

Thereafter, the levels of free-cysteine residues in sperm nucleoproteins were determined in the samples obtained by using the 2,2′-dithiodipyridine technique (2,2′-dipyridyl disulphide; Sigma; Saint Louis, USA) as described by Brocklehurst et al. (1979). This was determined by adding 10-μL of the resuspension to 990 μl of an aqueous solution of 0.4 mM 2,2′-dithiodipyridine. The mixture was incubated at 37 °C for 1 h. Afterwards, levels of free-cysteine residues in sperm nucleoproteins were determined through spectrophotometric analysis at a wavelength of 343 nm. The results obtained were then normalised through a parallel determination of total protein content of each sample through the Bradford technique (Bradford, 1976), after applying a commercial kit (BioRad; Hercules, CA, USA).

2.6. Statistical analysis

All statistical analyses were performed using the statistical package SPSS 20 and results were expressed as the mean ± S.E.M. The experiment was replicated six times (n = 6). Percentage data were arc sine transformed and analysed with ANOVA, and differences between means were determined using Duncan multiple tests. This was done to compare the various parameters across the incubation period. The independent sample t test was used to test for sperm parameters between treatments. The level of significance was set at $P < 0.05$.

3. Results

Once established that there was no significant difference ($P > 0.05$) between males used in this study, and that days of collection or replicates had no effect on the variables, the mean percentage of the five rams on each day was established and analysed. Results for CASA analysis are summarised in Tables 1 and 2. Results for pre-freeze samples on motion kinematics parameters are shown in Table 1. This was evaluated as regards having a clearer understanding on sperm motion parameters just before freezing between the treatments. There were no significant differences (P > 0.05) on almost all the sperm motion kinematic parameters studied except on TM, in which unwashed sperm was significantly higher (P < 0.05) than washed sperm (Table 1). Also, results immediately after thawing showed no significant difference between treatment on mean percentage values of TM, PM, VCL, ALH and BCF parameters (Table 2). However, the effect of treatment was observed (P < 0.05) on mean values of VSL, VAP, LIN and STR with unwashed sperm having higher values between treatments (Table 2). After 4 h pot-thawing incubation, no effect was seen on PM, VCL, VAP, STR, ALH and BCF mean values, regardless of treatment. Also, no effect was seen for unwashed sperm on TM, and LIN for washed sperm after 4 h. However, there was a significant decrease (P < 0.05) on LIN for unwashed sperm and also TM for washed sperm after 4 h

Table 1
Effect of the presence or absence of seminal plasma on pre-freeze sperm motion parameters.

Treatment	TM (%)	PM (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	ALH (μm/s)	BCF (Hz)
UW	55.63 ± 4.77[b]	25.53 ± 2.19	56.97 ± 3.20	17.54 ± 1.40	28.53 ± 1.84	34.30 ± 1.90	60.95 ± 2.00	2.90 ± 0.13	5.80 ± 0.30
W	46.67 ± 3.55[a]	21.33 ± 1.78	52.84 ± 1.41	15.56 ± 0.58	27.34 ± 0.83	33.56 ± 1.15	58.00 ± 1.87	2.71 ± 0.08	5.92 ± 0.30

UW: unwashed sperm; W: washed sperm; TM: total motility; PM: progressive motility; VCL: Curvilinear velocity; VSL: Linear velocity; VAP: Mean velocity; LIN: Linear coefficient; STR: Straightness coefficient; ALH: Lateral head displacement; BCF: Beat cross frequency. Data are shown as mean ± S.E.M. Different letters in the column indicates significant difference (P < 0.05) between treatments.

Table 2
Effect of 4 h thermal evaluation test on post-thawed sperm motion parameters frozen in the presence or absence of seminal plasma.

Time	TM (%)	PM (%)	VCL ($\mu m/s$)	VSL ($\mu m/s$)	VAP ($\mu m/s$)	LIN (%)	STR (%)	ALH ($\mu m/s$)	BCF (Hz)
0 h									
UW	40.33 ± 2.23[b]	19.53 ± 1.16	81.63 ± 4.04	33.70 ± 2.89[b]	54.0 ± 3.10[b]	42.12 ± 1.51[b]	60.71 ± 1.59[b]	3.28 ± 0.13	5.38 ± 0.29
W	36.30 ± 1.72[b]	16.33 ± 1.58	71.62 ± 3.72	21.17 ± 1.50[a]	35.78 ± 1.83[a]	30.28 ± 1.84[a]	53.93 ± 2.33[a]	3.23 ± 0.14	5.59 ± 0.28
4 h									
UW	38.70 ± 2.08[b]	16.67 ± 0.93	84.31 ± 4.31	30.17 ± 2.49[b]	49.33 ± 3.48[b]	35.39 ± 1.55[a]	56.23 ± 1.28[ab]	3.67 ± 0.15	6.12 ± 0.33
W	30.87 ± 2.61[a]	14.73 ± 1.24	74.33 ± 3.91	22.29 ± 1.76[a]	39.37 ± 2.35[a]	33.18 ± 2.27[a]	51.87 ± 2.59[a]	3.33 ± 0.17	5.67 ± 0.38

UW: unwashed sperm; W: washed sperm; TM: total motility; PM: progressive motility; VCL: Curvilinear velocity; VSL: Linear velocity; VAP: Mean velocity; LIN: Linear coefficient; STR: Straightness coefficient; ALH: Lateral head displacement; BCF: Beat cross frequency. Data are shown as mean ± S.E.M. Different letters in the column indicates significant difference ($P < 0.05$) between treatments or incubation time.

Table 3
Effect of 4 h thermal evaluation test on post-thawed sperm physiology frozen in the presence or absence of seminal plasma.

Time	Viability (%)	% Viable cells with intact acrosome and active mitochondria	% Viable cells with intact acrosome and inactive mitochondia	% Viable cells with damage acrosome and active mitochondria	Total acrosome damage (%)	ROS (GMFI)
0 h						
UW	25.83 ± 1.25	24.01 ± 0.38[b]	0.52 ± 0.17[b]	0.49 ± 0.21	36.30 ± 2.65	18.69 ± 7.03
W	24.47 ± 1.19	22.66 ± 0.49[b]	0.38 ± 0.12[b]	0.56 ± 0.39	34.97 ± 1.98	14.06 ± 1.66
4 h						
UW	23.13 ± 0.74	16.47 ± 3.70[a]	5.93 ± 0.72[a]	0.40 ± 0.42	39.10 ± 2.12	11.20 ± 0.75
W	21.83 ± 0.86	16.35 ± 3.31[a]	4.51 ± 0.30[a]	0.39 ± 0.32	38.17 ± 1.95	10.78 ± 1.41

UW: unwashed sperm; W: washed sperm. ROS: reactive oxygen species; GMFI: geometric mean fluorescence intensity of viable spermatozoa with high intracellular H_2O_2 levels. Data are shown as mean ± S.E.M. Different letters in the column indicates significant difference ($P < 0.05$) between treatments or incubation time.

post-thawing incubation (Table 2).

Flow cytometry results on the interaction between treatments and after 4 h post-thawing incubation on viable sperm populations are presented in Table 3. While viability and mitochondria status being good indicators of the overall integrity of sperm, there was no significant difference ($P > 0.05$) on the viability and other sperm populations immediately after thawing, irrespective of treatment. Similarly, the variable related to oxidative stress (ROS) showed no difference between treatments. However, after 4 h of incubation, we observed a significant decrease ($P < 0.05$) on the mean percentage of viable cells with intact acrosome and active mitochondria irrespective of treatments (Table 3). Also, there was a significant negative effect of post-thawing incubation on mitochondria functionality regardless of treatment. Nonetheless, mean percentage of viable cells with damage acrosome and active mitochondria, total acrosome damage and ROS were not affected either by treatment or incubation time.

Analysis of free cysteine radicals in sperm nucleoproteins at freeze-thawing showed no significant difference ($P > 0.05$) in the mean levels of cysteine residues in sperm nucleoproteins between treatments (unwashed and washed sperm) immediately after post thawing (0.47 ± 0.05 and 0.49 ± 0.03 nmol/µg protien) and or at 4 h post-thawing incubation (0.59 ± 0.07 and 0.56 ± 0.04 nmol/µg protien). Furthermore, incubation time had no significant effect the sperm cysteine residues regardless of treatment.

4. Discussion

The importance of establishing genetic resource banks of frozen semen from rare breeds is steadily being acknowledged, particularly for its potential towards environmental changes and utilization for AI (Holt, 1997; Yoshida, 2000). Nevertheless, preserving sperm quality after thawing for a longer period especially when subjected to thermo-evaluation test at 37 °C is a crucial aspect for fertilization processes (Bag et al., 2004). In the present study, an attempt was made to understand whether the presence or absence of seminal plasma prior to cryopreservation would exert any changes on post-thaw sperm quality and after 4 h of post-thawing incubation.

Results on the analysis of motility kinematics on pre-freeze sperm

showed that the removal of seminal plasma by centrifugation (washed sperm) significantly reduced the percentage of total motility (TM%) of sperm compared with sperm with seminal plasma (unwashed sperm). However, other pre-freeze sperm kinematics parameters were not affected between the treatments. This result agrees with other reports on pre-freeze sperm motility in goats (Leboeuf et al., 2000), in stallion (Hoogewijs et al., 2010) and in ram (Ahmed et al., 2016). Nonetheless, analysis on post thawed samples showed that unwashed sperm generally had higher values in all sperm kinematic motility with significant differences between treatments on VSL, VAP, LIN and STR motility parameters. Our results agree with other works on the importance of seminal plasma during ram sperm cryopreservation (Barrios et al., 2000; 2005), suggesting that seminal plasma seems to increase sperm resistance to cold shock thereby preserving motility and viability of ram thawed sperm. However, a previous study by our group demonstrated that sperm washing by centrifugation provided a beneficial effect on ram sperm kinematic parameters after thawing Garcia, (2014). A possible explanation for this discrepancy may be age related due to the fact that males used in the previous study were much younger (1–2 years) than males in the present study (5 years), and seems that the beneficial effect of seminal plasma may be evident in older males than younger ones (Mandiki et al., 1998; Maxwell et al., 1999). Furthermore, analysis of sperm kinematics after 4 h post-thawing incubation showed that sperm motility was negatively affected irrespective of the treatments. Despite this general negative effect on sperm motility, unwashed sperm had better kinematic parameters than washed sperm with significant differences on TM, VSL and VAP motility parameters. We suggest that the presence of seminal plasma proteins in the unwashed sperm may have had a beneficial effect in maintaining sperm motility (Turner, 2005). However, in the present study regarding post-thawing incubation effect on kinematic parameters, a significant decrease on sperm linearity (LIN) for unwashed sperm and total motility (TM) for washed sperm was observed. This observation agrees with similar results on motility during post-thawing incubation for ram sperm (Bag et al., 2004; Joshi et al., 2005), suggesting a gradual decrease in the ability of sperm to generate ATP from mitochondrial respiration due to mitochondrial ageing (Viswanath and Shannon, 1997). Another explanation could be as a result of dead cells releasing toxic effect of

membrane-bound aromatic amino acid oxidase enzyme thereby causing a decline in the motility of spermatozoa (Shannon and Curson, 1972).

Cryopreservation process has been reported to induce various forms of changes in sperm function such as loss of membrane permeability, sperm structural damage, reduced enzyme activity, viability and motility (Thundathil et al., 1999). Furthermore, the deterioration of sperm viability during post-thaw incubation as a result of freezing and thawing depends on factors such as the nature and components used as diluents during freezing (Fiser and Fairfull, 1989), or thawing (Holt and North, 1994). Therefore, the degree of damage during freezing and thawing will be exposed during incubation and ultimately determines the life span of frozen-thawed spermatozoa in the female genital tract. In this study, we observed no effect between treatments either immediately after post-thaw or after 4 h of incubation on the various sperm populations analysed by flow cytometry. The only changes observed was the adverse effect of the thermal incubation showing an increase on viable cells with intact acrosome and inactive mitochondria, irrespective of treatments. This finding is in agreement with other researchers demonstrating a decline of acrosome integrity and mitochondria functionality from prolonged incubation (Bag et al., 2004; Joshi et al., 2005). We suggest that the decrease in mitochondrial functionality further elucidated the decline in sperm motility during post thawing incubation due to mitochondrial ageing.

Regarding sperm nucleoprotein integrity, studies have demonstrated that oxidative stress caused by osmotic disturbances induces changes between nucleoproteins and DNA bonding by affecting the strength of disulfide bonds, ultimately destabilising nucleoprotein integrity (Yang et al., 2007; Flores et al., 2011). Furthermore, the weakening and disruption of disulfide bonds due to oxidative stress during cryopreservation, freeze-thawing or post-thawing incubation has been reported to cause damages in boar sperm nucleoprotein structure (Oganesyan et al., 2007; Yeste et al., 2013). Therefore, to the best of our knowledge, the inclusion of this methodology is the first attempt to determine the sperm head free cysteine radicals as a direct measure in the number of disulfide bonds in nucleoprotein-DNA structure ram sperm. Our results showed no significant difference between treatments regardless of post thawing sperm incubation time. However, although the determination on the level of free cystiene radicals was not done on fresh ejaculates prior to cryopreservation, we hypothesis that ram sperm nucleoprotein may be cryotolerant as regards its resistance to alteration in their structure during freeze-thawing as observed from their low values in our result. Nonetheless, we can only speculate about the significance of this structure but further investigation is needed in order to elucidate the strength of this hypothesis, as well as the consequences during the entire cryopreservation to freezing-thawing processes and thermal stress for ram sperm.

To this regard and in the present study, it seems that the presence of seminal plasma prior to freezing was only beneficial in maintaining sperm motility parameters, and such parameters have been considered as an important factor in correlation with fertility (Budworth et al., 1988; Aitken, 1990). Also, the lack of difference observed on mitochondrial activity between sperm treatments may indicate that the effect of seminal plasma is primarily on glycolysis metabolism of sperm flagellum and not on mitochondrial oxidative phosphorylation (Rovegno et al., 2013). Nevertheless, more studies are needed to determine the relationship between mitochondrial status and seminal plasma; however, the presence or absence of seminal plasma prior to sperm preservation is still a controversial topic (Muiño-Blanco et al., 2008). Therefore, from our result herein, further research should be aimed at evaluating the importance of adding seminal plasma to frozen-thawed sperm, especially to improve sperm kinematics towards fertilizing the ovum for cervical AI.

In conclusion, our result confirms that the presence of seminal plasma prior to cryopreservation assisted in the maintenance of sperm motility at post thaw and after a 4 h post-thawing incubation period. Following this pattern, further investigation is needed to assess the fertilizing capability through artificial insemination.

Conflict of interest

The authors declare no conflict of interest.

References

Ahmed, T., Islam, R., Lone, F.A., Malik, A.A., 2016. Effect of washing on the post-thaw quality of cryopreserved ram epididymal spermatozoa. Vet. World 9 (5), 519–523.

Aisen, E.G., Alvarez, H.L., Venturino, A., Grade, J.J., 2000. Effect of trehalose and EDTA on cryoprotective action of ram semen diluents. Theriogenology 53, 1053–1061.

Aitken, R.J., 1990. Motility parameters and fertility. In: Gagnon, C. (Ed.), Control of Sperm Motility: Biological and Clinical Aspects. CRS Press, Boca Raton p. 285–302.

Anel, L., Alvarez, M., Martinez-Pastor, F., Garcia-Macias, V., Anel, E., de Paz, P., 2006. Improvement strategies in ovine artificial insemination. Reprod. Domest. Anim. 41 (2), 30–42.

Bag, S., Joshi, A., Naqvi, S.M.K., Mittal, J.P., 2004. Effect of post-thaw incubation on sperm kinematics and acrosomal integrity of ram spermatozoa cryopreserved in medium-sized French straws. Theriogenology 62, 415–424.

Bailey, J., Morrier, A., Cormier, N., 2003. Semen cryopreservation: successes and persistent problems in farm species. Can. J. Anim. Sci. 83, 393–401.

Barrios, B., Pérez-Pé, R., Gallego, M., Tato, A., Osada, J., Muino-Blanco, T., Cebrián-Pérez, J.A., 2000. Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol. Reprod. 77, 1531–1537.

Barrios, B., Fernandez-Juan, M., Muiño-Blanco, T., Cebrián-Pérez, J.A., 2005. Immunocytochemical localization and biochemical characterization of two seminal plasma proteins that protect ram spermatozoa against cold shock. J. Androl. 26, 539–549.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brocklehurst, K., Stuchbury, T., Malthouse, J.P.G., 1979. Reactivities of neutral and cationic forms of 2, 2-dipyridyl disulphide towards thiolate anions: detection of differences between the active centres of actinidin, papain and ficin by a three-protonic-state reactivity probe. Biochem. J. 183, 233–241.

Budworth, P.R., Amann, R.P., Chapman, P.L., 1988. Relationships between computerized measurements of motion of frozen-thawed bull spermatozoa and fertility. J. Androl. 9, 41–53.

Dominguez-Rebolledo, A.E., Fernandez- Santos, M.R., Garcia-Alvarez, O., Maroto-Morales, A., Garde, J.J., Martinez-Pastor, F., 2009. Washing increases the susceptibility to exogenous oxidative stress in red deer spermatozoa. Theriogenology 72, 1073–1084.

Druart, X., Cognie, J., Baril, G., Clement, F., Dacheux, J.L., Gatti, J.L., 2009. In vivo imaging of in situ motility of fresh and liquid stored ram spermatozoa in the ewe genital tract. Reproduction 138, 45–53.

Fernandez-Juan, M., Gallego, M., Barrios, B., Osada, J., Cebrián-Pérez, J.A., Muiño-Blanco, T., 2006. Immunohistochemical localization of sperm-preserving proteins in the ram reproductive tract. J. Androl. 27, 588–595.

Fiser, P.S., Fairfull, R.W., 1989. The effect of glycerol-related osmotic changes on post-thaw motility and acrosomal integrity of ram spermatozoa. Cryobiology 26, 64–69.

Flores, E., Ramio-Lluch, I, Bucci, D., Fernandez-Novell, J., Pena, A., Rodriguez-Gil, J.E., 2011. Freezing-thawing induces alterations in histone H1-DNA binding and the breaking of protein-DNA disulfide bonds in boar sperm. Theriogenology 76, 1450–1464.

Gadea, J., Gumbao, D., Matas, C., Romar, R., 2005. Supplementation of the thawing media with reduced glutathione improves function and the in vitro fertilizing ability of boar spermatozoa after cryopreservation. J. Androl. 26, 749–756.

Garcia, W.C.V., 2014. Optimización de los protocolos de crioconservación de semen ovino de las razas autóctonas en peligro de extinción xisqueta y aranesa. (Ph.D. Thesis).

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

Holt, W.V., North, R.D., 1994. Effects of temperature and restoration of osmotic equilibrium during thawing on the induction of plasma membrane damage in cryopreserved ram spermatozoa. Biol. Reprod. 51, 414–424.

Holt, W.V., 1997. Alternative strategies for the long-term preservation of spermatozoa. Reprod. Fertil. Dev. 9, 309–319.

Hoogewijs, M., Rijsselaere, T., De Vliegher, S., Vanhaesebrouck, E., De Schauwer, C., Govaere, J., Thys, M., Hoflack, G., Soom, A.V., de Kruif, A., 2010. Influence of different centrifugation protocols on equine semen preservation. Theriogenology 74 (1), 118–126.

Joshi, A., Bag, S., Naqvi, S.M.K., Sharma, R.C., Mittal, J.P., 2005. Effect of post-thawing incubation on sperm motility and acrosomal integrity of cryopreserved Garole ram

semen. Small Rumin. Res. 56, 231–238.

