Effect Of Cholesterol-Loaded Cyclodextrins On Buck Sperm Quality After Cryopreservation With Different Extenders

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SUMMARY

Improvement of frozen-thawed buck sperm quality is important to increase results both in vivo and in vitro after using cryopreserved sperm. For this purpose, this study was contucted to determine the effects of cholesterol-loaded cyclodextrins (CLC) on quality of buck sperm frozen with different extenders. Buck sperm were treated prior to cryopreservation with 1 mg CLC/120 x 10^6 sperm and frozen with different freezing extenders Tris - egg yolk (20% or 2%) - glycerol (4%), skim milk – glucose - glycerol (7%) and Tris diluent-free egg yolk extender - glycerol (4%). CLC treatment improved post-thaw sperm motility and viability in all freezing extenders. Treating buck sperm with 1 mg CLC/120 x 10^6 sperm after elimination of seminal plasma increased post-thaw sperm motility (7-22 percentage points) and plasma membrane integrity (10-20 percentage points) compared with control groups ($P<0.05$). In addition sperm treated with CLC exhibited higher longevity during incubation for 150 min at 38 ^{o}C after thawing than control samples. Sperm frozen with 20% egg yolk or skim milk after seminal plasma elimination exhibited greater percentages of motility and plasma membrane integrity than sperm samples frozen with seminal plasma and 2% egg yolk or Tris-egg yolk free extenders. In conclusion, treating buck sperm with CLC (1 mg CLC/120 x 10^6 sperm) prior to cryopreservation provides improved sperm quality post – thawing and this technology can be easily transferred to the industry using current cryopreservation techniques.

RESUMEN

La mejora de la calidad del semen congelado-descongelado es importante para mejorar los resultados obtenidos tanto in vivo como in vitro tras el uso de semen crioconservado. Con este objetivo, este trabajo se desarrolló para determinar el efecto del tratamiento de los espermatozoides de machos cabríos con ciclodextrinas saturadas de colesterol (CLC) en la calidad del semen congelado tras el uso de distintos diluyentes de congelación. Los espermatozoides de machos cabríos fueron tratados previamente a la congelación con 1 mg de CLC/120 x 106 espermatozoides y fueron congelados con diversos diluyentes: Tris-yema de huevo (20% ó 2%)- glicerol (4%), leche descremada-glucosa-glicerol (7%), y Tris-glicerol (4%). El tratamiento con CLC mejoró la calidad (motilidad y viabilidad) del semen post-descongelación independientemente del diluyente de congelación utilizado. El tratamiento de los espermatozoides de esta especie con 1 mg de CLC/120 x 106 espermatozoides tras la eliminación del plasma seminal incrementó tanto el porcentaje de espermatozoides móviles (entre 7 y 22 puntos) y el de espermatozoides vivos (de 10 a 20 puntos) comparado con las muestras control ($P < 0,05$). Además, los espermatozoides tratados con CLC presentaron mayor longevidad durante incubación a 38ºC durante 150 minutos tras la descongelación que los espermatozoides control. Las muestras congeladas en diluyente con 20% de yema de huevo o en diluyente a base de leche descremada (que requieren la eliminación del plasma seminal) presentaron mayores porcentajes de espermatozoides móviles y mayor porcentaje de integridad de membrana plasmática que las muestras congeladas con diluyentes con 2% ó 0% de yema de huevo (no requieren el lavado del plasma seminal). En conclusión, el tratamiento de los espermatozoides de machos cabríos con CLC (1 mg de CLC/120 x 106 espermatozoides) antes de la congelación mejoró la calidad de los espermatozoides post-descongelación y esta tecnología puede ser fácilmente transferida al sector sin necesidad de modificar los protocolos de congelación utilizados.

1.   INTRODUCTION

1.1. THE ROLE OF ARTIFICIAL INSEMINATION ON GOAT PRODUCTION

Artificial insemination has been used since 1780 (Herman et al., 1994) and this technology supposed a great advance for reproductive biotechnology as well as for many other areas of animal science. One of the most important benefits obtained when artificial insemination is performed is that one single ejaculate can be used to impregnate several females. Additionally, artificial insemination allows the diffusion of desirable genes to meet the requirements of production and it accelerates the genetic progress in animal breeding.