Leahy, T., Marti, J., Evans, G., Maxwell, W., 2010. Seasonal variation in the protective effect of seminal plasma on frozen-thawed ram spermatozoa. Anim. Reprod. Sci. 119, 147–153.

Leboeuf, B., Restall, B., Salamon, S., 2000. Production and storage of goat semen for artificial insemination. Anim. Reprod Sci. 62 (1–3), 113–141.

Ledesma, A., Fernández-Alegre, E., Cano, A., Hozbor, F., Martínez-Pastor, F., Cesari, A., 2016. Seminal plasma proteins interacting with sperm surfacerevert capacitation indicators in frozen-thawed ram sperm. Anim. Reprod. Sci. 173, 35–41.

Mandiki, S.N.M., Derycke, G., Bister, J.L., Paquay, R., 1998. Influence of season and age on sexual maturation parameters of Texel, Suffolk and Ile-de-France rams: 1. Testicular size, semen quality and reproductive capacity. Small Rumin. Res. 28, 67–79.

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

Maxwell, W.M., Evans, G., Mortimer, S.T., Gillan, L., Gellatly, E.S., McPhie, C.A., 1999. Normal fertility in ewes after cervical insemination with frozen–thawed spermatozoa supplemented with seminal plasma. Reprod. Fertil. Dev. 11, 123–126.

Maxwell, W.M.C., de Graaf, S.P., El-Hajj Ghaoui, R, Evans, G., 2007. Seminal plasma effects on sperm handling and fertility. In: Juengel, J.I., Murria, J.F., Smith, M.F. (Eds.), Reproduction in Domestic Ruminants VI. Nottingham University Press, Nottingham UK, pp. 13–38.

Moore, A.I., Squires, E.L., Graham, J.K., 2005. Effect of seminal plasma on the cryopreservation of equine spermatozoa. Theriogenology 63, 2372–2381.

Muiño-Blanco, T., Pérez-Pé, R., Cebrián- Pérez, J.A., 2008. Seminal plasma proteins and sperm resistance to stress. Reprod. Domest. Anim. 4, 18–31.

Naqvi, S.M.K., Joshi, A., Das, G.K., Mittal, J.P., 2001. Development and application of ovine reproductive technologies: an Indian experience. Small Rumin. Res. 39, 199–208.

Oganesyan, N., Ankoudinova, I., Kim, S., Kimb, R., 2007. Effect of osmotic stress and heat shock in recombinant protein over expression and crystallization. Prot. Expr. Purif. 52, 280–285.

Palomo, M.J., García, W., Tabarez, A., 2017. Effect of seminal plasma and butylated hydroxytoluene (BHT) concentration on ram sperm freezability. Small Rum. Res. 153, 66–70.

Peterson, K., Kappen, M.A.P., Ursem, P.J.F., Nothling, J.O., Colenbrander, B., Gadella, B.M., 2007. Microscopic and flow cytometric semen assessment of Dutch AI-bucks: effect of semen processing procedures and their correlation to fertility. Theriogenology 67 (4), 863–871.

Rovegno, M., Beringui, W., Monteiro, A., Mota, C., Visintin, J.A., Ortiz, M.E., 2013. Assessment of post-thawed ram sperm viability after incubation with seminal plasma. Cell Tissue Bank. 14, 333–339.

Said, T., Gaglani, A., Agarwal, A., 2010. Implication of apoptosis in sperm cryoinjury. Reprod. Bio. Med. 21, 456–462.

Salamon, S., Maxwell, W.M.C., 1995. Frozen storage of ram semen. I. Processing, freezing, thawing and fertility after cervical insemination. Anim. Reprod. Sci. 37, 185–249.

Salamon, S., Maxwell, W.M.C., 2000. Storage of ram semen. Anim. Reprod. Sci. 62, 77–111.

Shannon, P., Curson, B., 1972. Toxic effect and action of dead sperm on diluted bovine semen. J. Dairy Sci. 55, 614–620.

Tabarez, A., García, W., Palomo, M.J., 2017. Effect of the type of egg yolk, removal of seminal plasma and donor age on buck sperm cryopreservation. Small Rum. Res. 149, 91–98.

Thundathil, J., Gil, J., Januskauskas, A., Larsson, B., Soderquist, L., Mapletoft, R., 1999. Relationship between the proportions of capacitated spermatozoa present in fro-zen?thawed bull semen and fertility with artificial insemination. Int. J. Androl. 22, 366–373.

Turner, R.M., 2005. Moving to the beat: a review of mammalian sperm motility regulation. Reprod. Fertil. Dev. 18, 25–38.

Viswanath, R., Shannon, P., 1997. Do sperm cells age?: A review of the physiological change in sperm during storage at ambient temperature. Reprod. Fertil. Dev. 9, 321–331.

Webb, G.W., Dean, M.M., 2009. Effect of centrifugation technique on post-storage characteristics of stallion spermatozoa. J. Equine Vet. Sci. 29 (9), 675–680.

Yang, Y., Song, Y., Loscalzo, J., 2007. Regulation of the protein disulfide proteome by mitochondria in mammalian cells. Proc. Nat. Am. Soc. 104, 10.

Yeste, M., Estrada, E., Casas, I., Bonet, S., Rodríguez-Gil, J.E., 2013. Good and bad freezability boar ejaculates differ in the integrity of nucleoprotein structure after freeze-thawing but not in ROS levels. Theriogenology 79, 929–939.

Yoshida, M., 2000. Conservation of sperms: current status and new trends. Anim. Reprod. Sci. 60–61, 349–355.

82

Study 4

**Assessment of post-thawed (washed *vs.* unwashed)
ram sperm quality after incubation
with autologous whole seminal plasma obtained
by electroejaculation and artificial vagina**

Abstract

This study was conducted to evaluate the effect of washing post-thawed sperm by centrifugation and incubating with seminal plasma (SP) obtained via electroejaculation (during non-breeding and breeding season) and, via artificial vagina (AV) (breeding season) for up to 90 min. The aim being that exposing these frozen-thawed sperm treatments to a thermal resistance test will provide useful information on their survivability over a period of time. Briefly, frozen-thawed semen from 5 males (5 years old) collected by AV, and split into two samples. One sample was diluted (1:5) in Tris-citric acid-glucose (TCG) solution and washed twice by centrifugation at $600 \times g$ for 10 min, while the other sample was kept unwashed. Each sample was further split into four treatment groups and supplemented with 20% of the various seminal plasma source and PBS (control), and incubated at $37^{o}C$ for up to 90min. Sperm kinematics were evaluated by CASA while plasma membrane integrity, acrosome integrity and mitochondria activity were analysed by flow cytometry at 0, 45 and 90 min of post-thawing incubation period. Sperm kinematics immediately after thawing showed that the addition of SP regardless its source on cyropreserved sperm (washed and unwashed) improved motility when compared with their controls. Sperm motility was generally affected by the incubation period. No difference was observed amongst treatment groups with SP on all sperm function parameters evaluated by flow cytometry immediately after thawing except on acrosome integrity and mitochondria functionality for washed sperm (+PBS/Control). This study demonstrates that supplementing frozen-thawed sperm with SP irrespective of its source was beneficial in maintaining sperm quality during a post-thawing incubation period. Furthermore, there was no identifiable beneficial effect of the different SP sources on sperm quality.

Introduction

In the ram, it is well known that cryopreservation process causes series of detrimental alterations in sperm functions such as in viability, motion characteristics, mitochondrial membrane potential, increase in reactive oxygen species (ROS) production and chromatin damages (Holt and North, 1994; Bailey et al., 2003; Said et al., 2010). Furthermore, an important aspect of cryo alteration is the untimely initiation of a capacitation-like process, also referred to as cryocapacitation (Bailey et al., 2003). All these alterations may result to less survival of cryopreserved sperm in the female genital tract thus reducing its capability to

fertilize the ovum after cervical insemination (Dominguez-Rebolledo et al., 2009; Mata-Campuzano et al., 2012). Therefore, the evaluation and maintenance of frozen-thawed sperm characteristics over an extended period of time at 37°C (thermo-resistance test) is paramount, and gives a better indication towards achieving high fertility rates (Bag et al., 2004).

However, several researchers on different species have reported that the longevity of frozen-thawed sperm during *in vitro* incubation is low due to various alterations in sperm membrane during cryopreservation (Gadea et al., 2005; Dominguez-Rebolledo et al., 2009; Mata-Campuzano et al., 2012). In spite of this, attempts to improve the quality of frozen-thawed semen in various species have been demonstrated; one such method is the addition of SP to frozen-thawed sperm (Ledesma et al., 2016). Despite studies reporting that adding SP after thawing reversed freeze–thaw effect on sperm, improved sperm resistance to thermal shock, increase sperm motility and its ability to migrate through the cervical mucus (Rebolledo et al., 2007; Rickard et al., 2014), others have demonstrated detrimental (Maxwell et al., 2007; Rovegno et al., 2013) or no effect (Morrier et al., 2003). These inconsistencies may be due to the complexity and variance of seminal plasma amongst species (Mandiki et al., 1998), donor age (Muiño-Blanco et al., 2008), seasons (Leahy et al., 2010) and or method of collection (Ledesma et al., 2014).

Furthermore, seasonal effect on SP protein profile and function on sperm has also been reported, showing better results from a breeding season when compared to a non-breeding season (Dominguez et al., 2008). Nevertheless, the role of SP remains a controversial topic and still a matter of speculation. Therefore, taking the beneficial role of SP factors into consideration, the present work is aimed at evaluating the effect of adding autologous whole SP collected via electroejaculation (breeding and non-breeding season) and artificial vagina (breeding season) on post-thawed washed and unwashed ram sperm quality. Furthermore, we tested the resilience of these frozen-thawed sperm treatments during a 90 minutes incubation period at 37°C.

Materials and Methods

Reagents and Media

Unless otherwise stated, all reagents were acquired from Sigma Chemical Co. (St. Louis, Mo, USA). Fluorescence probes and analysis kits (LIVE/DEAD® sperm viability kit (L-7011; SYBR-14 and PI) and Mitotracker deep red (M22426) were purchased from Invitrogen

(Barcelona, Spain). All fluorochrome solutions were kept in the dark at -20^0C. The freezing extender was prepared in our laboratory using powdered egg yolk as described by Palomo et al., (2017) obtained from NIVE (Nunspeet Holland Eiproducten). Incubation media used in this study was a modified phosphate buffer solution (PBS) (supplemented with 36μg/ml pyruvate and 0.5mg/ml BSA) with osmolarity of 280-300 mOsm and pH 7.3-7.4.

Animals and sample collection

Five adult male rams of *Aranesa* breeds (aged 5 years) were used in this study. They were maintained under an intensive management system in the *Institut de Recerca i Tecnologia Agroalimentaria* (IRTA) farm, Caldes de Montbui, Barcelona, Spain, where semen collection and initial assessment was carried out. Semen was collected twice weekly with two ejaculates per collection from each male via artificial vagina with a total of six replications. Prior to collection, the preputial hairs were shaved and the orifice was washed with clean water and dried to prevent contamination of samples. All ejaculates were collected during the breeding season and maintained in water bath at 37^0C following initial analysis. Volume, mass motility, progressive motility, sperm concentration and morphology were assessed immediately after collection through conventional methods. Only ejaculates of good quality were used and frozen (mass motility: ≥ 4; sperm concentration: ≥ 2500 x 10^6 sperm/mL: normal sperm morphology $\geq 70\%$).

Cryopreservation of semen

The basic extender used in this study was Tris (hydroxymethyl-aminoethane)-citric acid-glucose (TCG) solution as defined by Salamon and Maxwell (2000), consisting of Tris (0.3M), citric acid anhydrous (94.7 mM), and D(+)-glucose (27.75 mM). This solution was adjusted to a pH of 7.25 ± 0.05 and osmolarity 333 ± 2.80 mOsm. Thereafter, glycerol (5% v/v, final concentration) and antibiotics (1000 UI/mL sodium penicillin and 1mg/mL streptomycin sulfate) were added to the solution having a final pH 7.0–7.17 and 1327 ± 234 mOsm. Powdered egg yolk (PEY) was then added to a final concentration of 15% as described by Palomo et al., (2017). The first and second ejaculates from each male were mixed together following no differences in the semen characteristics, and diluted with the freezing extender (1:4). All extended samples were held for equilibration for 4 hours at 5°C. After cooling for 4 hours, the 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 and

sealed with polyvinyl alcohol. All straws were kept in liquid nitrogen vapor (5cm above the nitrogen level) for 10 minutes before been plunged into the liquid nitrogen and stored.

Seminal plasma collection and preparation

Seminal plasma recovery was done using two methods; electroejaculation (EE) and artificial vagina (AV). Seminal plasma was obtained via EE during the breeding season (B) and non-breeding season (NB) while via AV was during the breeding season (B). All seminal plasma used as diluents was obtained from the same males used in this study. Preparation of SP was similar for both methods of collection (EE and AV). Briefly, an aliquot of fresh semen was centrifuged at 5,000 x g for 10 min at 5ºC. The supernatant was collected and centrifuged again at 5,000 x g for 10 min at 5ºC to remove the remaining sperm and cell debris. Thereafter, the clear supernatant (SP) from each male was recovered, pooled and freeze dried in liquid nitrogen before stored at -80ºC until used.

Sperm incubation with seminal plasma

This was carried out to verify the longevity and quality of frozen-thawed treated sperm samples incubated with the various SP sources. Two straws per replicate (n=6) from five males were thawed, pooled and divided into two samples. One sample was washed twice by dilution (1:5) in TGC by centrifugation at 600 X g for 10 min to remove the SP/extender leaving the resulting sediment while the other sample was kept unwashed. Thereafter, each sample (washed and unwashed) were diluted in the modified PBS to 40×10^6 sperm/mL and divided into four aliquots. Furthermore, the various SP samples were thawed and added to their respective sperm aliquots to a final concentration of 20%. The following eight treatments were established:

- Washed sperm + SP collected by EE (Non-breeding season)
- Washed sperm + SP collected by EE (Breeding season)
- Washed sperm + SP collected by AV (Breeding season)
- Washed sperm + PBS (Control)
- Unwashed sperm+ SP collected by EE (Non-breeding season)
- Unwashed sperm + SP collected by EE (Breeding season)
- Unwashed sperm + SP collected by AV (Breeding season)
- Unwashed sperm + PBS (Control)

All aliquots were then incubated at 37°C in a dry bath in the dark. Evaluation on all post thawed parameters in this study was assessed after thawing at 0, 45 and 90 min of incubation.

Semen evaluation

Motility assessment by CASA

Motion characteristics of various post-thawed sperm treatments samples were assessed at 0, 45 and 90 min of incubation using the computer-assisted sperm analysis (CASA) system ISAS® (PROISER SL., Valencia, Spain). On analysis, all sperm samples were diluted (1:10) in PBS, and 5µl drop of sperm suspension was placed on a slide and covered with a cover-slip (24x24mm). Sperm motility was assessed at 37°C at x200 magnification using a phase contrast microscope (Olympus BH-2, Japan). For each treated sample, more than three fields per drop were analyzed and a minimum of 200 spermatozoa evaluated.

The percentage of total motility (TM), progressive motility (PM), curvilinear velocity (VCL, µm/s), linear velocity (VSL, µm/s), mean velocity (VAP, µm/s), linearity coefficient (LIN= [VSL/VCL]x100, %), straightness coefficient (STR= [VSL/VAP]x100, %), lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz) were 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 diluents and materials used for sperm analysis were maintained at 37°C.

Flow cytometry analysis

Flow cytometry was performed using the BD FACSCanto platform (BD Biosciences, USA), and data were analyzed by BD FACS DIVA software (BD Biosciences, USA). Plasma and acrosomal membrane integrity as well as mitochondrial function were evaluated by flow cytometry using a quadruple-staining technique as described by Tabarez et al., (2017). The following fluorescent probes were used: LIVE/DEAD® sperm viability kit (SYBR-14 and Propidium Iodide (PI; L-7011, Invitrogen SA)) for plasma membrane integrity (viability), Phycoerythrin-Peanut Agglutinin (PE-PNA; GTX01509, Antibody, Bcn SL) for acrosome integrity and Mitotracker deep red (M22426, SA Invitrogen) for the detection of mitochondrial activity.

This analysis was done 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 sulfate, 50 mM sodium phosphate and 0.05% sodium azide, pH 7.0 containing 1 mM [Ca2 +] and [Mn2 +] ions) and 1.5 nM of Mitotracker deep red (diluted in DMSO) with each treated sperm sample diluted in PBS to a final concentration of 1×10^6 sperm/mL. Samples were mixed and incubated at 37 °C for 10 min and then remixed just before analysis. Stained sperm suspensions were subsequently ran through the 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.

After evaluation, sperm populations taken into consideration for this study were sperm viability (total alive sperm cells), viable cells with intact acrosome and active mitochondria (SYBR14+/ IP-/PE-PNA-/Mitotracker +), viable cells with damaged acrosome and active mitochondria (SYBR14+/IP-/PE-PNA+/Mitotracker +) and viable cells with intact acrosome and inactive mitochondria (SYBR14$^+$/IP$^-$/ PE-PNA-/Mitotracker$^-$). Also, the total acrosome damage was recorded.

Statistical analysis

Statistical analysis was performed using the statistical package SPSS 20 and results were expressed as the mean ± S.E.M. The data obtained from the analysis of all sperm parameters were subjected to tests of normality and homoscedasticity Shapiro-Wilk and Levene, respectively. Where necessary, data were arc sine transformed. Sperm parameters were compared by ANOVA, and differences between means were determined using Duncan multiple tests. Data were considered as statistically significance at $P<0.05$.

Results

Once established that there was no significant difference (P >0.05) between days of collection and males on the studied parameters, a mean percentage for each sperm treatment was established and analysed. Results for CASA analysis are summarised in Table 4.1. Sperm kinematic parameters varied considerably across various treatments and incubation time in this study. Analysis immediately after thawing (0 min) showed that the addition of SP regardless the source on washed and unwashed sperm had a significant effect (P < 0.05) on

TM percentages when compared with the controls. However, no difference was observed on PM regardless the sperm (washed or unwashed) or the addition of the various sources of SP.

A significant difference was observed for VCL, VAP, LIN, STR, ALH and BCF percentages in all sperm with SP addition when compared with the control for washed sperm (without any SP). There was no significant difference between washed and unwashed sperm controls (+PBS) on percentage values of TM, VAP, VSL, LIN, STR, ALH and BCF immediately after thawing (Table 4.1). Furthermore, after 90 min of incubation, there was a negative effect on washed and unwashed sperm incubated with SP obtained via EE in the non-breeding season for VCL, VSL, VAP, ALH and only on BCF percentage values in washed sperm. Incubation time also had an effect on TM and PM (washed sperm) and on STR percentage values (unwashed sperm) incubated with SP collected via AV during the breeding season. Nonetheless, the highest percentages on TM and PM values after 90 min of incubation was observed on unwashed sperm incubated with SP obtained via EE (breeding season) while the lowest was seen in washed sperm incubated in PBS (control).

Flow cytometry analysis on the effect of the various sources of SP additions on post-thawed sperm (washed and unwashed) and incubation on sperm physiological parameters is presented in Table 4.2. While plasma membrane integrity and mitochondria status being good indicators of the overall integrity of sperm, there was no significant difference ($P > 0.05$) on the viability and percentage of viable cells with intact acrosome and active mitochondria immediately after thawing (0 min), irrespective of treatments. However, percentage of viable cells with intact acrosome and inactive mitochondria was significantly higher ($P < 0.05$) in washed sperm (+PBS/Control) when compared to sperm treatments for washed incubated with SP via EE (non-breeding season) and unwashed sperm incubated with SP via EE (non-breeding season) and SP via EE and AV (breeding season).

Furthermore, there was a significant increase ($P < 0.05$) in the percentage of viable cells with damaged acrosome and high mitochondria activity on washed sperm (+PBS/Control) when compared to the other sperm treatments, immediately after thawing (Table 4.2). In this study, we considered this sperm population as "cryocapacitated" sperm. Also, a significant increase was observed on the total acrosome damage for washed sperm (+PBS/Control) compared to the other sperm treatments. As regards the effect of incubation time within each sperm treatment, there was no statistical difference observed on viability as well as on viable cells with intact acrosome and active mitochondria except for washed sperm (+PBS/Control)

which had low values after 90 min of incubation. Also, no significant effect of incubation time on the total acrosome damage was noted on the various sperm treatments except for washed sperm (+PBS/Control). However, incubation effect was seen on the percentages of viable cells with intact acrosome and inactive mitochondria as well as on damaged acrosome with active mitochondria irrespective of sperm treatments (Table 2).

Discussion

Many studies have tested the effects of adding seminal plasma to frozen thawed sperm, with variable results (El-Rajj Ghaoui et al., 2007; Dominguez et al., 2008; Rovengo et al., 2013). In this study, an attempt was made to analyse possible effects of adding whole seminal plasma (20%) in respect to seasons and method of collection on thawed cyropreserved washed (washing by centrifugation) and unwashed sperm. Furthermore, we evaluated whether the presence or absence of these various seminal plasma sources would exert any changes on sperm quality following a 90 minute incubation period at 37°C (thermo-resistance test).

Results on sperm kinematics by CASA immediately after thawing showed that the addition of seminal plasma regardless its source on cyropreserved sperm (washed and unwashed) generally had a positive effect on sperm motility parameters when compared with their controls. This was notably observed by the lower values in the various sperm controls (+PBS). Our result agrees with a previous study demonstrating that the beneficial effect of seminal plasma may be on sperm motility (Turner, 2005). Furthermore, analysis of sperm kinematics after 45 and 90 min of post-thawing incubation (thermo-resistance test) showed that motility was negatively affected irrespective of the treatments. Results herein agree with various studies on sperm motility during post-thawing incubation (Bag et al., 2004; Joshi et al., 2005). We suggest that the gradual decrease in motility could be as a result of dead cells releasing toxic effect of membrane-bound aromatic amino acid oxidase enzyme thereby causing a decline in the motility of spermatozoa (Shannon and Curson, 1972).

Nonetheless, there was no significant difference observed on total and progressive motility parameters on sperm incubated with seminal plasma regardless its source. Our results contradicts a similar work by Dominguez et al., (2008) who reported that seminal plasma obtained during the breeding season improved motility, and this may be due to the low molecular weight proteins and higher total protein content in seminal plasma obtained via EE

(Ledesma et al., 2014). Although seminal plasma characteristics were not tested in this study, we suggest that this inconsistency may be due to the complexity and variance of seminal plasma amongst species (Mandiki et al., 1998). However, our result agrees with Morrier et al., (2003) who also found no effect. As regards to sperm qualitative function analysed by the flow cytometry, cryopreservation process has been reported to induce changes in sperm function such as loss of membrane permeability, sperm structural damage, reduced enzyme activity, viability and motility (Thundathil et al. 1999). In this study, the addition of seminal plasma immediately after thawing showed no statistical effect on viability and viable cells with intact acrosome and active mitochondria, irrespective of the various sperm treatments. The results herein contradict a previous work by Barrios et al., (2000), who demonstrated that post-thaw dilution with seminal plasma concentration ranging from 20-50% restored sperm plasma membrane integrity induced by cold shock. Nonetheless, the evident change in our result with the beneficial presence of seminal plasma, irrespective the sperm treatment was only observed on acrosome damage due to the significant differences seen in washed sperm (control).

However, after 90 min of post-thawing incubation, the presence of seminal plasma irrespective of treatments maintained sperm viability and viable cells with intact acrosome and active mitochondria. The only changes observed in relation to the thermal incubation period was in washed sperm incubated with PBS which showed a decrease in viability and viable cells with intact acrosome and active mitochondria. Our result agrees with El-Rajj Ghaoui et al., (2007) but contradicts a recent work by Rovegno et al., (2013) who reported an increased apoptotic sperm cell rate after 60 min of incubation with seminal plasma. This discrepancy may be due to fact that whole seminal plasma is a complex mixture of organic and inorganic components, as well as proteins which has both positive and negative effects (Perez-Pe et al., 2001) thus making results hardly conclusive.

Table 4.1. Effect of addition of seminal plasma collected by electroejaculation or artificial vagina and incubation time (0, 45 and 90 min) on motility parameters of post-thaw (washed and unwashed) sperm

Parameter (unit)	Washed sperm				Unwashed sperm			
	+SP/EE-NB	+SP/EE-B	+SP/AV-B	+PBS/Control	+SP/EE-NB	+SP/EE-B	+SP/AV-B	+PBS/Control
TM (%)								
0	30.40 ± 2.17^{ab}	37.20 ± 3.12^{b}	38.40 ± 5.33^{b1}	26.40 ± 3.27^{a}	37.10 ± 6.28^{b}	40.60 ± 5.69^{b}	34.60 ± 6.97^{ab}	27.20 ± 5.63^{a}
45	31.20 ± 1.88^{ab}	28.80 ± 3.18^{ab}	29.40 ± 2.48^{ab12}	25.20 ± 2.22^{a}	36.80 ± 7.49^{b}	28.80 ± 8.58^{ab}	35.40 ± 3.75^{b}	28.40 ± 7.86^{ab}
90	23.12 ± 1.79^{ab}	29.60 ± 2.40^{ab}	27.40 ± 1.89^{ab2}	20.60 ± 1.36^{a}	30.40 ± 8.21^{b}	32.60 ± 8.18^{b}	30.20 ± 2.13^{b}	25.80 ± 2.73^{ab}
PM (%)								
0	23.20 ± 1.22	22.00 ± 1.95	23.40 ± 5.54^{1}	17.40 ± 0.75	24.60 ± 4.95	25.00 ± 0.89	22.00 ± 1.76	18.60 ± 1.60
45	17.40 ± 1.19^{ab}	20.80 ± 1.93^{ab}	16.00 ± 1.05^{ab12}	12.00 ± 0.55^{a}	22.00 ± 5.52^{b}	21.80 ± 5.21^{b}	21.40 ± 3.36^{b}	15.00 ± 1.98^{ab}
90	15.20 ± 1.12^{ab}	19.20 ± 1.59^{ab}	15.00 ± 1.41^{ab2}	11.80 ± 0.92^{a}	19.20 ± 2.94^{ab}	23.00 ± 6.17^{b}	18.60 ± 2.60^{ab}	13.80 ± 1.59^{a}
VCL (μm/s)								
0	77.46 ± 5.23^{b1}	69.08 ± 10.21^{b}	78.22 ± 4.31^{b}	52.36 ± 13.99^{a}	71.28 ± 13.25^{b1}	81.76 ± 11.67^{b}	72.32 ± 17.33^{b}	68.92 ± 18.29^{b}
45	56.42 ± 4.88^{ab1}	75.76 ± 9.21^{b}	60.88 ± 10.68^{b}	37.08 ± 4.39^{a}	87.02 ± 11.38^{b12}	55.74 ± 22.68^{ab}	80.72 ± 11.44^{b}	59.10 ± 18.71^{ab}
90	20.78 ± 5.71^{a2}	82.50 ± 7.67^{b}	65.23 ± 8.04^{b}	40.58 ± 6.31^{ab}	48.84 ± 18.35^{ab1}	67.44 ± 18.93^{b}	80.40 ± 21.29^{b}	74.48 ± 18.04^{b}
VSL (μm/s)								
0	30.56 ± 3.40^{ab1}	30.90 ± 6.14^{ab}	38.90 ± 7.85^{b}	15.76 ± 7.16^{a}	48.30 ± 12.69^{b1}	48.80 ± 4.04^{b}	22.70 ± 6.72^{a}	22.88 ± 7.91^{a}
45	29.54 ± 2.84^{b1}	40.52 ± 4.96^{b}	26.26 ± 6.89^{b}	8.38 ± 1.20^{a}	36.40 ± 7.80^{b1}	29.26 ± 13.29^{b}	39.66 ± 6.23^{b}	19.60 ± 7.39^{ab}
90	18.50 ± 3.20^{ab2}	40.88 ± 3.20^{b}	29.48 ± 8.61^{b}	9.48 ± 2.01^{a}	19.64 ± 8.09^{ab2}	40.74 ± 13.98^{b}	32.72 ± 8.17^{b}	35.98 ± 8.33^{b}
VAP (μm/s)								
0	40.92 ± 3.12^{b1}	44.16 ± 8.05^{b}	52.78 ± 7.64^{bc}	29.42 ± 10.85^{a}	66.12 ± 12.24^{bc1}	70.24 ± 7.39^{c}	41.12 ± 9.66^{ab}	34.66 ± 10.91^{ab}
45	38.22 ± 3.40^{ab1}	52.42 ± 6.08^{b}	36.74 ± 8.53^{ab}	17.52 ± 1.79^{a}	50.74 ± 6.67^{b12}	37.32 ± 16.12^{ab}	53.02 ± 8.37^{b}	30.62 ± 10.87^{ab}
90	12.26 ± 3.91^{a2}	55.70 ± 5.32^{c}	40.82 ± 8.27^{bc}	18.68 ± 1.40^{ab}	27.90 ± 10.42^{ab2}	48.00 ± 16.00^{bc}	46.62 ± 10.60^{bc}	49.52 ± 12.25^{bc}
LIN (%)								
0	40.40 ± 2.17^{a}	40.66 ± 3.28^{a}	43.54 ± 6.10^{a}	30.12 ± 4.21^{b}	44.16 ± 8.15^{a}	47.50 ± 2.82^{a}	41.70 ± 3.72^{a}	25.76 ± 9.32^{b}
45	50.34 ± 2.47^{a}	49.58 ± 3.27^{a}	38.58 ± 3.42^{ab}	27.26 ± 1.38^{bc}	42.18 ± 3.94^{ab}	37.76 ± 11.90^{b}	47.82 ± 3.14^{a}	21.50 ± 6.37^{c}
90	26.82 ± 2.92	45.42 ± 1.43	43.16 ± 5.46	24.26 ± 4.43	29.84 ± 8.30	42.14 ± 12.27	41.36 ± 6.09	47.54 ± 8.77
STR (%)								
0	69.08 ± 2.63^{a}	62.54 ± 2.32^{a}	64.64 ± 5.48^{a}	50.58 ± 3.85^{b}	66.22 ± 6.80^{a}	67.38 ± 2.47^{a}	57.64 ± 2.53^{a1}	43.40 ± 14.44^{b}
45	73.50 ± 3.33^{a}	72.00 ± 4.51^{a}	62.10 ± 3.31^{a}	47.78 ± 1.95^{b}	67.10 ± 5.73^{a}	41.67 ± 17.46^{b}	71.26 ± 1.75^{a2}	40.70 ± 10.99^{b}
90	40.90 ± 3.61	64.98 ± 1.87	65.40 ± 4.40	44.56 ± 5.92	48.88 ± 12.91	62.22 ± 16.22	64.00 ± 3.47^{2}	66.56 ± 5.78
ALH (μm/s)								
0	3.18 ± 0.19^{ab1}	2.98 ± 0.36^{ab}	3.16 ± 0.15^{ab}	2.52 ± 0.38^{a}	4.38 ± 0.37^{b1}	4.22 ± 0.51^{b}	3.28 ± 0.69^{ab}	3.30 ± 0.84^{ab}
45	2.58 ± 0.18^{1}	2.96 ± 0.30	2.74 ± 0.39	2.06 ± 0.20	3.62 ± 0.29^{12}	2.30 ± 0.82	3.52 ± 0.36	2.70 ± 0.85
90	1.14 ± 0.22^{a2}	3.34 ± 0.31^{b}	2.91 ± 0.22^{ab}	2.42 ± 0.41^{ab}	2.18 ± 0.72^{ab2}	2.44 ± 0.67^{ab}	3.58 ± 0.89^{b}	3.14 ± 0.69^{b}
BCF (Hz)								
0	6.88 ± 0.39^{a1}	5.58 ± 0.31^{ab}	6.38 ± 0.75^{a}	3.12 ± 0.55^{b}	7.56 ± 1.58^{a}	7.00 ± 0.82^{a}	5.20 ± 0.59^{ab}	4.46 ± 1.19^{ab}
45	5.76 ± 0.40^{ab1}	6.02 ± 0.59^{ab}	4.44 ± 0.71^{ab}	2.94 ± 0.25^{a}	6.96 ± 1.03^{b}	5.90 ± 1.73^{ab}	7.14 ± 0.73^{b}	3.60 ± 1.33^{a}
90	1.84 ± 0.49^{a2}	6.12 ± 0.67^{bc}	4.43 ± 0.64^{abc}	2.58 ± 0.48^{ab}	5.18 ± 1.93^{abc}	7.20 ± 2.26^{c}	4.68 ± 1.39^{abc}	3.98 ± 0.86^{abc}

SP: seminal plasma; EE: electroejaculation; AV: artificial vagina; NB: non-breeding season; B: breeding season; PBS: phosphate buffer saline; TM: total motility; PM: progressive motility; VCL: Curvilinear velocity; VSL: Linear velocity; VAP: Mean velocity; LIN: Linear coefficient; STR: Straightness coefficient; ALH: Lateral head displacement; BCF: Beat cross frequency. Data are shown as mean ± S.E.M. Different numbers (1, 2) in the column within the same treatment indicates significant difference ($P < 0.05$) during incubation period. Different superscripts (a, b,c) for the same parameter across the same row indicate a significant difference amongst treatments ($P < 0.05$).

Table 4.2. Effect of addition of seminal plasma collected by electroejaculation or artificial vagina following incubation (0, 45 and 90 min) on quality parameters evaluated by flow cytometry of post-thaw (washed and unwashed) sperm

Parameter (unit)	Time (min)	Washed sperm				Unwashed sperm			
		+SP/EE-NB	+SP/EE-B	+SP/AV-B	+PBS/Control	+SP/EE-NB	+SP/EE-B	+SP/AV-B	+PBS/Control
Viability (%)	0	21.50 ± 1.71	21.75 ± 2.56	21.75 ± 1.25	20.79 ± 0.85	24.25 ± 1.65	23.10 ± 1.11	24.00 ± 1.16	22.00 ± 2.16
	45	19.50 ± 1.56	20.50 ± 0.87	20.25 ± 0.49	19.77 ± 2.38	22.50 ± 1.50	22.00 ± 0.58	22.50 ± 0.29	21.25 ± 1.11
	90	18.75 ± 1.11^{ab}	21.50 ± 3.07^{a}	19.50 ± 0.87^{ab}	16.31 ± 1.55^{b}	20.75 ± 1.65^{a}	22.67 ± 1.19^{a}	20.50 ± 1.19^{a}	20.00 ± 2.04^{a}
% Viable cells with intact acrosome and active mitochondria	0	20.64 ± 3.62	20.47 ± 1.45	20.56 ± 3.54	18.25 ± 3.75^{1}	23.56 ± 4.95	22.21 ± 3.89	23.09 ± 2.76	20.85 ± 6.60
	45	15.73 ± 5.39	17.38 ± 1.73	16.23 ± 3.05	12.11 ± 1.55^{12}	18.33 ± 5.52	17.80 ± 5.21	19.18 ± 3.36	17.90 ± 3.98
	90	11.34 ± 2.12	16.72 ± 1.52	15.55 ± 2.31	6.40 ± 0.92^{2}	15.75 ± 2.94	16.95 ± 2.17	16.45 ± 2.64	12.85 ± 1.59
% Viable cells with intact acrosome and inactive mitochondia	0	0.40 ± 0.13^{a1}	0.91 ± 0.71^{ab1}	0.86 ± 0.41^{ab1}	1.17 ± 0.19^{b1}	0.15 ± 0.11^{a1}	0.46 ± 0.37^{a1}	0.43 ± 0.13^{a1}	0.83 ± 0.19^{ab1}
	45	3.63 ± 1.18^{a2}	2.90 ± 0.89^{a2}	3.89 ± 1.27^{a2}	6.28 ± 2.59^{b2}	4.02 ± 2.18^{a2}	4.03 ± 1.63^{a2}	3.20 ± 1.74^{a2}	3.25 ± 1.71^{a2}
	90	7.21 ± 3.91^{a3}	4.78 ± 1.37^{b2}	3.71 ± 1.14^{b12}	7.72 ± 0.37^{a2}	4.87 ± 1.33^{b2}	5.61 ± 2.93^{ab2}	3.87 ± 1.29^{b2}	7.01 ± 2.74^{a3}
% Viable cells with damage acrosome and active mitochondria	0	0.41 ± 0.23^{a1}	0.34 ± 0.14^{a1}	0.31 ± 0.11^{a1}	1.35 ± 0.17^{b1}	0.36 ± 0.19^{a1}	0.35 ± 0.14^{a1}	0.46 ± 0.22^{a1}	0.30 ± 0.01^{a1}
	45	0.10 ± 0.01^{a2}	0.21 ± 0.16^{a1}	0.10 ± 0.00^{a2}	1.37 ± 0.20^{b12}	0.13 ± 0.00^{a2}	0.10 ± 0.10^{a2}	0.11 ± 0.03^{a2}	0.10 ± 0.03^{a2}
	90	0.14 ± 0.13^{a2}	0.00 ± 0.00^{a2}	0.10 ± 0.02^{a2}	2.18 ± 0.11^{b2}	0.10 ± 0.00^{a2}	0.10 ± 0.07^{a2}	0.15 ± 0.11^{a2}	0.10 ± 0.00^{a2}
Total acrosome damage (%)	0	42.00 ± 4.14^{a}	40.50 ± 4.09^{a}	41.00 ± 3.67^{a}	60.25 ± 9.18^{b1}	39.00 ± 3.77^{a}	41.25 ± 3.57^{a}	44.67 ± 3.67^{a}	35.50 ± 4.67^{a}
	45	45.00 ± 4.14^{a}	41.75 ± 3.52^{a}	42.50 ± 4.53^{a}	65.25 ± 4.27^{b1}	41.50 ± 3.57^{a}	41.67 ± 4.91^{a}	44.25 ± 2.59^{a}	38.25 ± 5.59^{a}
	90	44.50 ± 6.40^{a}	42.25 ± 2.32^{a}	43.25 ± 4.01^{a}	83.25 ± 4.45^{b2}	43.75 ± 2.43^{a}	43.67 ± 4.63^{a}	42.75 ± 3.61^{a}	39.00 ± 5.43^{a}

SP: seminal plasma; EE: electroejaculation; AV: artificial vagina; NB: non-breeding season; B: breeding season; PBS: phosphate buffer saline. Data are shown as mean ± S.E.M. Different numbers (1, 2, 3) in the column within the same treatment indicates significant difference (P < 0.05) during incubation period. Different superscripts (a, b, c) for the same parameter within each incubation time across the same row indicate a significant difference amongst treatments (P < 0.05).