Goat production has importance in agriculture economy worldwide. However, selection programs specific for goat production have been developed relatively recently, compared to those developed for dairy cattle breeding. When the objective is the genetic improvement of a particular population, it is mandatory to develop a selection program and in this context the use of artificial insemination is essential for the connection of the herds to diffuse high quality genetics of sires between organizations and breeders. Nevertheless, most of the goat breeders are not using selection programs at this moment. Nowadays there are a small number of selected and well characterised breeds, but most of the census are undefined populations and local breeds that in general lack of selection schemes and organisation (Dubeuf and Boyazoglu, 2009).

The success of artificial insemination will depend on the management of semen collection, storage and use (Leboeuf et al., 2000). The use of fresh or refrigerated semen is recommended when semen is stored for short periods of time or when it is used in small areas. However, frozen-thawed semen present some advantages over fresh or refrigerated semen, since this type of semen can be used for an indefinite period of time and it can be used worldwide. Unfortunately, sperm accumulate damages during the cryopreservation process and, as a consequence, the fertilizing ability of frozen-thawed sperm is lower than that of fresh or refrigerated semen. A reduction in sperm motility and viability can be

observed after cryopreservation (Salamon and Maxwell, 1995).Nevertheless, the damages provoked by the cryopreservation process are due to several factors, such as the presence of seminal plasma, type of cryoprotectant and its concentration, and the stage of the cryopreservation process (Leboeuf et al. 2000; Watson, 2000; Purdy, 2006).

1.2. SPERM MOTILITY AND VIABILITY

Sperm will have to get to the oviducts on time in order to fertilize the oocytes. Therefore, right after semen is deposited in the female genital tract, sperm will start to migrate through the female genital tract until arriving to the oocyte. Although muscular contractions of the female genital tract will help during this migration, it will mostly depend on the sperm motility. Even when motility is essential for fertility, each motile spermatozoon may not have fertilizing ability (Hafez, 1993), since fertility does not rely exclusively upon this parameter and each sperm must possess several attributes to be fertile. Nevertheless, sperm motility can be used as a parameter to check semen quality, and may relate to other sperm quality parameters and fertility. With this respect, correlations between sperm motility and fertility (0.15-0.84; Rodríguez-Martínez, 2003; Graham, 2001), and sperm viability and fertility (0.33-0.66; Graham, 2001) are variable between studies. However, not only sperm motility, but also the movement characteristics of the sperm can be important and correlated to the in vivo fertility (in bulls; Januskauskas et al., 2000). Gillan et al. (2008) demonstrated that in vitro diagnostic assesments (including assessment of motility, morphology, concentration, viability, acrosomal and chromatin integrity conducted immediately post-thaw and after swim-up, in conjunction with membrane status (CTC staining) and migration in an artificial cervical mucus) of bull sperm correlated with fertility and additionally by combining the results of these sperm assays, models were obtained showing a high correlation with fertility.

Although relations between motility and other sperm quality parameters as well as field fertility vary from low to high, motility is an important parameter and its evaluation is relatively simple, therefore it is used widely to evaluate fresh or frozen semen. The

evaluation of sperm quality prior to be cryopreserved or inseminated is essential, and in some studies it has been reported that the percentages of motile sperm in fresh semen are the best predictors of total post-thaw viability parameters (R=0.655, Dorado et al., 2009).

Traditionally, the sperm motility is assessed visually under contrast microscopy (Dorado et. al., 2009). In that context, subjective motility evaluation performed by optical microscopic analysis presents variations from 30 to 60% for the same ejaculates (Verstegen et. al., 2002). Several systems were developed in order to prevent these biases and, among them, the Computer-Assisted Sperm Analysis (CASA) system is one of the most important. Computer-assisted sperm analysis was first proposed by Doth and Faster 20 years ago (Verstegen et. al., 2002). Computer-assisted sperm analysis (CASA) is a valuable tool for sperm evaluation, since it allows the evaluation of sperm motion parameters objectively and more quickly if compared with manual methods. Assessment of sperm motility characteristics are important to evaluate the in-vivo or in-vitro fertilizing ability of the semen samples, and motility evaluated by CASA system provided repeatable estimates of many criteria of sperm movements (Holt et. al., 1994; Verstegen et. al., 2002; Gillan et al., 2008).

Progressive motility and total motility of the sperm are motion parameters obtained by CASA widely used for sperm evaluation. Total motility refers to the sperm that display any type of movement while it is considered that a progressively motile spermatozoon swims forward in an essentially straight line (Rouge, 2003).

To estimate more accurately the sperm quality of a sample (fresh, cooled or frozen-thawed), several laboratory assays should be performed. Sperm membrane integrity (or sperm viability) is a parameter widely used to estimate sperm quality. For the estimation of sperm membrane integrity, samples are stained and then they are evaluated under bright field or fluorescence microscopy, depending on the stains used. Stains used for bright field evaluation are cheap and they allow the estimation of this parameter with simple equipments. However, when fluorescence stains are used, samples can be evaluated with flow cytometry. Flow cytometry measures and analyzes simultaneously multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light (Anonym 2, 2000). Sperm analysis by flow cytometry allows the objective,

rapid and simultaneous analysis of a large number of spermatozoa and the results obtained with these analyses may allow the estimation of the fertility of a semen sample (Peterson et. al., 2007). Flow cytometry analysis by using one or more fluorescent stains can be applied for several sperm analysis such as sperm count, viability, apoptotic-like markers, motility, sperm acrosome reaction or DNA content and integrity (Cordelli et al. 2005).

Controversy exists between studies concerning to the best sperm quality parameter and correlations between assays of sperm quality in vitro and field fertility are variable between studies. This is mostly due to the differences between studies concerning to the animals used, the extenders, semen handling procedures, etc. On the other hand, fertility does not depend exclusively on the sperm, but many other factors related to the females and the artificial insemination (estrous detection and person performing the insemination) are also important (for a review, see Mocé and Graham, 2008).

When several parameters of sperm quality are evaluated, a more accurate estimation of the fertilizing potential of a sample can be obtained. However, this would be time consuming and expensive, so in practice only a few laboratory analyses are performed. Assays for determination of live sperm, particularly viability assays and motility assays appear to be most informative, as they consistently appear in multivariate correlations with fertility (Mocé and Graham, 2008).

1.3. CRYOPRESERVATION OF SPERM

Due to the development of semen collection, storage and artificial insemination techniques, donor males have a great impact on reproductive success, since each of the ejaculates of the males can be used to inseminate many females. To increase the storage period of the semen collected, it is necessary the development of optimal cryopreservation protocols is necessary.

Cryopreservation is a way of preserving germplasm that has applications in many areas of science (Barbas y Mascarenhas, 2008). Cryopreservation is used to establish

genetic resource banks for conservation of endangered species or breeds, and this allows maintaining the genetic diversity. Moreover, cryopreservation of the sperm carrying desired genes is substantially important in commercial livestock production, and the creation of companies specialized in reproductive techniques or centers which provide selected animals is becoming more habitual. Nowadays, artificial insemination centers are specialized in collecting, handling, and storing the sperm and they diffuse desired genes by means of artificial insemination. Cryopreservation permits the utilization of semen outside the sexual season in seasonal species, and it permits covering the demand of seminal doses in seasons where the seminal quality of the males decreases. Therefore, cryopreserved semen may have a great economic impact for breeders and animal production industries.

Sperm cryopreservation studies were mostly conducted with bull semen, but cryopreserved buck sperm presents relatively lower fertilizing ability than bull sperm. Frozen-thawed buck sperm present a wide fertility range, varying from 3% to 70% (Gacitua and Arav, 2005). For this reason, the commercial use of frozen-thawed buck semen can be limited in goat reproduction (Batista et al., 2009).

The cryopreservation of mammalian sperm is a complex process that is influenced by several factors that are inter-related. Therefore, for the development of a protocol for sperm freezing all the factors should be considered (Purdy, 2006). Furthermore, there are important differences between species with regard to the freezing procedure. These differences result from some factors such as the physiology and biochemistry of the spermatozoa themselves (Holt, 2000a). Thus, a protocol optimized for freezing of sperm of one species may not be appropriate for the sperm of a different species (Purdy, 2006). For these reasons, the specific requirements of each particular species should be taken into account when trying to develop a freezing protocol.