As regards mitochondria activity, our results showed that the presence of seminal plasma regardless the sperm treatments after thawing, assisted in maintaining mitochondrial functionality up to 45 min of incubation. This is evidenced by the significant difference in the percentage of viable sperm with intact acrosome and inactive mitochondria observed in only washed sperm (+PBS) after 45 min of incubation. The major changes observed was the adverse effect thermal incubation time which showed an increase on viable cells with intact acrosome and inactive mitochondria, irrespective of treatments. This finding is in agreement with other researchers demonstrating a decline in mitochondria functionality from prolonged incubation (Joshi et al., 2005). We suggest that the decrease in mitochondrial functionality during post thawing incubation may be as a result of the inability of sperm to generate ATP from mitochondrial respiration due to mitochondrial ageing (Viswanath and Shannon, 1997).

Regarding capacitation, it has been hypothesised that freeze/thawing procedure may increase the proportion of capacitated and acrosome reacted sperm (Maxwell and Watson, 1996). In the present study, there was no effect amongst treatments with seminal plasma on sperm capacitation irrespective of incubation time except for washed sperm (+PBS). This suggests that the presence of seminal plasma has the ability to delay capacitation-like changes and acrosome reaction. Also, the low values observed on various sperm with seminal plasma throughout course of post-thawing incubation agrees with a previous study demonstrating that seminal plasma has some decapacitating proteins that suppress capacitation and acrosome reaction (El-Rajj Ghaoui et al., 2007). This further explains the significant difference observed on total acrosome damage between all sperm samples with seminal plasma when with washed sperm incubated with only PBS, irrespective of the incubation period.

To this regard, it seems that the presence of seminal plasma was generally beneficial in maintaining sperm motility and regulating sperm functions during a prolong incubation time in respect to sperm without seminal plasma (washed sperm +PBS). Furthermore, the lack of a clear distinctive difference from other sources of seminal plasma on sperm quality was interesting. This may be due to the low amount of seminal plasma added (20%), however, conflicting studies still abounds on concentration of seminal plasma inclusion in improving sperm quality (Maxwell et al., 2007). Another explanation to the lack of difference may be attributed to pooled effect on seminal plasma used in this study which may have eliminated

the individual variation on seminal plasma quality of different males (Muiño-Blanco et al., 2008).

Moreso, under the condition of this study, the addition of seminal plasma irrespective of its source to washed frozen-thawed sperm did not improve sperm quality when compared to the unwashed sperm. We suggest that washing by centrifugation is not necessary and may only cause loss or damage of sperm as well as other vital components of seminal plasma or extender which helps in maintaining sperm quality (Azeredo et al., 2001). Nevertheless, we can only speculate about the physiological significance of seminal plasma but further investigation is needed in order to determine, quantify and purify seminal plasma based on individual male characteristics in relation to its beneficial effect towards improving sperm quality for cervical insemination.

In conclusion, our result confirms that supplementing frozen-thawed sperm with seminal plasma maintained sperm functions and motility characteristics during a post-thawing incubation period. Furthermore, there was no beneficial effect of washing by centrifugation or removal of freezing component or seminal plasma after thawing. Following this pattern, further investigation is needed to assess the fertilizing capability through artificial insemination.

References

Azeredo, G.A., Esper, C.R., Resende, K.T., 2001. Evaluation of plasma membrane integrity of frozen-thawed goat spermatozoa with or without seminal plasma. Small Rumin. Res. 41, 257–263.

Bag, S., Joshi, A., Naqvi, S.M.K., Mittal, J.P., 2004. Effect of post-thaw incubation on sperm kinematics and acrosomal integrity of ram spermatozoa cryopreserved in medium-sized French straws. Theriogenology 62, 415-424.

Bailey, J., Morrier, A., Cormier, N., 2003. Semen cryopreservation: successes and persistent problems in farm species. Can. J. Anim. Sci.83, 393–401.

Dominguez, M.P., Falcinelli, A., Hozbor, F., Sanchez, E., Cesari, A., Alberio, R.H., 2008. Seasonal variations in the composition of ram seminal plasma and its effect on frozen–thawed ram sperm. Theriogenology 69, 564–573.

Domınguez-Rebolledo, A.E., Fernandez- Santos, M.R., Garcıa-Alvarez, O., Maroto- Morales, A., Garde, J.J., Martınez-Pastor, F., 2009. Washing increases the susceptibility to exogenous oxidative stress in red deer spermatozoa. Theriogenology 72, 1073– 1084.

El-Rajj Ghaoui, R., Thomson, P., Leahy, T., Evans, G., Maxwell, W., 2007. Autologous whole ram seminal plasma and its vesicle free fraction improve motility characteristics and membrane status but not in vivo fertility of frozen thawed spermatozoa. Reprod. Domest. Anim. 42, 541–549.

Feitosa, W.B., Mendes, C.M., Milazzotto, M.P., Rocha, A.M., Martins, L.F., Simoes, R., Paula-Lopes, F.F., Visintin, J.A., Assumpcao, M.E., 2010. Exogenous DNA uptake by bovine spermatozoa does not induce DNA fragmentation. Theriogenology 74, 563–568

Fernandez-Juan, M., Gallego, M., Barrios, B., Osada, J., Cebrián-Pérez, J.A., Muiño-Blanco, T., 2006. Immunohistochemical localization of sperm-preserving proteins in the ram reproductive tract. J. Androl. 27, 588-595.

Gadea, J., Gumbao, D., Matas, C., Romar, R., 2005. Supplementation of the thawing media with reduced glutathione improves function and the in vitro fertilizing ability of boar spermatozoa after cryopreservation. J. Androl. 26, 749–756.

Holt W, North R, 1994. Effects of temperature and restoration of osmotic equilibrium during on the induction of plasma membrane damage in cryopreserved ram spermatozoa. Bio. Reprod. 51, 414-424.

Leahy, T., Marti, J., Evans, G., Maxwell, W., 2010. Seasonal variation in the protective effect of seminal plasma on frozen-thawed ram spermatozoa. Anim. Reprod. Sci. 119, 147–153.

Ledesma, A., Manes, J., Cesari, A., Alberio, R., Hozbor, F., 2014. Electroejaculation increases low molecular weight proteins in seminal plasma modifying sperm quality in Corriedale rams. Reprod. Domest. Anim. 49, 324–332.

Ledesma, A., Fernández-Alegre, E., Cano, A., Hozbor, F., Martínez-Pastor, F., Cesari, A., 2016. Seminal plasma proteins interacting with sperm surface revert capacitation indicators in frozen-thawed ram sperm. Anim. Reprod. Sci. 173, 35-41.

Mandiki, S.N.M., Derycke, G., Bister, J.L., Paquay, R., 1998. Influence of season and age on sexual maturation parameters of Texel, Suffolk and Ile-de-France rams: 1. Testicular size, semen quality and reproductive capacity. Small Rumin. Res. 28, 67–79.

Mata-Campuzano, M., Alvarez-Rodríguez, M., del Olmo, E., Fernández- Santos, M.R., Garde, J.J., Martínez-Pastor, F., 2012. Quality, oxidative markers and DNA damage (DNA) fragmentation of red deer thawed spermatozoa after incubation at 37^0C in presence of several antioxidants. Theriogenology 78, 1005–1019.

Maxwell, W.M.C., de Graaf, S.P., Ghaoui Rel, H., Evans, G., 2007. Seminal plasma effects on sperm handling and female fertility. Soc. Reprod. Fertil. Suppl. 64, 13–38.

Mori, C., Nakamura, N., Welch, J.E., Gotoh, H., Goulding, E.H., Fujioka, M., Eddy, E.M., 1998. Mouse spermatogenic cell-specific type 1 hexokinase (mHk1-s) transcripts are expressed by alternative splicing from the mHk1 gene and the HK1-S protein is localized mainly in the sperm tail. Mol. Reprod. Dev. 49, 374–385

Morrier, A., Castonguay, F., Bailey, J.L., 2003. Conservation of fresh ram spermatozoa at 5^0C in the presence of seminal plasma. Can. J. Anim. Sci. 83, 221–227.

Muiño-Blanco, T., Pérez-Pé, R., Cebrián- Pérez, J.A., 2008. Seminal plasma proteins and sperm resistance to stress. Reprod. Domest. Anim., 4, 18-31.

Muiño-Blanco, T., Pérez-Pé, R., Cebrián- Pérez, J.A., 2008. Seminal plasma proteins and sperm resistance to stress. Reprod. Domest. Anim., 4, 18-31.

Palomo, M., García, W., Tabarez, A., 2017. Effect of seminal plasma and butylated hydroxytoluene (BHT) concentration on ram sperm freezability. Small Rumin. Res. 153, 66–70.

Perez-Pe, R., Barrios, B., Muino-Blanco, T., Cebrian, J.A., 2001. Seasonal differences in ram seminal plasma revealed by partition in an aqueous two phase system. J. Chromatogr. Biomed. Sci. Appl. 760 (1), 113–121.

Rebolledo, A.D., Sierra, L.N., Tamayo, A.C., Loria, A.A., Denis, S.E., Oses, R.B., Parra, E.G., Monsreal, L.P., Ugalde, J.R., 2007. Fertility in hair sheep inseminated with freeze spermatozoa rediluted with seminal plasma. Rev. Cient 17, 73–76.

Rickard, J., Pini, T., Soleilhavoup, C., Cognie, J., Bathgate, R., Lynch, G., Evans, G., Maxwell, W., Druart, X., de Graaf, S., 2014. Seminal plasma aids the survival and cervical transit of epididymal ram spermatozoa. Reproduction 48, 469–478.

Rovegno, M., Beringui, W., Monteiro, A., Mota, C., Visintin, J.A., Ortiz, M.E., 2013. Assessment of post-thawed ram sperm viability after incubation with seminal plasma. Cell Tissue Bank 14, 333–339.

Said, T., Gaglani, A., Agarwal, A., 2010. Implication of apoptosis in sperm cryoinjury. Reprod. Bio. Med. 21, 456–462.

Salamon, S., Maxwell, W., 2000. Storage of ram semen. Anim. Reprod. Sci. 62, 77–111.

Tabarez, A., García, W., Palomo, M., 2017. Effect of the type of egg yolk, removal of seminal plasma and donor age on buck sperm cryopreservation. Small Rumim. Res. 149, 91–98.

Turner, R.M., 2005. Moving to the beat: a review of mammalian sperm motility regulation. Reprod. Fertil. Dev. 18, 25–38.

Chapter V

GENERAL DISCUSSION

The importance of establishing genetic resource banks of frozen semen for endangered breeds is steadily being acknowledged, particularly for its potential towards environmental changes and utilization for AI (Yoshida, 2000). Therefore, the main aim- being the establishment of a semen bank for endangered native Catalonian breed of rams (*Aranesa* and *Xisqueta*) towards its successful for AI. To this regards, various cryopreservation protocols/techniques towards improving the quality of both fresh and frozen-thawed sperm were attempted. This includes the evaluation of melatonin implantation during a non-breeding season on chilled liquid stored semen, the efficacy of collection methods (electroejaculation vs. artificial vagina) on the froozen-thawed sperm quality and various sperm treatments prior to cryopreservation (sperm washing) or during post-thawing (addition of different seminal plasma sources). Therefore, to fully address this topic, sperm motility was evaluated through conventional methods as well as objectively, by using CASA which provides precise and accurate information on sperm kinematics (Gravance and Davis, 1995). In addition, qualitative or functional characteristics of the sperm were analyzed using the flow cytometry, which further provides a more accurate prediction on sperm functionality (Graham, 2001; Gillan et al., 2005). Furthermore, an attempt was made to evaluate the sperm nucleoprotein integrity by determining the sperm head free cysteine radicals as a direct measure in the number of disulfide bonds in nucleoprotein-DNA structure. This technique was adopted from a published work on the determination of free cysteine radicals in boar sperm nucleoproteins (Flores et al., 2011). Therefore, with the abovementioned reasons, a wealth of information on the qualitative characteristics and predicting the fertilizing capacity of the sperm may be achieved before its use for artificial insemination.

Due to the improvement in sperm cryobanking, storage of semen in liquid nitrogen has become a standard in developed countries but where access to facilities for modern cryobanking is not readily available, the need for an alternative approach for sperm quality maintenance is necessary. However, the use of refrigerated extended semen seems to be an alternative, either for transporting sperm to freezing facilities or for artificial insemination (Karagiannidis et al., 2000). Therefore, in this thesis, we evaluated the sperm qualitative characteristics as regards to the influence of season and melatonin implantation during a prolonged chilled liquid storage, the quality of post-thawed sperm collected via different methods (electroejaculation and artificial vagina) as well as post-thawed sperm quality of different sperm treatments prior to and after cryopreservation using routine objective analysis techniques such as CASA and flow cytometry. Thus, the aim of this PhD thesis is to further

provide more information as regards *Aranesa* and *Xisqueta* ram sperm processes and quality during and after cryopreservation before testing its usefulness for further application.

In this thesis, we evaluated the seasonal effect and melatonin implantation on chilled liquid stored sperm. Our results showed that melatonin implantation to semen donors during a non-breeding season generally reversed seasonal effect and maintained sperm parameters during chilled liquid storage. This was achieved by directly comparing sperm parameters from ram sperm donors' in a non-breeding season (melatonin treated and untreated), and with sperm donors in a breeding season. Analysis on various semen parameters from fresh ejaculates varied considerably among the three sperm donor groups. In this study, it was evident that melatonin treatment in the non-breeding season improved fresh semen quality by reversing the negative effect on sperm production or quality usually observed during a non-breeding season (Casao et al., 2008; Rosa et al., 2012). However, melatonin administration did not exert any beneficial influence on viability and normal morphology in fresh ejaculates but improved the viability and normal sperm morphology during chilled liquid storage, and having no significant differences with sperm obtained during the breeding season. Also, results on sperm kinematics parameters by CASA showed that the melatonin group displayed better values than other sperm donor groups during 4hr of refrigeration. The only evident significant changes in all kinematic parameters were observed in untreated/non-breeding season sperm donor group showing very low values after 24h.

Regarding sperm nucleoprotein integrity, our results showed no effect of chilled liquid storage in all donor groups but a significant difference between seasons. To the best of our knowledge, this is the first study towards the evaluation of ram sperm nucleoprotein integrity by assessing disulfide bonds rupture, and as such, comparison with any similar study is not available. Nonetheless, the significant difference between seasons may indicate that ram sperm chromatin package with regards to nucleoprotein-DNA compactness maybe be correlated with seasonal variation but this area requires further investigation. In this study, it may be suggested that the adverse effect of seasonality on sperm may have been reversed by the increased antioxidant enzyme activities in the seminal plasma (Reiter et al., 2009; Casao et al., 2010) during storage. Therefore, it was concluded in this chapter that melatonin implantation to sperm donor rams during the non-breeding season enhanced sperm quality during chilled liquid storage. Future studies should be aimed at investigating the mechanism of action of melatonin implants on sperm function during liquid storage.

In small ruminants, semen collection via artificial vagina (AV) or electroejaculation (EE) are commonly used methods. Although, AV is the preferred method (Leboeuf et al. 2000), semen collection from large numbers of untrained rams makes sperm bank creation difficult as this technique requires previous training periods (Wulster-Radcliffe et al. 2001). Alternatively, EE could be a useful procedure for semen collection especially from untrained males (Jiménez-Rabadán et al. 2012). However, several works have suggested that the effect of collection methods on pre and or post-thawed sperm quality varies in small ruminants thus showing inconsistent results depending on species and or breeds (Marco-Jiménez et al. 2008). Therefore, on the basis of a quicker conservation program towards the creation of a sperm bank for this endangered breed, a study was conducted at evaluating the efficacy of two semen collection methods (EE vs. AV) on the quality of *Aranesa* ram sperm. This is due to the fact that for a rapid constitution of a sperm bank especially from a large number of untrained males, the use of an effective semen collection and evaluation technique is an integral aspect to achieving this goal. In this study, we directly compared the effect of two semen collection methods (EE vs AV) on fresh and frozen-thawed sperm quality of endangered *Aranesa* ram using an established cryopreservation protocol in our laboratory (Palomo et al., 2017). Our results showed that fresh ejaculates via EE had larger seminal volume with lesser sperm concentration than those obtained via AV (Jiménez-Rabadán et al., 2016), suggesting that the larger volume is as a result of increased seminal plasma (SP) from the accessory sex glands due to the electric stimulation (Marco-Jiménez et al., 2008).

Furthermore, the total sperm count and functional integrity of sperm membrane obtained via EE were lower compared to AV (Jiménez-Rabadán et al., 2016). Also, no effect was observed on sperm viability, morphology and total acrosome damage between the two methods (Marco-Jiménez et al., 2008; Jiménez-Rabadán et al., 2012). Most motility parameters on pre-freeze samples were significantly higher in EE samples than in AV, and we suggest that this may be due to the increase and changes in protein composition of seminal plasma obtained via EE which improved their motility characteristics (Marco-Jiménez et al., 2008; Mata-Campuzano et al., 2015). However, post-thawed sperm motility showed no difference between collection methods (Marco-Jiménez et al. 2008; Jiménez-Rabadán et al. 2016), and both were negatively affected from the freeze-thawing process (Jiménez-Rabadán et al., 2012; Ledesma et al., 2014). The qualitative analysis of post-thawed sperm by the flow cytometry showed no differences between methods on viability and ROS production (Álvarez et al., 2012) but contradicts Marco-Jiménez et al., (2005) who reported

better values in sperm collected by EE than via AV. It may be suggestted that this discrepancy may be due to the cryopreservation protocol used (Jiménez-Rabadán et al., 2016).

In addition, we also observed no effect on viable cells with intact acrosome and active mitochondria as well as in damaged acrosome with inactive mitochondria, irrespective of collection methods. These results contradict similar work by Jiménez-Rabadán et al., 2016, who demonstrated that ram sperm collected via AV were more resistance to cold shock with better sperm quality than via EE. However, Marco-Jiménez et al., (2005) reported a higher sperm cryoresistance quality in EE than AV samples. An explanation to this may be due the complexity and variance of seminal plasma amongst species or breeds (Leahy et al., 2010). Furthermore, EE samples had lower percentage of viable cells with damaged acrosome and active mitochondria (cryo-capacitation) and total acrosome damage than those via AV (Marco-Jiménez et al., 2005). This may suggests that EE frozen-thawed sperm seemed to display better stability during cryopreservation, and that this stability may be due to the increase in some seminal plasma proteins induced by electrical stimulation from the accessory glands thus suppressing acrosome reaction, and preserving the functional characteristics of spermatozoa during freezing (Barrios et al. 2000; El-Rajj Ghaoui et al. 2007). As regards sperm nucleoprotein integrity, our results showed no significant difference between methods on post-thawed sperm suggesting that ram sperm chromatin package in relation to nucleoprotein-DNA compactness between EE and AV may be similar but further investigation is needed. Therefore, results from this subject supports the inclusion of EE as a viable alternative and swift method for semen collection especially from a large number of untrained *Aranesa* rams towards the establishment of a sperm bank.