1.4.   DAMAGES INDUCED IN THE SPERM AFTER CRYOPRESERVATION

Many authors have reported the detrimental effect that cryopreservation has on sperm quality (Curry, 2000; Holt, 2000a; O'Connel et. al., 2002; Purdy, 2006; Hidalgo et. al., 2007). Cryopreservation negatively changes several sperm parameters such as motility and morphology, and it also provokes structural and functional changes on the sperm, as well as sperm death. All these unfavorable effects will provoke a loss of fertilizing ability of the cryopreserved sperm. With this respect, it has been estimated that the kindling rate obtained with fresh semen was approximately 12.1% higher than that observed for frozen-thawed sperm in Damascus goats (Karatzas et al., 1997).

During the cryopreservation process, several stresses are induced in the sperm that can be briefly summarized in temperature changes, and osmotic and toxic stresses resulting from the addition and elimination of cryprotectants as well as from the formation and dissolution of ice in the extracellular environment (Watson, 2000). Freezing and thawing of sperm produce alterations on sperm membranes and these alterations induce rearrangement of membrane lipids and proteins.

The most critical steps in the cryopreservation process are the temperature decrease and the return to physiological temperature. When ungulate sperm are rapidly cooled from 30 ºC to 0 ºC, lethal stress (named cold shock) is induced in some of the cells, therefore cooling to 4-5 ºC should be carefully conducted (Watson, 1981, 2000). When temperature decreases, plasma membrane is altered. Cold shock alters cellular functions such as selectivity in membrane permeability, which is lost, and causes irreversible loss of motility upon rewarming (Medeiros et. al., 2002). Membrane permeability is increased after cooling due to an increase in the membrane leakiness or to effects on specific protein channels (Watson, 2000). Not only cooling rate to 5ºC, but also the freezing rate has a great importance for the success of cryopreservation. Depending on the freezing rate, ice crystals may be formed or the cells may suffer due to hyperosmotic conditions resulting from unbalanced dehydration. Moreover, the addition and removal of the cryoprotectants will cause osmotic stress to the sperm, and its importance will depend on the relative permeability of the cryoprotectant used (Gao et al., 1993).

It is evident that the success of a cryopreservation protocol depends on multiple factors. To mitigate some of the detrimental effects derived from the cryopreservation process, it is necessary to establish adequate semen handling and freezing protocols as well as to use the adequate extenders.

1.5. BUCK SPERM FREEZING

1.5.1. REMOVAL OF SEMİNAL PLASMA

The first studies on buck sperm freezing were performed in the 1950's (Smith and Polge, 1950; Barker, 1957 for a review, see Leboeuf et. al., 2000). Nevertheless, fertility varied greatly between studies until Corteel (1974) discovered the detrimental effect of seminal plasma on sperm cryopreservation in this species (Leboeuf et. al., 2000). Molecular composition of seminal plasma is very complex, and great variations exist between species concerning to the anatomy of accessory glands, as well as the chemical composition and functions of their secretions (La Falci et al., 2002). Seminal plasma is produced by cauda epididymis and by the male accessory sex glands, and it exerts beneficial as well as harmful effects on the sperm. Seminal plasma can both inhibit and stimulate sperm function and fertility through the multifunctional actions of organic and inorganic components (Maxwell et. al., 2007). From the observations of Corteel (1974), it was determined that the protocol for freezing buck sperm needed an additional step to eliminate seminal plasma prior to the dilution with the extenders containing egg yolk or skim milk. This is due to the negative interactions between some ingredients from the freezing extenders (egg yolk and skim milk) and some enzymes from the seminal plasma originated by the bulbourethral gland secretions.