On the basis of creating a sperm bank for this breed as described above, the quality and effective use of frozen-thawed sperm is of siginificant importance. However, cryopreservation adversely impairs ram semen quality by altering sperm function, and these alterations may affect the post thawing fertilizing capabilities as a result of reduced survivability of the frozen thawed spermatozoa (Salamon and Maxwell, 1995: Ledesma et al., 2016). Therefore, in providing some information on the sperm post-thawed quality after cryopreservation and prior to testing its usefulness for further application, the maintenance of sperm quality after thawing for a longer period especially when subjected to thermo-evaluation test at 37^0C is a crucial aspect for fertilization processes (Bag et al., 2004). In this

thesis, an attempt was made to understand whether the removal of seminal plasma (washing by centrifugation) prior to cryopreservation would exert any changes on post-thaw sperm quality and after a 4 h of post-thawing incubation. Results on pre-freeze sperm motility showed that the removal of seminal plasma by centrifugation (washed sperm) prior to cryopreservation significantly reduced the percentage of total motility of sperm when compared to sperm with seminal plasma (unwashed sperm). This result agrees with other reports on pre-freeze sperm motility in goats (Leboeuf et al., 2000), in stallion (Hoogewijs et al., 2010) and in ram (Ahmed et al., 2016). Also, the kinematic parameters of unwashed post-thawed sperm had better values than that of washed sperm thus indicating the importance of seminal plasma during ram sperm cryopreservation (Barrios et al., 2000; 2005).

However, a previous study by our group demonstrated that sperm washing by centrifugation provided a beneficial effect on ram sperm kinematic parameters after thawing (Garcia, 2014). In the present work, we suggest that this discrepancy may be age related due to the fact that males used in the previous study were much younger (1-2years) than males in this study (5 years), and seems that the beneficial effect of seminal plasma may be evident in older males than younger ones (Mandiki et al., 1998; Maxwell et al., 1999). Furthermore, after 4 h post-thawing incubation, all sperm kinematic parameters were negatively affected irrespective of the treatments (Bag et al., 2004; Joshi et al., 2005), and despite this general negative effect on sperm motility, unwashed sperm had better kinematic parameters than washed sperm. We suggest that the presence of seminal plasma proteins in the unwashed sperm may have had a beneficial effect in maintaining sperm motility (Turner, 2005). An explanation could be as a result of dead cells releasing toxic effect of membrane-bound aromatic amino acid oxidase enzyme thereby causing a decline in the motility of spermatozoa (Shannon and Curson, 1972). As regards the analysis of various sperm populations analyzed by flow cytometry, we observed no effect between treatments either immediately after post-thaw or after 4 h of incubation period. The only change observed was the adverse effect of the thermal incubation on mitochondrial functionality, irrespective of treatments (Bag et al., 2004; Joshi et al., 2005). Furthermore, the decrease in mitochondrial functionality further elucidates the decline in sperm motility during post thawing incubation due to gradual decrease in the ability of sperm to generate ATP from mitochondrial respiration due to mitochondrial ageing (Viswanath et al., 1997).

Although studies have demonstrated that oxidative stress affects the strength of disulfide bonds which ultimately destabilising nucleoprotein integrity (Yang et al., 2007; Flores et al., 2011), in this study, no significant difference was observed between treatments regardless of post thawing sperm incubation time on sperm nucleoprotein integrity. It may be suggested that ram sperm nucleoprotein may be cryotolerant as regards its resistance to alteration in their structure during freeze-thawing but further investigation is needed, since this was the first attempt to determine the sperm head free cysteine radicals as a direct measure in the number of disulfide bonds in nucleoprotein-DNA structure in ram sperm. Therefore, we can conclude from the results in this study that the presence of seminal plasma prior to cryopreservation assisted in the maintenance of sperm motility at post-thaw and after a 4 h post-thawing incubation period, and such parameter have been considered as an important factor in correlation with fertility (Budworth et al., 1988; Aitken, 1990). Following this pattern, another study was proposed at evaluating the importance of adding seminal plasma to frozen-thawed sperm with the aim of maintaining sperm kinematics and quality towards fertilizing the ovum during cervical artificial insemination.

Due to the positive influence of seminal plasma during cryopreservation and sperm post-thawed quality, another work in this thesis was aimed at evaluating possible effects of adding autologous whole seminal plasma (20%) in respect to seasons and method of collection to frozen-thawed sperm (washed and unwashed) during a 90 minutes incubation period at $37^{o}C$ (thermo-resistance test). Results on sperm kinematics immediately after thawing showed that the addition of seminal plasma regardless its source on cyropreserved sperm (washed and unwashed) generally had a positive effect on sperm motility parameters when compared with their controls. This was notably observed due to the low motility values in their various sperm controls (+PBS), and in agreement with a previous study demonstrating that the beneficial effect of seminal plasma may be on sperm motility (Turner, 2005). Also, motility was negatively affected after 45 and 90 min of post-thawing incubation (thermo-resistance test), irrespective of the treatments (Bag et al., 2004; Joshi et al., 2005). Nonetheless, there was no significant difference observed on total and progressive motility parameters on sperm incubated with seminal plasma regardless its source. Our results contradicts a similar work by Dominguez et al., (2008) who reported that seminal plasma obtained during the breeding season improved motility, and this may be due to the low molecular weight proteins and higher total protein content in seminal plasma obtained via EE (Ledesma et al., 2014). Although the characteristic of seminal plasma was not tested in this study, we suggest that

this inconsistency may be due to the complexity and variance of seminal plasma amongst species (Mandiki et al., 1998). However, our result agrees with Morrier et al., (2003) who also found no effect.

As regards sperm functionality, the addition of seminal plasma immediately after thawing showed no statistical effect on viability and viable cells with intact acrosome and active mitochondria, irrespective of the various sperm treatments (Barrios et al., 2000). However, after 90 min of post-thawing incubation, the presence of seminal plasma irrespective of treatments maintained sperm viability and viable cells with intact acrosome and active mitochondria, and significantly differed from washed sperm incubated with only PBS (control). Our result agrees with El-Rajj Ghaoui et al., (2007) but contradicts Rovegno et al., (2013), who reported an increased apoptotic sperm cell rate after 60 min of incubation with seminal plasma. Regarding mitochondrial functionality, the only changes observed was the adverse effect of incubation time which showed a decrease, irrespective of treatments, and is in agreement with Joshi et al., (2005). The worst result as regards mitochondria functionality during the incubation period was seen in washed sperm (+PBS), and this led to increased sperm death. Furthermore, there was no effect amongst treatments with seminal plasma on sperm capacitation, irrespective of incubation time except for washed sperm (+PBS/control). This suggests that the presence of seminal plasma may have delayed capacitation-like changes and acrosome reaction (El-Rajj Ghaoui et al., 2007).

Therefore, it seems that the presence of seminal plasma was generally beneficial in maintaining sperm motility and regulating sperm functions during a prolong incubation time, regardless its source. It is also interesting to note the lack of a clear distinctive difference on sperm quality after the addition of the different sources of seminal plasma. It may be hypothesised that this non-significant difference amongst sperm quality may be due to the low amount of seminal plasma added (20%), or the pooled effect of whole seminal plasma used which may have eliminated individual variation of seminal plasma characteristics from different males (Muiño-Blanco et al., 2008). Another explanation may be that the addition of seminal plasma prior to freezing gives a better frozen-thawed sperm quality than when added after thawing (Petyim et al., 2014). Nevertheless, the physiological significance of seminal plasma is still a mater of speculation, and further investigation is needed to determine, quantify and purify seminal plasma based on individual male characteristics in relation to its

beneficial use as a supplement towards improving post-thawed sperm quality for cervical insemination.

Results herein confirm that supplementing frozen-thawed sperm with seminal plasma maintained sperm functions and motility characteristics during a post-thawing incubation period. Also, there was no beneficial effect of washing by centrifugation or the removal of freezing component before the addition of seminal plasma on frozen-thawed sperm quality.

Chapter VI

CONCLUSIONS

1. Melatonin implantation to sperm donor rams during a non-breeding season generally reversed seasonal effect and maintained sperm parameters during 24h of chilled liquid storage, in addition, no difference on semen quality was observed between donor rams from melatonin treated (non-breeding season) when compared to sperm donors in breeding season.

2. The sperm obtained by electroejaculation showed higher motility after 4h of refrigeration (pre-freezing) than those obtained via artificial vagina, whereas, at post-thaw, no difference was observed between both methods. However, frozen-thawed sperm obtained via electoejaculation displayed better stability during cryopreservation due to its low capacitation or acrosome reaction status than those obtained via artificial vagina.

3. The inclusion of electroejaculation as a viable alternative and swift methodology for semen collection especially from a large number of untrained males towards the ongoing conservation program and establishment of *Aranesa* sperm cryobank is highly recommendable.

4. The presence of seminal plasma prior to cryopreservation assisted in the maintenance of sperm motility and mitochondrial functionality despite the adverse effect of a 4h thermal incubation period compared to sperm frozen without seminal plasma.

5. Semen washing by centrifugation prior to cryopreservation is not necessary due to its non-beneficial and negative effect on post-thawed sperm quality.

6. Supplementing frozen-thawed sperm with seminal plasma assisted in maintaining sperm functions and motility characteristics during a post-thawing incubation period.

7. The presence or addition of seminal plasma irrespective of season or method of collection on frozen-thawed sperm delayed capacitation-like changes and acrosome reaction during the post-thawing thermal incubation period.

8. There is no beneficial effect of washing by centrifugation or removal of freezing component of frozen-thawed sperm before supplementing with seminal plasma, and this process may only cause loss or damage of sperm as well as other vital components which helps in maintaining sperm quality.

9. Results in this study indicate that the sperm motility parameters evaluated by CASA, sperm functional characteristics analyzed using flow cytometry, determination of sperm head free cysteine radicals and a thermal resistance test may be useful in predicting the fertilizing capacity of sperm prior to further application.

Chapter IX

REFERENCES

Abecia, J.A., Valares, J.A., Forcada, F., Palac´ın, I., Martin, S., Martino, A., 2007. The effect of melatonin on the reproductive performance of three sheep breeds in Spain. Small Rumin. Res. 69, 10–16.

Agarwal, A., Saleh, R.A., Bedaiwy, M.A., 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril. 79, 829-843.

Agarwal, A., Virk, G., Ong, C., du Plessis, S.S., 2014. Effect of Oxidative Stress on Male Reproduction. World J. Mens Health, 32(1), 1-17.

Ahmed, T., Islam, R., Lone, F.A., Malik, A.A., 2016. Effect of washing on the post-thaw quality of cryopreserved ram epididymal spermatozoa. Veterinary World, 9 (5), 519-523.

Aisen, E. G., Veturino, A., 2004. Recolección y evaluación del semen. En Reproducción ovina y caprina. Ed. Aisen, E. Inter-Medica. Buenos Aires, Argentina. pp. 55-69.

Aisen, E.G., Alvarez, H.L., Venturiono, A., Grade, J.J., 2000. Effect of trehalose and EDTA on cyroprotective action of ram semen diluents. Theriogenology 53, 1053-1061.

Aitken, R., Ryan, A., Baker, M., McLaughlin, E., 2004. Redox activity associated with the maturation and capacitation of mammalian spermatozoa. Free Radic. Biol. Med. 36, 994–1010.

Aitken, R J., 1990. Motility parameters and fertility. In: Gagnon C, editor. Control of sperm motility: biological and clinical aspects. Boca Raton: CRS Press; p. 285–302.

Aitken, R.J., Smith, T.B., Jobling, M.S., Baker, M.A., De Iuliis, G.N., 2014. Oxidative stress and male reproductive health. Asian J Androl. 16(1), 31–38.

Alghamdi, A.S., Foster, D.N., Troedsson, M.H.T., 2004. Equine seminal plasma reduces sperm binding to polymorphonuclear neutrophils and improves fertility of fresh semen inseminated into inflamed uteri. Reproduction 127, 593-600.

Allai, L., Druart, X., Contell, J., Louanjli, N., Moula, A.B., Badi, A., Essamadi, A., Nasser, B., Amiri, B., 2015. Effect of argan oil on liquid storage of ram semen in Tris orskim milk based extenders. Anim. Reprod. Sci. 160, 57–67.

Alvarez, J.G.; Storey, B.T., 1984. Assessment of cell damage caused by spontaneous lipid peroxidation in rabbit spermatozoa. Biology of Reproduction. 30, 323-331.

Alvarez, J.G., Storey, B.T., 1992. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J. Androl. 13, 232-241.

Álvarez, M., Tamayo-Canul, J., Martínez-Rodríguez, C., López-Urue˜na, E., Gomes-Alves, S., Anel, L., Martínez-Pastor, F., De Paz, P., 2012. Specificity of the extender used for freezing ram sperm depends of the spermatozoa source (ejaculate, electroejaculate or epididymis). Anim Reprod Sci 132, 145–154.

Amann, R.P., 1999. Cryopreservation of sperm. En: Knobil E and Neill JD (ed), Encyclopaedia of Reproduction, Vol. 1. Academic Press San Diego, pp. 773-783.

Anger, J., Ronot, X., Dadoune, J.P., 1989. Human sperm mitochondrial function related to motility: a flow and image cytometric assessment. J. Androl. 10, 439- 448.

Asher, G.W., Kraemer, D.C., Magyar, S.J., Brunner, M., Moerbe, R., Giaquinto, M., 1990. Intrauterine insemination of farmed fallow deer (Dama dama) with frozen-thawed semen via laparoscopy. Theriogenology, 34, 569-577.

Ashworth, P.J., Harrison, R.A., Miller, N.G., Plummer, J.M. & Watson, P.F., 1994. Survival of ram spermatozoa at high dilution: protective effect of simple constituents of culture media as compared with seminal plasma. Reprod. Fertil. Dev. 6, 173-180.

Avellanet, R., Arangune-Méndez, J.A., Jordana, J., 2005. La raza ovina Xisqueta en España: Caracterizacion estructural de las explotaciones. Boletín de información sobre Recursos Genéticos Animales., 37, 21-30.

Awad, H., Halawa, F., Mostafa, T., Atta, H., 2006. Melatonin hormone profile in infertile males. J. Androl. 29, 409–413.

Awda, B.J., Mackenzie-Bell, M., Buhr, M.M., 2009. Reactive oxygen species and boar sperm function. Biol. Reprod. 81 (3), 553–561.

Axner, E., Linde Forsberg, C., 2007. Sperm morphology in the domestic cat, and its relation with fertility: a retrospective study. Reprod Domest Anim. 42: 282- 91.

Backman, T., Bruemmer, J.E., Graham, J.K., Squires, E.L., 2004. Pregnancy rates of mares inseminated with semen cooled for 18 h and then frozen. J. Anim. Sci. 82, 690–694.

Bag, S., Joshi, A., Naqvi, S.M.K., Mittal, J.P., 2004. Effect of post-thaw incubation on sperm kinematics and acrosomal integrity of ram spermatozoa cryopreserved in medium-sized French straws. Theriogenology 62, 415-424.

Bailey, J., Morrier, A., Cormier, N., 2003. Semen cryopreservation: successes and persistent problems in farm species. Can. J. Anim. Sci.83, 393–401.

Bains, H.K., Pabst, M.A., Bawa, S.R., 1993. Changes in the lecitin binding sites of the testicular, epididymal, vas, and ejaculated spermatozoon surface of dog. Andrologia. 25, 19-24.

Balhorn R., 2007. The protamine family of sperm nuclear proteins. Genome Biol, 8, 227–238.

Barbas, L., Mascarenhas, S., 2009. Cryopreservation of domestic animal sperm cells Cell Tissue Bank. 10, 49–62.

Barrios, B., Fernandez-Juan, M., Muiño-Blanco, T., Cebrián-Pérez, J.A., 2005. Immunocytochemical localization and biochemical characterization of two seminal plasma proteins that protect ram spermatozoa against cold shock. J. Androl. 26, 539-549.

Barrios, B., Pérez-Pé, R., Gallego, M., Tato, A., Osada, J., Muino-Blanco, T., Cebrián-Pérez, J.A., 2000. Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol. Reprod. 77, 1531-1537.

Baumber, J., Ball, B.A., Gravance, C., Medina, V., Davies-Morel, M., 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. J. Androl. 21, 895-902.

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

Bellvé, A.R., O´Brien, D.A., 1983. The mammalian spermatozoon: structure and temporal assembly. In: Mechanism and control of animal fertilization. Ed JF Hartman. Academic Press, 55-137.

Beltrán de Heredia, I., 2009. Reproducción y control reproductivo en el macho. En Ovinotecnia (Produccion y Economia de la Especie). Eds: Sañudo, C.; Cepero Briz, R. Prensas Universitarias de Zaragoza. Zaragoza, España. pp: 105-114.

Bilodeau, J.F., Blanchette, S., Cormier, N., Sirard, M.A., 2002. Reactive oxygen species-mediated loss of bovine sperm motility in egg yolk Tris extender: Protection by pyruvate, metal chelators and bovine liver or oviductal fluid catalase. Theriogenology. 57, 1105-1122.

Bilodeau, J.F., Blanchette, S., Gagnon, C., Sirard, M.A., 2001. Thiols prevent H_2O_2-mediated loss of sperm motility in cryopreserved bull semen. Theriogenology. 56, 275-286.

Bochenek, M., Smorag, Z., Pilch, J., 2001. Sperm chromatin structure assay of bulls qualified for artificial insemination. Theriogenology. 56:557-67.

Boland, M.P., Al-Kamali, A.A., Crosby, T.F., Haynes, N.B., Howles, C.M., 1985. The influence of breed, season and photoperiod on semen characteristics, testicular size, libido and plasma hormone concentration in rams. Anim Reprod Sci. 9, 241-252

Boyers, S.P., Davis R., Katz D.F., 1989. Automated semen analysis. Curr. Probl. Obstet. Gynecol. Fertil. 12, 172-200.

Brewer, L., Corzett, M., Lau, E.Y., Balhorn, R., 2003. Dynamics of protamine-1 binding to single DNA molecules. J. Biol. Chem. 278, 42403–42408.

Brito, L.F.C., Silva, A.E., Rodrigues, L.H., Veira, F.V., Deragon, L.A., Kastelic, J.P., 2002: Effects of environmental factors, age and genotype on sperm production and semen quality in *Bos indicus* and *Bos taurus* AI bulls in Brazil. Anim. Reprod. Sci. 70, 181-190.

Bucak, M.N., Sarıozkan, S., Tuncer, P.B., Uluta, P.A., 2009. Effect of antioxidants on microscopic semen parameters, lipid peroxidation and antioxidant activities in Angora goat semen following cryopreservation. Small Rum. Res. 81, 90–95.

Budworth, P.R., Amann, R.P., Chapman, P.L., 1988. Relationships between computerized measurements of motion of frozen-thawed bull spermatozoa and fertility. J. Androl. 9, 41–53.

Cabrera, F., González, F., Batista, M., Calero, P., Medrano, A., Gracia, A., 2005. The effect of removal of seminal plasma, egg yolk level and season on sperm freezability of canary buck (Capra hircus). Reprod. Domest. Anim. 40, 191-195.

Casao, A., Mendoza, N., Pérez-Pé, R., Grasa, P., Abecia, J.A., Forcada,F., Cebrián-Pérez, J.A., Mui˜no-Blanco, T., 2010. Melatonin prevents capacitation and apoptotic-like changes of ram spermatozoa and increases fertility rate. J. Pin. Res. 48, 39–46.

Casao, A., Vega, S., Palacín, I., Pérez-Pe, R., Lavi˜na, A., Quintín, F.J., Sevilla, E., Abecia, J.A., Cebrián-Pérez, J.A., Forcada, F., Mui˜no-Blanco, T., 2008. Effects of melatonin implants during non-breeding season on sperm motility and reproductive parameters in Rasa Aragonesa rams. Reprod. Domest. Anim. 45, 425–432.

Casas I, Sancho S, Briz M, Pinart E, Bussalleu E, Yeste M, et al., 2009. Freezability prediction of boar ejaculates assessed by functional sperm parameters and sperm proteins. Theriogenology, 72, 930–948.

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

Chemineau, P., 1986. Sexual behaviour and gonadal activity during the year in the tropical Creole meat goat. 2. Male mating behaviour, testis diameter, ejaculate characteristics and fertility. Reprod. Nutr. Develop. 26, 453-460.

Chemineau, P., Malpaux, B., Delgadillo, J.A., Guerin, Y., Pavault, J.P., Thimonier, J., Pelleiter, J., 1992. Control of sheep and goat reproduction: use of light and melatonin. Anim. Reprod. Sci. 30, 157-184.

Cheryl, A.T., Garner, D.L., Dejarnette, J.M., Clifton, E.M., 1997. Fluorometric assessments of acrosomal integrity and viability in cryopreserved bovine spermatozoa. Biol. Reprod. 56, 991-998.

Christensen, P., J.P. Stenvang, and W.L. Godfrey. 2004. A flow cytometric method for rapid determination of sperm concentration and viability in mammalian and avian semen. J Androl. 25:255-64.

Coloma, M.A., Toledano-Díaz, A., Castaño, C., Velázquez, R., Gómez-Brunet, A., López-Sebastián, A., Santiago-Moreno, J., 2011. Seasonal variation in reproductive physiological status in the Iberian ibex (*Capra pyrenaica*) and its relationship with sperm freezability. Theriogenology 76, 1695-1705.

Cooper, T.G., 1986. The epididymis, sperm maduration and fertilisation. Springer verlag, Heidelberg, Alemania.

Correa, L.M., Thomas, A., Meyers, S.A., 2007. The macaque sperm actin cytoskeleton reorganizes in response to osmotic stress and contributes to morphological defects and decreased motility. Biol Reprod. 77, 942–953.

Corteel, J.M., 1981. Goat Production: Collection, Processing and Artificial Insemination of Goat semen. Ed. Academic Press, London, 171-191.

Cross, N., Morales, P., Overstreet, J., Hanson, F., 1986. Two simple methods for detecting acrosome-reacted human-sperm. Gamete Res. 15, 213–226.

Cumming, R.C., Andon, N.L., Haynes, P.A., Shubert, D., 2004. Protein Disulfide Bond Formation in the Cytoplasm during Oxidative Stress. Journal of Biological Chemistry, 279(21), 21749-58.

Curry, M.R., 2000. Cryopreservation of semen from domestic livestock. Reviews of Reproduction 5, 46-52.

D'Alessandro, A.G., Martemucci, G., 2003. Evaluation of seasonal variations of season freezability in Leccese ram. Anim. Reprod. Sci. 79, 93–102.

DAADER, A. H., MARAI, I. F. M., EL-JIBOURI, S. A. H. and EL-KERABY, F., 1985. Ram semen characteristics affected by some climatic elements in sub-tropical conditions. Egyptian J. Anim. Prod., 25 (1): 105-116.

David, I., Bodin, L., Lagriffoul, G., Leymarie, C., Manfredi, E., Robert-Granié, C., 2007. Genetic analysis of male and female fertility after artificial insemination in sheep: comparison of single trait and joint models. J. Dairy Sci. 90, 3917-23.

Davis, R.O., Katz, D.F., 1993. Operational standards for CASA instruments. J. Androl., 14, 385-394.

De Leeuw, F.E., De Leeuw, A.M, Den Dass, J.H.G., Colenbrander, B., Verkleij, A.J., 1993. Effects of various cryoprotective agents and membrane-stabilizing compounds on bull sperm membrane integrity after cooling and freezing. Cryobiology 30, 32-44.

Deka, B.C., Rao, A.R., 1986. Effect of glycerol level in Tris-based extender and equilibration period on quality of frozen goat semen. Theriogenology 26, 231-238.

Delgadillo, J. A., 2004. Características anatómicas y funcionales del sistema reproductor del macho. En: Reproduccion Ovina y Caprina. Ed. Aisen, E. Inter-Medica. Buenos Aires, Argentina. pp: 1-9.

Delgadillo, J.A., Leboeuf, B., Chemineau, P., 1991. Decrease in the seasonality of sexual behavior and sperm production in bucks by exposure to short photoperiodic cycles. Theriogenology. 36(5), 755-770.

Dominguez, M.P., Falcinelli, A., Hozbor, F., Sanchez, E., Cesari, A., Alberio, R.H., 2008. Seasonal variations in the composition of ram seminal plasma and its effect on frozen–thawed ram sperm. Theriogenology 69, 564–573.

Domınguez-Rebolledo, A.E., Fernandez- Santos, M.R., Garcıa-Alvarez, O., Maroto- Morales, A., Garde, J.J., Martınez-Pastor, F., 2009. Washing increases the susceptibility to exogenous oxidative stress in red deer spermatozoa. Theriogenology 72, 1073– 1084.

Dorado, J., Muñoz-Serrano, A., Hidalgo, M., 2010. The effect of cryopreservation on goat semen characteristics related to sperm freezability. Anim. Reprod. Sci. 121, 115–123.

Dorigo, B., Schalch, T., Kulangara, A., Duda, S., Schroeder, R.R., Richmond, T.J., 2004. Nucleosome arrays reveal the two-start organization of the chromatin fiber. Science, 306, 1571–1573.

Drobnis, E.Z., Crowe, L.M., Berger, T., Anchordoguy, T.J., Overstreet, J.W., Crowe, J.H., 1993. Cold shock damage is due to lipid phase-transitions in cell membranes – a demonstration using sperm as a model. J. Exp. Zool. 265, 432-437.

Egerszegi, I., Sarlós, P., Rátkya, J., Solti, L., Faigl, V., Kulcsár, M., Cseh, S., 2014. Effect of melatonin treatment on semen parameters and endocrine function in Black Racka rams out of the breeding season. Small Rumin. Res. 116, 192-198.

El-Bahrawy, K.A., 2010. Effect of seminal plasma centrifugation for viscosity elimination on cryopreservation of dromedary camel semen. Nature and Science 8, 196-202.

El-Rajj Ghaoui, R., Thomson, P., Leahy, T., Evans, G., Maxwell, W., 2007. Autologous whole ram seminal plasma and its vesicle free fraction improve motility characteristics and membrane status but not in vivo fertility of frozen thawed spermatozoa. Reprod. Domest. Anim. 42, 541–549.

Encyclopaedia of Reproduction, Vol. 1. Academic Press San Diego, pp. 773-783.

Ericsson, S.A., Garner, D.L., Redelman, D., Ahmad, K., 1989. Assessment of the viability and fertilizing potencial of cryopreserved bovine spermatozoa using dual fluorescent staining and two-flow cytometric systems. Gamete Res. 22, 355-368.

ESHRE andrology special interest group. European society for human reproduction and embryology, 1998. Guideliness on the application of CASA technology in the analysis of spermatozoa. Hum. Reprod., 13: 142-145.

Esteban, C., 2003. Razas ganaderas españolas ovinas. Feagas, editor. MAPA.

Evans, G., Maxwell, W.M.C., 1987a. Frozen storage of semen. En: Salamon's Artificial Insemination of Sheep and Goats. Butterworths, Wellington, pp. 122-141.

Evans, G., Maxwell, W.M.C., 1987b. Salamon's Artificial Insemination of Sheep and Goats, Butterworths, Sydney, 107–141.

Evenson, D.P., Darzynkiewicz, Z., Melamed, M.R., 1982. Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. J. Histochem. Cytochem. 30, 279-280.

FAO, 2007. Animal production and health guidelines: Breeding strategies for sustainable management of animal genetic resources. Rome.

Farlin, F.M., Jasko, D.J., Graham, J.K., Squires, E.L., 1992. Assessment of *Pisum sativum* agglutinin in identifying acrosomal damage in stallion sperm. Mol. Reprod. Dev., 32: 23-27.

Farrell, P.B., Foote, R.H., McArdle, M.M., Trouern-Trend, V.L., Tardiff, A.L. 1996. Media and dilution procedures tested to minimize handling effects on human, rabbit and bull sperm for computer-assisted sperm analysis (CASA). J. Androl., 17, 293-300.

Farrell, P.B., Trouern-Trend, V., Foote, R.H., Douglas Hamilton, D., 1995. Repeatibility of measurements on human, rabbit, and bull sperm by computerassisted sperm analysis when comparing individual fields and means of 12 fields. Fertil. Steril. 64(1), 208-210.

Fernadez-Abella, D., Villegas, N., Echeverria, D., Robaina, J., 1993. Evaluation of the effect of seasonal variation on semen production in rams of four breeds. Boletin, Tecnico-deciencias-Biologicas-Universidad-de-la- Republica, Salto. 3(1): 23-34.

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

Ferreira, A.L.A., Matsubara, L.S., 1997. Free radicals: concepts, relationed diseases, defence system and oxidative stress. Rev. Assoc. Med. Bras. 43 (1), 1–16.

Fiser, P,S., Hansen, C., Underhill, H., Marcus, G.J., 1991. New thermal stress test to assess the viability of cryopreserved boar sperm, Cryobiology 28, 454-459.

Fiser, P.S., Batra, T.R., 1984. Effect of equilibration time at 5 ◦C and photoperiod on survival of ram spermatozoa frozen in straws. Can. J. Anim. Sci. 64, 777–780.

Fiser, P.S., Fairfull, R.W., 1983. Effects of changes in photoperiod on freezability of ram spermatozoa. Cryobiology 20, 684-689.

Fiser, P.S., Fairfull, R.W., 1984. The effect of glycerol concentration and cooling velocity on cryosurvival of ram spermatozoa frozen in straws. Cryobiology 21, 542-551.

Fitzgerald, J.A., Stellflug, J.N., 1991. Effects of melatonin on seasonal changes in reproduction of rams. J. Anim. Sci., 69, 264–275.

Flajshans M, Cosson J, Rodina M, Linhart O., 2004. The application of image cytometry to viability assessment in dual fluorescence-stained fish spermatozoa. Cell Biol Int. 28: 955–959.

Flesch, F.M., Voorhout, W.F., Colenbrander, B., van Golde, L.M., Gadella, B.M., 1998. Use of lectins to characterize plasma membrane preparations from boar spermatozoa: a novel technique for monitoring membrane purity and quantity. Biol. Reprod. 59, 1530-1539.

Flores, E., Fernández-Novell, J.M., Peña, A., Rigau, T., Rodríguez-Gil, J.E., 2011. Cryopreservation –induced alterations in boar spermatozoa mitochondrial function are related changes in the expression and location of midpiece mitofusion-2 and actin network. Theriogenology, 74, 354-363.

Folch, J., 2000. Manejo del morueco. XXV Jornadas Científicas SEOC, Producción ovina y caprina. pp: 61-64.

Foote, R.H., 1978. Factors influencing the quantity and quality of semen harvested from bulls, rams, boars and stallions. J. Anim. Sci., 47(2), 1-11.

Foote, R.H., Kaproth, M.T., 2002. Large batch freeing of bull semen: effect of time of freezing and fructose on fertility. J. Dairy Sci. 85, 453–456.

Forouzanfar, M., Sharafi, M., Hosseini, S.M., Ostdadhosseini, S., Hajian, M., Hosseini,L., Abedi, P., Nili, N., Rahmani, H.R., Nasr-Esfahani, M.H., 2010. In vitro comparison of egg yolk–based and soybean lecithin–based extenders for cryopreservation of ram semen. Theriogenology 73, 480–487.

Franca, L.R., Becker-Silva, S.C., Chiarini-García, H., 1999. The length of the cycle of seminiferous epithelium in goats (Capra hircus). Tissue and Cell, 31(3), 274-280.

Fraser L, Dziekońska A, Strzeżek R, Strzeżek J, 2007. Dialysis of boar semen prior to freezing-thawing. Its effects on post-thaw sperm characteristics. Theriogenology 67, 994-1003.

Fraser, L., Gorszczaruk, K., Strzezek, J., 2001. Relationship between motility and membrane integrity of boar spermatozoa in media varying in osmolatily. Reprod Dom Anim., 36, 325-329.

Fuerst-Waltl, B., Schwarzenbacher, H., Perner, C., Slkner, J., 2006. Effects of age and environmental factors on semen production and semen quality of Austrian Simmental bulls. Anim. Reprod. Sci. 95, 27-37.

Gadea, J., 2003. Semen extenders used in the artificial insemination of swine. A review. Span. J. Agric. Res., 1, 17–27.

Gadea, J., 2005. Sperm factors related to in vitro and in vivo porcine fertility. Theriogenology, 63: 431–444.

Gadea, J., Gumbao, D., Matas, C., Romar, R., 2005. Supplementation of the thawing media with reduced glutathione improves function and the in vitro fertilizing ability of boar spermatozoa after cryopreservation. J. Androl. 26, 749–756.

Gadea, J., Sellés, E., Marco, M.A., Coy, P., Matás, C., Romar, R., Ruiz, S., 2004. Decrease in glutathione content in boar sperm after cryopreservation Effect of the addition of reduced glutathione to the freezing and thawing extenders Theriogenology, 62, 690–701.

Gao, D.Y., Lin, S., Watson, P.F., Critser, J.K., 1995. Fracture phenomena in an isotonic salt solution during freezing and their elimination using glycerol. Cryobiology 32, 270-284.

García, V., 2014: Optimización de los protocolos de crioconservación de semenovino de las razas autóctonas en peligro de extinción Xisqueta y Aranesa. Universidad Autónoma de Barcelona, Barcelona, Tesis doctoral.

Garde, J.J., Soler, A.J., Cassinello, J., Crepo, C., Malo, A.F., Espeso, G., Gomenndio, M., Roldán, E.R.S., 2003. Sperm Cryopreservation in Three Species of Endangered Gazelles (Gazella cuvieri, G. dama mhorr, and G. dorcas neglecta). Biol Reprod 69, 602-611.

Garner, D., Dobrinsky, J., Welch, G., Johnson, L., 1996. Porcine sperm viability, oocyte fertilization and embryo development after staining spermatozoa with SYBR-14. Theriogenology. 45, 1103–1113.

Garner, D., Johnson, L., 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol. Reprod., 53, 276–84.

Garner, D., Pinkel, D., Johnson, L., Pace, M., 1986. Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analyses. Biol. Reprod., 34, 127-138.

Garner, D., Johnson, L., Allen, L.A., Palencia, C.H., Chambers, C. S., 1996. Comparison of seminal quality in Holstein bulls as yearlings and as mature sires. Theriogenology 45, 923-934.

Garner, D.L., C.A. Thomas, H.W. Joerg, J.M. DeJarnette, and C.E. Marshall. 1997. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol. Reprod. 57:1401-6.

Garner, D.L., Hafez, E.S.E. 1993. Spermatozoa and seminal plasma. En: Reproduction in Farm Animals, 6th edition. Hafez ESE (ed). Lea and Febiger. (Philadelphia. USA). 165-187.

Garner, D.L., Johnson, L.A., Yue, S.T., Roth, B.L., Haugland, R.P., 1994. Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. J. Androl. 15:620-9.

Gatti, J.L., Castella, S., Dacheux, F., Ecroyd, H., Métayer, S., Thimon, V., Dacheux, J.L., 2004. Post-testicular sperm environment and fertility. Anim. Reprod. Sci. 82, 321-39.

Gillan, L., Evans, G., Maxwell, W.M.C., 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology, 63, 445-457.

Glover, T.D., Barrat, C.L.R., Tyler, J.P.P., Hennessey, J.F., 1990. Sperm production and its control, En: Human Male Fertility and Semen Analysis, Academic Press, London.

González Urdiales, R., Tejerina, F., Domínguez, J.C., Alegre, B., Ferreras, A., Peláez, J., Bernal, S., Cárdenas, S., 2006. Técnicas de análisis rutinario de la calidad espermática: motilidad, vitalidad, concentración, resistencia osmótica y morfología espermática. En: "Manual de técnicas de reproducción asistida en porcino". Eds: Bonet, S.; Martínez, E.; Rodríguez, J.E.; Barrera, X. Universidad de Gerona y Red temática nacional de reproducción porcina, Gerona, España. pp: 19-38.

González-Recio, O., Alenda, R., 2005. Genetic parameters for female fertility traits and a fertility index in Spanish dairy cattle. J. Dairy Sci. 88, 3282-3289.

Graham, J., 2001. Assessment of sperm quality: a flow cytometric approach. Anim Reprod Sci., 68, 239–247.

Graham, J.K., Kunze, E., Hammerstedt, R.H., 1990. Analysis of sperm cell viability, acrosomal integrity and mitochondrial function using flow cytometry. Biol. Reprod., 43, 55-64.

Gravance, C.G., Davis, R.O., 1995. Automated sperm morphometry analysis (ASMA) in the rabbit. J. Androl. 16, 88–93.

Greyling, J., Gobbelaar, J., 1983. Seasonal variation in semen quality of Boer and Angora goat rams using different collection techniques. S. Afr. J. Anim. Sci. 13, 250–252.

Guerra, M.M.P., Evans, G., Maxwell, W.M.C., 2004. The role of oxidants and antioxidants on andrology. Rev. Bras. Reprod. Anim. 28 (4), 187–195.

Gundogan, M., 2006. Some reproductive parameters and seminal plasma constituents in relation to season in Akkaraman and Awassi Rams. Turk. J. Vet. Anim. Sci. 30, 95-100.

Guthrie, H.D., Welch, G.R., 2005. Impact of storage prior to cryopreservation on plasma membrane function and fertility of boar sperm. Theriogenology 63, 396–410.

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

Hafez, B. & Hafez, E. S. E., 2000. Reproduction in Farm Animals. 7th edition. Lippincott Williams and Wilkens, New York. pp. 509.

Hammerstedt, R.H., Graham, J.K., Nolan, J.P., 1990. Cryopreservation of mammalian sperm: what we ask them to survive. J Androl 11, 73-88.

Henkel, R.R., 2011. Leukocytes and oxidative stress: dilemma for sperm function and male fertility. Asian J Androl. 13, 43–52.

Hinshaw, D.B., Sklar, L.A., Bohl, B., Schraufstatter, I.U., Hyslop, P.A., Rossi, M.W., Spragg, R.G., Cochrane, C.G., 1986. Cytoskeletal and morphologic impact of cellular oxidant injury. Am J Pathol, 123: 454– 464.

Holt, W., Pickard, R., 1999. Role of reproductive technologies and genetic resource banks in animal conservation. Rev. Reprod. 4, 143–150.

Holt, W.V, North, R.D., 1994. Effects of temperature and restoration of osmotic equilibrium during thawing on the induction of plasma membrane damage in cryopreserved ram spermatozoa. Biol. Reprod. 51, 414-424.

Holt, W.V., 2000a. Fundamental aspects of sperm cryobiology. The importance of species and individual differences. Theriogenology 53, 47-58.

Holt, W.V., 2000b, Basic aspects of frozen storage of semen. Anim. Reprod. Sci. 62, 3-22.

Holt, W.V., Brien, J.O., Abaigar, T., 2007. Applications and interpretation of computer assisted sperm analyses and sperm sorting methods in assisted breeding and comparative research. Reprod. Fertil. Dev., 19: 709-718.

Holt, W.V., Head, M.F, North, R.D., 1992. Freeze-induced membrane damage in ram spermatozoa is manifested after thawing: observations with experimental cryomicroscopy. Biol. Reprod. 46, 1086-1094.

Holt, W.V., Watson, P., Curry, M., Holt, C., 1994. Reproducibility of computer-aided semen analysis: comparison of five different systems used in practical workshop. Fertil. Steril. 62, 1277-1282.

Hoogewijs, M., Rijsselaere, T., De Vliegher, S., Vanhaesebrouck, E., De Schauwer, C., Govaere, J., Thys, M., Hoflack, G., Soom, A.V. and de Kruif, A., 2010. Influence of different centrifugation protocols on equine semen preservation. Theriogenology 74 (1), 118-126.