Egg yolk and skim milk are the most common non-permeant cryoprotectants used for buck sperm freezing (Leboeuf et. al., 2000; Purdy, 2006; Barbas and Mascarenhas, 2008). These cryoprotectants exert a beneficial effect in protecting the plasma membrane integrity. However, negative interactions occur between seminal plasma enzymes and egg

yolk components when egg yolk is used at high rates (20%) in the freezing extenders. An enzyme originating from bulbourethral gland secretion (BUS) called egg yolk coagulating enzyme (EYCE) degrades egg yolk components (Roy, 1957; Iritani et al., 1961; for a review, see Purdy, 2006). This enzyme was identified as phospholipase A and it acts as a catalyst that hydrolyzes egg yolk lecithin into fatty acids and lysolecithin (Iritani and Nishikawa, 1961, 1963; for a review, see Purdy, 2006). Consequently, lysolecithin is toxic for buck spermatozoa (Aamdal et al., 1965; Leboeuf et. al., 2000) and additionally this hydrolysis causes the sperm membranes to be more fusogenic, thereby inducing the acrosome reaction (Upreti et al., 1999), and chromatin decondensation (Sawyer and Brown, 1995).

The deleterious effect observed when skim milk extenders were used for freezing were due to a protein fraction contained in the bulbourethral gland secretion (Nunes, 1982). That protein fraction, named SBUIII from goat BUS (BUSgp60), has been identified as a monomeric 55-60 kDa glycoprotein lipase (Pellicer-Rubio et al., 1997). BUSgp60 displays triacylglycerol hydrolysis activity and sperm-toxic fatty acids that are produced from hydrolysis of skim milk triglycerides by BUS lipase cause several damages on the sperm, such as a decrease in the percentage of motile sperm, deterioration in the movement quality, breakage of acrosomes and death of sperm cells (Pellicer-Rubio et al., 1997; Leboeuf et. al., 2000).

To overcome these deleterious effects derived from the interaction between seminal plasma and egg yolk and skim milk components, several strategies were developed. Since seminal plasma had a detrimental effect, the first strategy tested was to wash seminal plasma by centrifugation. Therefore, many protocols to freeze buck semen include a centrifugation step in which the sperm are washed either once or twice, each for 10–15 min at 550–950 x g (Purdy, 2006). Washing of spermatozoa in a physiological solution increases the percentage of motile sperm before and after freezing and maximizes acrosomal integrity (Corteel, 1974; Drobnis et al., 1980; Ritar and Salamon, 1982; Memon et al., 1985). In addition, washing increases the longevity of sperm after freezing and thawing (Pellicer-Rubio et al., 1997). However, it should be noted that the washing is a time-consuming process, and also causes some loss of spermatozoa (Corteel, 1981). To

avoid the centrifugation step, reduction of egg yolk concentration in the extenders was also tested. Thus, it is possible to avoid detrimental effects of seminal plasma when egg yolk is included in the freezing extender at a concentration of 1.5 % (Salamon and Ritar, 1982).

In addition, several alternative extenders can be used to minimize the negative interactions between sperm and lipase such as adding of BUSgp60 lipase inhibitors, using lipid-free cowmilk, using a triglyceride-free diluent containing the milk protein casein or using milk from species other than dairy cows (for a review, see Purdy, 2006). It should be emphasized that goat is a seasonal species that shows variations in semen quality throughout the year and those variations may cause varying results on processed and stored semen (Cabrera et. al., 2005).

1.5.1. FREEZING EXTENDERS FOR BUCK SPERM

Following semen collection it is necessary to maintain maximum sperm quality under in vitro conditions. Semen extenders play an important role on sperm survival, therefore they should be carefully chosen. Extenders provide energy sources for the sperm, protect them from damage related to the decrease of temperature and maintain a suitable environment for sperm to survive temporarily (Purdy, 2006). That suitable environment has to have determined pH and osmolality conditions that depend on the biological characteristics of species. The extender pH value which maximizes the oxygen uptake, respiration and motility of buck sperm is between 7.0-7.5, therefore, it was suggested that an optimal extender for goat sperm survival in vitro should have a pH value of 7.2 (Fukuhara and Nishikawa, 1973). Large changes in semen pH value can result in sperm damage, infertility or sperm mortality (Purdy, 2006) and extender pH values for mammalian sperm range from 7.2-7.8 (Barbas y Mascarenhas, 2008). The pH values of egg yolk or milk-based extenders for goat sperm normally range between 6.75 and 6.8 (Purdy, 2006).