HOWARTH, B., 1969. Fertility in ram following exposure to elevated ambient temperature and humidity. J. Reprod. Fertil., 19 (1): 179– 183.

Hu, L., Wong, P., Moore, P., Bian, J., 2007. Hydrogen sulfide attenuates lipopolysaccharideinduced inflammation by inhibition of p38 mitogen-activated protein kinase in microglia. J. Neurochem, 100, 1121–8.

Jane, E.J., William, R. B. & Dawn, W. B., 1996. Manual versus Computer-automated semen analyses. Determination or the working range of a computer-automated semen analyzer. Fertil. Steril. 65, 156-159.

Janett, F., Thun, R., Niederer, K., Burger, D., Hässig, M., 2003. Seasonal changes in semen quality and freezability in the Warmblood stallion. Theriogenology 60, 453-461.

Jennings, J.J., Mcweeney, J., 1976. Effect of frequent ejaculation on semen characteristics in rams. Vet. Rec., 98, 230-233.

Jeyendran, R.S., Van der Ven, H.H., Perez-Pelaez, M., Crabo, B.G., Zaneveld, L.J.D., 1984. Development of an assays to asses the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil. 70, 219-228.

Jiménez-Rabadán, P., Ramón, M., García-Álvarez, O., Maroto-Morales, A., Del Olmo, E., Pérez-Guzmán, M.D., Bisbal, A., Fernández-Santos, M.R., Garde, J.J., Soler, A.J 2012. Effect of semen collection method (artificial vagina *vs.* electroejaculation), extender and centrifugation on post-thaw sperm quality of Blanca-Celtibérica buck ejaculates. Anim Reprod Sci 132, 88-95.

Jiménez-Rabadán, P., Soler, A., Ramón, M., García-Álvarez, O., Maroto-Morales, A., Iniesta-Cuerda, M., Fernández-Santos, M., Montoro, V., Pérez-Guzmán, M., Garde, J., 2016. Influence of semen collection method on sperm cryoresistance in small ruminants. Anim Reprod Sci 167, 103–108.

Joshi, A., Bag, S., Naqvi, S.M.K., Sharma, R.C., Mittal, J.P., 2005. Effect of post-thawing incubation on sperm motility and acrosomal integrity of cryopreserved Garole ram semen. Small Rumin. Res. 56, 231-238.

Juyema, N., Stelletta, C., 2012. Seminal Plasma: An Essential Attribute to Spermatozoa. J. Androl. 33 (4): 536-550.

Kankofer, M., Kolm, G., Aurich, J., Aurich, C., 2005. Activity of glutathione peroxidase, superoxide dismutase and catalase and lipid peroxidation intensity in stallion semen during storage at 5 ºC. Theriogenology, 63, 1354-1365.

Karagiannidis, A., Varsakeli, S., Alexopoulos, C., Amarantidis, I., 2000. Seasonal variation in semen characteristics of Chios and Friesian rams in Greece. Small Rumin. Res. 37, 125–130.

Kasimanickam, R., Kasimanickam, V., Tibary, A., Pelzer, K., 2011. Effect of semen extender on sperm parameter of ram during liquid storage at 4ºC. Small Rumin. Res. 99, 208–213.

Kaya, A., Aksoy, M., Baspinar, N., Yildiz, C., Ataman, M.B., 2001. Effect of melatonin implantation to sperm donor rams on post-thaw viability and acrosomal integrity of sperm cells in the breeding and nonbreeding season. Reprod. Domest. Anim. 36, 211–215.

Kaya, A., Aksoy, M., Tekeli, T., 2002. Influence of ejaculation frequency on sperm characteristics, ionic composition and enzymatic activity of seminal plasma in rams. Small Rumin Res. 44, 153-158.

Keeler, K.D., Mackenzie, N.M., Dresser, D.W., 1983. Flow microfluorometric analysis of living spermatozoa stained with Hoechst 33342. J. Reprod. Fert. 68, 205-212.

Keij, J.F., Bell-Prince, C., Steinkamp, J.A., 2000. Staining of mitochondrial menbranes with 10-nonil acridine orange, MitoFluor Green, and Mitotracker Green is affected by mitochondrial membrane potential altering drugs. Cytometry, 41, 148.

Kim, S.H., Yu, D.H., Kim, Y.J., 2010. Apoptosis-like change, ros, and DNA status in cryopreserved canine spermrecovered by glass wool filtration and percoll gradient centrifugation techniques. Anim Reprod Sci. 119, 106–114.

Kozdrowski, R., Dubiel, A., Bielas, W., Dzieciol, M., 2007. Two protocols of cryopreservation of goat semen with the use of computer-assisted semen analysis system. Acta Vet. Brno. 76, 601-604.

Krause, W., 1995. The significance of computer-assisted semen analysis (CASA) for diagnosis in andrology and fertility prognosis. Hum. Reprod. 10, 60-66.

Krause, W., Viethen, G., 1999. Quality assessment of computer-assisted semen analysis (CASA) in the andrology laboratory. Andrologia, 31, 125-129.

Kruger, T.F., Coetzee, K., 1999. The role of sperm morphometry in assisted reproduction. Human Reprod. Update, 5, 172–78.

Lahlou-Kassi, A., Marie, M., 1985. Sexual and ovarian function of the D'Man ewe. En Genetics of reproduction in sheep. R.B. Land and D.W. Robinson, ed., Publ. Butterworths, Londres. 245-260.

Lahnsteiner, F., Berger, B., Weismann, T., Patzner, R., 1996. The influence of various cryoprotectants on semen quality of rainbow trout (*Oncorhynchus mykiss*) before and after cryopreservation. J. Appl. Ichthyol. 12, 99-106.

Langford, G. A., Ainsworth, L., Marcus G. J. & Shrestha, J. N., 1987. Photoperiod entrainment of testosterone, luteinising hormone, follicle- stimulating hormone, and prolactin cycles in rams in relation to testis size and semen quality. Biol. Reprod. 37, 489-499.

Leahy, T., Marti, J., Evans, G., Maxwell, W., 2010. Seasonal variation in the protective effect of seminal plasma on frozen-thawed ram spermatozoa. Anim Reprod Sci 119, 147–153.

Leboeuf, B., Restall, B., Salamon, S., 2000. Production and storage of goat semen for artificial insemination. Anim Reprod Sci 62, 113–141.

Ledesma, A., Fernández-Alegre, E., Cano, A., Hozbor, F., Martínez-Pastor, F., Cesari, A., 2016. Seminal plasma proteins interacting with sperm surfacerevert capacitation indicators in frozen-thawed ram sperm. Anim. Reprod. Sci. 173, 35-41.

Ledesma, A., Manes, J., Cesari, A., Alberio, R., Hozbor, F., 2014. Electroejaculation increases low molecular weight proteins in seminal plasma modifying sperm quality in Corriedale rams. Reprod. Domest. Anim. 49, 324–332.

Lincoln, G.A., 1996. Melatonin regulation of gonadotropin and prolactin secretion in the ram: the dual site hypothesis. Acta Neurobiol. Exp., 56: 846.

Lindsay, D.R., Pelletier, J., Pisselet, C., Courot, M., 1984. Changes in photoperiod and nutrition and their effect on testicular growth of rams. J. Reprod. Fertil., 71: 351–356.

Lymberopoulos, A.G., Tsakmakidis, I.A., Khalifa, T.A.A., 2010. Effect of ram age on structural and functional competence of frozen–thawed spermatozoa in dairy sheep. Reprod Dom Anim., 45, 572–578.

Mahfouz, R., Sharma, R., Lackner, J., Aziz, N., Agarwal, A., 2008. Evaluation of chemiluminescence and flow cytometry as tools in assessing production of hydrogen peroxide and superoxide anion in human spermatozoa. Fertil. Steril. 92(2), 819-27.

Malejane, C.M., Greyling, J.P., Raito, M.B., 2014. Seasonal variation in semen quality of Dorper rams using different collection techniques. S. Afr. J. Anim. Sci., 44 (1): 26-32.

Mandiki, S.N.M., Derycke, G., Bister, J.L., Paquay, R., 1998. Influence of season and age on sexual maturation parameters of Texel, Suffolk and Ile-de-France rams: 1. Testicular size, semen quality and reproductive capacity. Small Rumin. Res. 28, 67–79.

Marai, I.F., El-Darawany, A.A., Fadiel, A., Abdel-Hafez, M.A.M., 2007. Physiological traits as affected by heat stress in sheep- a review. Small Rumin. Res., 71(1), 1-12.

Marco-Jiménez, F., Puchades, S., Gadea, J., Vicente, J., Viudes-de-Castro, M., 2005. Effect of semen collection method on pre- and post-thaw Guirra ram spermatozoa. Theriogenology 64, 1756–1765.

Marco-Jiménez, F., Vicente, J., Viudes-de-Castro, M., 2008. Seminal plasma composition from ejaculates collected by artificial vagina and electroejaculation in Guirra ram. Reprod. Domest. Anim. 43, 403–408.

Martinez-Pastor, F., M. Mata-Campuzano, M. Alvarez-Rodriguez, M. Alvarez, L. Anel, and P. de Paz., 2010. Probes and techniques for sperm evaluation by flow cytometry. Reprod Domest Anim. 45 Suppl 2:67-78.

Mata-Campuzano, M., Alvarez-Rodríguez, M., del Olmo, E., Fernández- Santos, M.R., Garde, J.J., Martínez-Pastor, F., 2012. Quality, oxidative markers and DNA damage (DNA) fragmentation of red deer thawed spermatozoa after incubation at 37^0C in presence of several antioxidants. Theriogenology 78, 1005–1019.

Mata-Campuzano, M., Soleilhavoup, C., Tsikis, G., Martinez-Pastor, F., de Graaf, S.P., Druart, X., 2015. Motility of liquid stored ram spermatozoa is altered by dilution rate independent of seminal plasma concentration. Anim. Reprod. Sci. 162, 31–36.

Mathevon, M., Dekkers, J.C.M., Buhr, M.M., 1998. Environmental, management and genetic factors affecting semen production in French Montbéliard bulls. Livest Prod Sci 55, 65- 77.

Matos, C.A.P., Thomas, D.L., 1992. Physiology and genetics of testicular size in sheep: a review. Livestock Prod. Sci., 32(1), 1-30

Matyus, L., Szabo, G.J., Resli, I., Gaspar, R.J., Damjanovich, S., 1984. Flow cytometric analysis of viability of bull sperm cells. Acta Biochim. Biophys. Acad. Sci. Hung., 19: 209–14.

Maxwell, W.M., Evans, G., Mortimer, S.T., Gillan, L., Gellatly, E.S., McPhie, C.A., 1999. Normal fertility in ewes after cervical insemination with frozen–thawed spermatozoa supplemented with seminal plasma. Reprod. Fertil. Dev. 11, 123–126.

Maxwell, W.M.C., de Graaf, S.P., Ghaoui Rel, H., Evans, G., 2007. Seminal plasma effects on sperm handling and female fertility. Soc. Reprod. Fertil. Suppl. 64, 13–38.

Maxwell, W.M.C., Watson, P.F., 1996. Recent progress in the preservation of ram semen. Anim. Reprod. Sci. 42, 55-65.

Maxwell, W.M.C., Welch, G.R., Johnson, L.A., 1997. Viability and membrane integrity of spermatozoa after dilution and flow cytometric sorting in the presence or absence of seminal plasma. Reprod. Fertil. Dev. 8, 1165-1178.

Mazur, P., 1980. Fundamental aspects of the freezing of cells with emphasis on mammalian ova and embryos. 9th Inter Congr. Animal Reprod. Artif. Insem. Madrid, pp 99-114.

Mazur, P., 1985. Basic concepts in freezing cells. In: Deep freezing of boar semen (edited by LA Johnson and K Larsson) Swedish University of Agricultural Sciences, Uppsala, Sweden, pp 91-111.

Hossain, S., Johannisson, A., Wallgren, M., Nagy, S., Siqueira, A.P., Rodriguez-Martinez, H., 2011. Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. Asian Journal of Andrology 13, 406–419.

Medrano, A., Terrazas, A., Soto, R., 2010. Principles and perspectives for the conservation of goat buck spermatozoa. Small Rumin. Res. 89, 140-143.

Mehta, P.R., Toshniwal, S.N., Honmode, J., 1972. Norms of semen of rams of Russian Merino, Malpura and their crossbreds reared under semi-arid conditions. Indian Vet. J., 1000-1007.

Memon, A.A., Wahid, H., Rosnina, Y., Goh, Y., Ebrahimi, M., Nadia, F., 2013. Effect of ascorbic acid concentrations, methods of cooling and freezing on Boer goat semen cryopreservation. Reprod. Domest. Anim. 48, 325-330.

Miranda-Vilela, A.L., Alves, P.C., Akimoto, A.K., Pereira, L.C., Nazaré Klautau-Guimarães, M., Grisolia, C.K., 2010. The effect of hydrogen peroxide-induced oxidative stress on leukocytes depends on age and physical training in healthy human subjects carrying the same genotypes of antioxidant enzymes' gene polymorphisms. Am. J. Hum. Biol. 22, 807–812.

Moore, A.I., Squires, E.L., Graham, J.K., 2005. Effect of seminal plasma on the cryopreservation of equine spermatozoa. Theriogenology 63, 2372–2381.

Moradi, A.R., Malekinejad, H., Farrokhi-Ardabili, F., Bernousi, I., 2013. Royal jelly improves the sperm parameters of ram semen during liquid storage and serves as an antioxidant source. Small Rumin. Res. 113, 346-352.

Morrier, A., Castonguay, F., Bailey, J.L., 2003. Conservation of fresh ram spermatozoa at 5^0C in the presence of seminal plasma. Can. J. Anim. Sci. 83, 221–227.

Mortimer, D., Curtis, E.F., Dravland, J.E., 1987. Specific labelling by peanut agglutinin of the outer acrosomal membrane of the human spermatozoon. J. Reprod. Fertil., 81, 127-135.

Mortimer, S.T., 2000. CASA practical aspects. J. Androl. 21, 515-524.

Moudgal, N.R., Sairam, M.R., 1998. Is there a true requirement for follicle stimulating hormone in promoting spermatogenesis and fertility in primates? Hum. Reprod.13 (04), 916-919.

Muiño-Blanco, T., Pérez-Pé, R., Cebrián- Pérez, J.A., 2008. Seminal plasma proteins and sperm resistance to stress. Reprod. Domest. Anim., 4, 18-31.

Nagy, S., Hallap, T., Johannisson, A., Rodríguez-Martínez, H., 2004. Changes in plasma membrane and acrosome integrity of frozen-thawed bovine spermatozoa during a 4 h incubation as measured by multicolor flow cytometry. Anim Reprod Sci. 80, 225-235.

Nagy, S., Jansen, J., Topper, E.K., Gadella, B.M., 2003. A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biol. Reprod. 68:1828-35.

Naing, S.W., Haron, A.W., Goriman, M.A.K., Yusoff, R., Abu Bakar, M.Z., Sarsaifi, K., Bukar, M.M., Thein, M., Kyaw, T., San, M.M., 2011. Effect of seminal plasma removal, washing solutions, and centrifugation regimes on Boer goat semen cryopreservation. Pertanika J. Trop. Agric. Sci., 34, 271-279.

Nair, S.J., Brar, A.S., Ahuja, C.S., Sangha, S.P., Chaudhary, K.C., 2006. A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. Anim Reprod Sci. 96, 21-29.

Natali, L., 2011. Sperm preparation techniques for artificial insemination – comparison of sperm washing, swim up, and density centrifugation methods. In: Artificial Insemination in Farm Animals.

Nikolovski, M., Atanasov, B., Dovenska, M., Petkov, V., Dovenski, T., 2013. Variations in semen quality parameters of ovchepolian pramenka rams according to the method of collection and the meteorological season. Mac Vet Rev 35, 79-86.

Nolan, J.P., Magargee, S.F., Posner, R.G., Hammerstedt, R., 1995. Flow cytometric analysis of transmembrane phospholipid movement in bull sperm. Biochemistry, 33, 9968-74.

O' Meara, C., Hanrahan, J., Donovan, A., Fair, S., Rizos, D., Wade, M., Boland, M., Evans, A., Lonergan, P., 2005. Relationship between in vitro fertilization of ewe oocytes and the fertility of ewes following cervical artificial insemination with frozen thawed semen. Theriogenology, 64, 1797–1808.

O' Meara, C., Hanrahan, J.P., Prathalingam, N.S., Owen, J.S., Donovan, A., Fair, S., Ward, F., Wade, M., Evans, A.C.O., Lonergan, P., 2008. Relationship between in vitro sperm functional tests and in vivo fertility of rams following cervical artificial insemination of ewes with frozen-thawed semen. Theriogenology, 69, 513–522.

O'Flaherty, C., Beconi, M., Beorlegui, N., 1997. Effect of natural antioxidants, superoxide dismutase and hydrogen peroxide on capacitation of frozen-thawed bull spermatozoa. Andrologia, 29, 269-275.

Osinowo, O.A., Ahmed, M.S., Ekpe, G.A., 1988. Semen quality and sperm output of Yankasa rams at different ages. Theriogenology, 29, 381–386.

Palacín, I., Abecia, J.A., Forcada, F., Casao, A., Cebrian-Perez, J.A., Muino-Blanco, T., Palacios, C., Pontes, J.M., 2008. Effect of exogenous melatonin treatment on out of season ram fertility. Ital. J. Anim. Sci. 7, 199–206.

Palomo, M., García, W., Tabarez, A., 2017. Effect of seminal plasma and butylated hydroxytoluene (BHT) concentration on ram sperm freezability. Small Rum Res 153, 66–70.

Parés, P.M., 2008. Caracterització estructural i racial de la raça ovina aranesa. Tesis Doctoral. Universitat Autònoma de Barcelona.

Parinaud, J., Le Lannou, D., Vieitez, G., Griveau, J.F., Milhet, P., Richoilley, G., 1997. Enhancement of motility by treating spermatozoa with an antioxidant solution (Sperm-Fit) following ejaculation. Hum Reprod. 12, 2434-2436.

Parkinson, T., 2009. Veterinary Reproduction and Obstetrics, 9th ed. Saunders Elsevier, London.

Pedigo, N.G., Vernon, M.W., Curry, T.E., 1989. Characterization of a computerized semen analysis system. Fertil. Steril. 52, 659-666.

Pelletier, J., Chemineau, P., Delgadillo, J.A., 1988. Seasonnality of sexual activity and its photoperiodic control in the adult ram and he-goat. Proc. 11th Int. Cong. Anim. Reprod. AI, Dublin, 5, 211-219.

Pena, F., Rodrıguez-Martinez, H., Tapia, J., Ortega-Ferrusola, C, Gonzalez-Fernandez, L., et al., 2009. Mitochondria in mammalian sperm physiology and pathology: a review. Reprod. Domest. Anim. 44: 345–9.

Peña, F.J., Rodriguez, H., 2006. Citometria de flujo: aplicaciones en el estudio del espermatozide porcino. En: Manual de Tecnicas de Reproducción Asistida en Porcinos. Eds: Bonet, S.; Martínez, E.; Rodríguez, J.E.; Barrera, X. Universidad de Gerona y Red temática nacional de reproducción porcina, Gerona, España. Pp: 133-143.

Perez, B., Mateos, E., 1996. Effect of photoperiod on semen production and quality in bucks of Verata and Malagueña breeds. Small Rumin. Res. 23 (1), 23-28.

Perez-Pe, R., Barrios, B., Muino-Blanco, T., Cebrian, J.A., 2001. Seasonal differences in ram seminal plasma revealed by partition in an aqueous two phase system. J. Chromatogr. Biomed. Sci. Appl. 760 (1), 113–121.

Peris, S.I., Bilodeau, J.F., Dufour, M., Bailey, J.L., 2007. Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation, and functional parameters in ram sperm. Mol. Reprod. Dev. 74, 878-892.

Pintado, B., De la Fuente, J., Roldan, E.R.S., 2000. Permeability of boar and bull spermatozoa to the nucleic acid stains propidium iodide or Hoechst 33258 or to eosin: accuracy in the assessment of cell viability. J. Reprod. Fertil. 118,145-152.

Poot, M., Zhang, Y.Z., Kramer, J.A., Wells, K.S., Jones, L., Hanzel, D.K., Lugade, A.G., Singer, V.L., 1996. Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. J. Histochem. Cytochem. 44, 1363-1372.

Pratap, N., Reddy, V.N.V., Sarma, P.A., Honnappa, T.G., 2000. Employment of the hypoosmotic swelling test (HOST) to evaluate sperm membrane integrity of fresh and frozen buffalo (*Bubalus bubalis*) bull semen. Buffalo J. 16, 207-213.

Purdy, P.H., 2006. The post-thaw quality of ram sperm held for 0 to 48h at 5ºC prior to cryopreservation. Anim. Reprod. Sci. 93, 114-123.

Purdy, P.H., Moce, E., Stobart, R., Murdoch, W.J., Moss, G.E., Larson, B., Ramsey, S., Graham, J.K., Blackburn, H.D., 2010. The fertility of ram sperm held for 24h at 5^0C prior to cryopreservation. Anim. Reprod. Sci. 118, 231-235.

Rabinovitch, L., 2006. Comparison of the sperm quality analyzer SQA-Vb and CASA for bull semen evaluation. Medical Electronic Systems Ltd.

Ramón, M., Pérez-Guzmán, M.D., Jiménez-Rabadán, P., Esteso, M.C., García-Álvarez, O., Maroto-Morales, A., Anel-López, L., Soler, A.J., Fernández-Santos, M.R., Garde, J.J., 2013. Sperm cell population dynamics in ram semen during the cryopreservation process. PLoS One 8, e59189.

Rasola, A., Bernardi, P., 2007. The mitochondrial permeability transition pore and its involvement in cell death and in disease pathogenesis. Apoptosis 12, 815–33.

Rebolledo, A.D., Sierra, L.N., Tamayo, A.C., Loria, A.A., Denis, S.E., Oses, R.B., Parra, E.G., Monsreal, L.P., Ugalde, J.R., 2007. Fertility in hair sheep inseminated with freeze spermatozoa rediluted with seminal plasma. Rev. Cient 17, 73–76.

Rege, J.E.O., Toe, F., Mukasa-Mugerwa, E., Tembely, S., Anindo, D., Baker, R.L., Lahlou-Kassi, A., 2000. Reproductive characteristics of Ethiopian highland sheep: II. Genetic parameters of semen characteristics and their relationships with testicular measurements in ram lambs. Small Ruminant Res., 37(3), 173-187.

Reiter, R.J., Tan, D.X., Manchester, L.C., Paredes, S.D., Mayo, J.C., Sainz, R.M., 2009. Melatonin and reproduction revisited. Biol. Reprod. 81, 445–456.

Rickard, J., Pini, T., Soleilhavoup, C., Cognie, J., Bathgate, R., Lynch, G., Evans, G., Maxwell, W., Druart, X., de Graaf, S., 2014. Seminal plasma aids the survival and cervical transit of epididymal ram spermatozoa. Reproduction 48, 469–478.

Rigau, T., Piedrafita, J., Reverter, J., Canal, M., Rodríguez-Gil, J.E., 1996. The rate of l-lactate production: a feasible parameter for the fresh diluted boar semen quality analysis. Anim. Reprod. Sci., 43, 161– 172.

Rijsselaere, T., van Soom, A., Maes, D., de Kruif, A., 2003. Effect of technical settings on canine semen motility parameters measured by the Hamilton-Thorne analyzer. Theriogenology, 60, 1553-1568.

Rodríguez, I., Dorado, J., Hidalgo, M., Pérez, C.C., Corral, S., Sanz, J., 2003. Influencia de la circunferencia escrotal y de los factores ambientales sobre la producción seminal del macho de raza Florida. IV Congreso Ibérico de Reproducción Animal, LasPalmas de Gran Canaria, España. Pp: 67.

Rodriguez-Gil, J.E., 2006. Mammalian sperm energy resources management and survival during conservation in refrigeration. Reprod. Domest. Anim. 41, 11–20.

Rodrıguez-Martınez H. Can we increase the estimated value of semen assessment? Reprod Domest Anim 2006; 41 (Suppl 2): 2–10

Rodríguez-Martínez, H., 2003. Laboratory semen assessment and prediction of fertility: still utopia? Reprod.Dom. Anim. 38, 312-318.

Roldán, E.R.S., Gomendio, M., Garde, J.J., Espeso, G., Ledda, S., Berlinguer, F., del Olmo, A., Soler, A.J., Arregui, L., Crespo, C., González, R., 2006. Inbreeding and reproduction in endarangered ungulates: Preservation of genetic variation through the organization of genetic resource banks. Reprod. Dom. Anim. 41, 82-92.

Rosa, H.J.D., Silva, C.C., Bryant, M.J., 2012. The effect of melatonin treatment in rams on seasonal variation of testicular size and semen production parameters. Small Rum. Res. 102, 197–201.

Rovegno, M., Beringui, W., Monteiro, A., Mota, C., Visintin, J.A., Ortiz, M.E., 2013. Assessment of post-thawed ram sperm viability after incubation with seminal plasma. Cell Tissue Bank 14, 333–339.

Rowe, P.J., Comhaire, F.H., Hargreave, T.B. & Mellows, H.J., 1993. WHO Manual for the standard investigation and the diagnosis of the infertile couple. Cambridge, United Kingdom: Cambridge University Press.

Said, T., Gaglani, A., Agarwal, A., 2010. Implication of apoptosis in sperm cryoinjury. Reprod. Bio. Med. 21, 456–462.

Salamon, S., Maxwell, W.M.C., 1995. Frozen storage of ram semen I. Processing, freezing, thawing and fertility after cervical insemination. Anim. Reprod. Sci. 37, 185-249.

Salamon, S., Maxwell, W.M.C., 2000. Storage of ram semen. Anim. Reprod. Sci. 62, 77-111.

Salazar, J.L., Teague, S.R., Love, C.C., Brinsko, S.P., Blanchard, T.L., Varner, D.D., 2011. Effect of cryopreservation protocol on postthaw characteristics of stallion sperm. Theriogenology 76, 409-418.

Saleh, R.A., Agarwal, A., Sharma, R.K., Nelson, D.R., Thomas, A.J., 2002. Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: a prospective study. Fertil Steril., 78, 491–499.

Sanchez, R., Risopatron, J., Schulz, M., Villegas, J., Isachenko, V., Kreinberg, R., Isachenko, E., 2011. Canine sperm vitrification with sucrose. Effect on sperm function. Andrologia 43, 233-241.

Santiago-Moreno, J., Coloma, M.A., Dorado, J., Pulido-Pastor, A., Gómez-Guillamón, F., Salas-Vega, R., Gómez-Brunet, A., López-Sebastián, A., 2009. Cryopreservation of Spanish ibex (Capra pyrenaica) sperm obtained by electroejaculation outside the rutting season. Theriogenology 71, 1253-1260.

Shackell, G.H., Kelly, R.W., Allison, A.J., 1977. Seasonal variation in libido of rams. N.Z Journal of Experimental Agric. 5,121-123.

Shannon, P., Vishwanath, R., 1995. The effect of optimal and suboptimal concentrations of sperm on the fertility of fresh and frozen bovine semen and a theoretical model to explain the fertility differences. Anim. Reprod. Sci. 39, 1-10.

Shannon, P., Curson, B., 1972. Toxic effect and action of dead sperm on diluted bovine semen. J. Dairy Sci. 55, 614–620.

Sherins, R.J., 1991. Clinical use and misuse of automated semen analysis. Ann. N.Y. Acad. Sci., 637, 424-435.

Shipley, C., Buckrell, B., Mylne, M., 2007. Current Therapy Large Animal Theriogenology, 2nd ed. Saunders-Elsevier, St. Louis.

Sikka, S.C., 2001. Relative impact of oxidative stress on male reproductive function. Curr Med. Chem. 8, 851-862.

Silva, P.F., Gadella, B.M., 2006. Detection of damage in mammalian sperm cells. Theriogenology, 65(5), 958–978.

Smith, J.F., Parr, J., Murray, G.R., McDonald, R.M., Lee, R.S.F., 1999. Seasonal changes in the protein content and composition of ram seminal plasma. In: Proceedings of the New Zealand Society of Animal Production, vol. 59, pp. 223-225.

Soler, A.J., García, A.J., Fernández-Santos, M.R., Esteso, M.C., Garde, J.J., 2003. Effects of thawing procedure on postthawed in vitro viability and in vivo fertility of red deer epididymal spermatozoa cryopreserved at -196^{0}C. J. Androl. 24, 746-756.

Soler, C., Pérez-Sánchez, F., Schulze, V., Bergmann, M., Oberpenning, F., Yeung, C.H., Cooper, T.G., 2000. Objective evaluation of the morphology of human epididymal sperm heads. Int. J. Androl., 23, 77–84.

Somsin Petyim, Chanon Neungton, Isarin Thanaboonyawat, Pitak Laokirkkiat, Roungsin Choavaratana, 2014. Sperm preparation before freezing improves sperm motility and reduces apoptosis in post-freezing-thawing sperm compared with post-thawing sperm preparation. J Assist Reprod Genet 31:1673–1680.

Stålhammer, E.M., Janson, L., Philipsson, J., 1989. Genetic studies on fertility in AI bulls I. Age, season and genetic effects on semen characteristics in young bulls. Anim Reprod Sci 19, 1-17.

Strzezek, J., 2002. Secretory activity of boar seminal vesicle glands. Biol. of Reprod. 2, 243-66.

Strzezek, J., Hopfer, E., Zaborniak, A, 1987. Zinc-ion dependent protein in boar semen. II. Effects on sperm motility and antibacterial properties. Anim. Reprod. Sci. 13: 133-142.

Sutovsky, P., Ramalho-Santos, J., Moreno, R. D., Oko, R., Hewitson, L., Schatten, G., 1999. On-stage selection of single round spermatids using a vital, mitochondrion-specific fluorescent probe Mito Traker (TM) and his resolution differential interference contrast microscoy. Hum Reprod. 14, 2301-2312.

Sztein, J.M., Noble, K., Farley, J.S., Mobraaten, L.E., 2001. Comparison of permeating and nonpermeating cryoprotectants for mouse sperm cryopreservation. Cryobiology 41, 28-39.

Tabarez, A., García, W., Palomo, MJ., 2017. Effect of the type of egg yolk, removal of seminal plasma and donor age on buck sperm cryopreservation. Small Rum. Res. 149, 91–98.

Taha, T.A., Abdel-Gawad, E.I. & Ayoub, M.A., 2000. Monthly variations in some reproductive parameters of Barki and Awassi rams throughout 1 year under subtropical conditions. 2 Biochemical and enzymatic properties of seminal plasma. Anim. Sci. 71, 325-332.

Tejerina, F., Domínguez, J.C., González Urdiales, R., Alegre, B., Alegre, E., Castejón, M., Ferreras, A., Cárdenas, S., Bernal, S., Campos, J., 2005. Morphometric characterization of the boar sperm head by using a Computer-Assisted Semen Analysis (CASA) programme. Reprod. Dom. Anim. 40, 368-369.

Thibier, M., Wagner, H.G., 2002. World statistics for artificial insemination in cattle. Livest. Prod. Sci. 74, 203-212.

Thibier, M., Guerin, B., 2000. Hygienic aspects of storage and use of semen for artificial insemination. Anim Reprod Sci, 62: 233–251.

Thomas, C., Garner, D., DeJarnette, J., Marshall, C., 1997. Fluorometric assessments of acrosomal integrity and viability in cryopreserved bovine spermatozoa. Biol. Reprod. 56, 991–998.

Thuwanut, P., Chatdarong, K., Techakumphu, M., Axner, E., 2008. The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of frozen-thawed epididymal cat spermatozoa. Theriogenology, 70, 233-240.

Toe, F., Rege, J. E., Mukasa-Mugerwa, E., Tembely, S., Anindo, D., Baker, R.L., Lahlou-Kassi, A., 2000. Reproductive characteristics of Ethiopian highland sheep. Genetic parameters of testicular measurements in ram lambs and relationship with age at puberty in ewe lambs. Small Rumin. Res. 36, 227-240.

Topfer-Petersen, E., Januschke, E., Schmoeckel, C., Schill, W.B., 1984. Ultrastructural localization of lectin binding sites of the acrosomal membrane system of boar spermatozoa. Andrologia, 16, 322-332.

Troedsson, M.H.T., Desvousges, A., Alghamdi, A.S., Dahms, B., Dow, C.A., Hayna, J. Valesco, R., Collahan, P.T., Macpherson, M.L., Pozor, M., Buhi, M.C., 2005. Components in seminal plasma regulating sperm transport and elimination. Anim Reprod Sci. 89, 171–186.

Tuli, R.K., Holtz, W., 1994. Effect of glycerolization procedure and removal of seminal plasma on post-thaw survival and GOT-release from boer goat spermatozoa. Theriogenology 42, 547-555.

Tuli, R.K., Scmidt-Baulain, R., Holtz, W., 1991. Influence of thawing temperature on viability and release of glutamic oxaloacetic transaminase in frozen semen from Boer goats. Anim. Reprod. Sci. 25, 125-131.

Turner, R.M., 2005. Moving to the beat: a review of mammalian sperm motility regulation. Reprod. Fertil. Dev. 18, 25–38.

Vázquez, J.M., Martínez, E.A., Martínez, P., García-Artiga, C., Roca, J., 1997. Hypoosmotic swelling of boar spermatozoa compared to other methods for analysing the sperm membrane. Theriogenology, 47, 913-922.

Vazquez, L., Martinez, F., Alcaide, M., Diazvubero, C., 1986. Congelación del semen de morueco. XI Jornadas Científicas de la S.E.O.C. Madrid, España. pp: 169-176.

Verstegen, J., Iguer-Ouada, M., Onclin, K., 2002. Computer assisted semen analyzer in andrology research and veterinary practice. Theriogenology, 57, 149-179.

Vilagran I, Yeste M, Sancho S, Castillo J, Oliva R, Bonet S., 2015. Comparative analysis of boar seminal plasma proteome from different freezability ejaculates and identification of Fibronectin 1 as sperm freezability marker. Andrology, 3, 345–356.

Vishwanath, R., Shannon, P., 1997. Do sperm cells age? A review of physiological changes in sperm cells during storage at ambient temperature. Reprod Fertil Dev 9, 321-331.

Waberski, D., 2007. Espermatrozoide y plasma seminal. En: Manual de inseminación artificial de los animales domésticos y de explotación zootéctnica. Eds. Bush, W.; Waberski, D. Acribia, S.A. Zaragoza (España). pp: 91-97.

Walkden-Brown, S.W., Restall, B.J., Taylor, W.A., 1994. Testicular and epididymal sperm content in grazing Cashmere bucks: seasonal variation and prediction from measurements in vivo. Reprod. Fertil., 6(6): 727-736.

Watson, P.F., 1979. The preservation of semen in mammals. In: C.A. Finn (Eds.), Oxford Reviews of Reproductive Biology. Vol. I. Clarendon Press, Oxford, pp. 283-350.

Watson, P.F., 1990. Artificial insemination and the preservation of semen. En: "Marshall´s physiology of reproduction. 4th edition. Vol. 2: Reproduction in the male". Ed: Lamming, G.E. Churchill Livingstone, New York, EEUU. pp: 747-869.

Watson, P.F., 1995. Recent develpoments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod. Fert. Dev. 7, 871-891.

Watson, P.F., 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60-61, 481-492.

Welzels, A.M., Janssen, H.J., Goverde, H.J., Bastiaans, B.A., Takahashi, K., Rolland, R., 1993. The influence of sperm density on the motility characteristics of washed human spermatozoa. Int. J. Androl., 16, 15-19.

White, I.G., Goh, P. & Voglmayr, J.K., 1987. Effect of male reproductive tract fluids and proteins on the metabolism and motility of ram spermatozoa. Arch. Androl.19, 115-125.

Wiemer, K.E., Ruttle, J.L., 1987. Semen characteristics, scrotal circumference and bacterial isolates of fine wool range rams. Theriogenology 28, 625–637.

Woelders, H., 1990. Overview of in vitro methods for evaluation of semen quality. Reprod. Dom. Anim., 145, 164.

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

Wulster-Radcliffe, M., Wiliams, M., Stellflug, J., Lewis, G., 2001. Technical note: artificial vagina vs. a vaginal collection vial for collecting semen from rams. J. Anim. Sci. 79, 2964-2967.

Yanagimachi R. Fertility of mammalian spermatozoa: its development and relativity. Zygote 1994; 2: 371–2.

Yanagimachi, R., 1988. Mammalian fertilization. In: The physiology of reproduction. Ed. E Knobil and JD Neill. Raven Press, New York. pp: 135-185.

Yanagimachi, R., 1994. Mammalian fertilization. In: The physiology of reproduction. Second Edition. Ed. E Knobil and JD Neill. Raven Press Ltd, New York. pp: 189-317.

Yang, Y., Song, Y., Loscalzo, J., 2007. Regulation of the protein disulfide proteome by mitochondria in mammalian cells. Proceedings of the National Academy of Sciences 104(26), 10813-7.

Yeste, M., Fernández-Novell, J.M., Ramió-Lluch, L., Estrada, E., Rocha, L.G., Cebrián-Pérez, J.A., et al., 2015. Intracellular calcium movements of boar spermatozoa during 'in

vitro' capacitation and subsequent acrosome exocytosis follow a multiple-storage place, extracellular calcium dependent model. Andrology, 3, 729–747.

Yeste, M., 2016. Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. Theriogenology 85, 47–64.

Yeste, M., Flores, E., Estrada, E., Bonet, S., Rigau, T., Rodríguez-Gil J.E., 2013. Reduced glutathione and procaine hydrochloride protect the nucleoprotein structure of boar spermatozoa during freeze-thawing by stabilising disulfide bonds. Reprod. Fertil. Dev. 25, 1036–1050.

Yoshida, M., 2000. Conservation of sperms. Current status and new trends. Anim. Reprod. Sci. 60-61, 349-355.

Yuan, Y.Y., Chen, W.Y., Shi, Q.X., Mao, L.Z., Yu, S.Q., Fang, X., Roldan, E.R., 2003. Zona pellucida induces activation of phospholipase A2 during acrosomal exocytosis in guinea pig spermatozoa. Biol Reprod 68, 904-913.

Zaneveld, L.J.D., Jeyendran, R.S., 1990. Hypoosmotic swelling test. En: "Handbook of the laboratory diagnosis and treatment of infertility". Eds: Keel, B.A. y Webster, B.W. CRC press, Boca Ratón, Florida, EEUU. pp: 91-110.

Zarazaga, L.A., Malpaux, B., Bodin, L., Chemineau, P., 1998. The large variability in melatonin blood levels in ewes is under strong genetic influence. American Journal of Physiology-Endocrinology and Metabolism 37, 607-610.