In general, a goat sperm cryopreservation extender includes a non-penetrating cryoprotectant (skim milk or egg yolk), a penetrating cryoprotectant (glycerol, ethylene

glycol, or dimethyl sulfoxide), a buffer (Tris or Test), one or more sugars (glucose, fructose, lactose, raffinose,sucrose or trehalose), salts (sodium citrate, citric acid) and antibiotics (penicillin, streptomycin) (Evans and Maxwell, 1987; for a review, see Purdy, 2006). The extenders most widely used for goat sperm freezing are reconstituted skim cow milk-glucose (Corteel, 1974, 1975; for a review, see Leboeuf et al., 2000) and tris–glucose–citric acid–egg yolk (Salamon and Ritar, 1982). Diluents slightly hypertonic are frequently used for freezing and diluting buck or ram semen (Barbas and Mascarenhas, 2008). Among the sugars, post-thaw motility was greater when the Tris buffered extender was combined with glucose or fructose than when lactose or raffinose were added to the extender (Salamon and Ritar, 1982). In addition, the Tris concentration recommended to optimize sperm cryosurvival was 300, 375 or 450 mM and the concentration of the sugars required was 0–62.4 mM (Salamon and Ritar, 1982).

Egg yolk is one of the common non-penetrating cryoprotectant used for cryopreservation of buck semen and it provides protection against cold shock during freezing and thawing (Holt, 2000a; Aboagla et al., 2004; Purdy, 2006; Barbas and Mascarenhas, 2008). The effect of egg yolk occurs on the sperm membrane and plays a role on the phase transition of the sperm membrane. The protective mechanisms of the egg yolk are determined by the phospholipids (lecithin) and low density lipoproteins that contains (Holt, 2000a; Medeiros et al. 2002; Aboagla et al., 2004; Purdy, 2006; Barbas and Mascarenhas, 2008; Varela Junior et. al., 2009). Another cryoprotectant widely used in freezing extenders for buck semen is skim milk (Leboeuf et. al., 2000; Purdy, 2006; Barbas and Mascarenhas, 2008), its protein fractions are responsible of minimizing cold shock damage (Medeiros et al., 2002).

Glycerol was discovered as a cryoprotectant by Polge et al. (1949) and it is widely used for sperm cryopreservation in many species. Glycerol is a penetrating cryoprotectant that minimizes damage of the sperm during freezing. Penetrating cryoprotectants such as glycerol or dimethyl sulfoxide cause membrane lipid and protein rearrangement, resulting in increased membrane fluidity, greater dehydration at lower temperatures and reduced intracellular ice formation and, as a consequence, these cryoprotectants increase sperm

cryosurvival rates (Holt, 2000b). Additionally, penetrating cryoprotectants are solvents that dissolve sugars and salts in the cryopreservation media (Purdy, 2006).

Although glycerol has beneficial effects during cryopreservation through its cryoprotectant activity, addition of glycerol may damage the sperm. This detrimental effect of glycerol varies between species, and depends on several factors, such as the temperature at which it is added, the concentration used or the incubation time of the sperm with the cryoprotectant. Not only the addition of glycerol can induce osmotic damage (Purdy, 2006), but also it may be toxic for sperm (Medeiros et al., 2002). Nevertheless, buck sperm are relatively tolerant to these negative effects of glycerol and can withstand exposure to this cryoprotectant. Different glycerol concentrations, addition step and temperature of addition were tested in several studies (Salamon and Ritar, 1982; Tuli and Holtz, 1994) and they concluded that glycerol can be added to the sperm in a single step (Purdy, 2006) at an optimum concentration of 4-8% in the diluted semen (Leboeuf et. al., 2000). In the method developed by Corteel, skim milk media supplemented with 14 % glycerol is used for freezing and it is added at 4 $^{\circ}$C in three steps at 10 min intervals, resulting in 7% final glycerol concentration for 400 to 500×10^{6} sperm/mL (Leboeuf et. al., 2000).

1.5.2. FREEZING PROTOCOL FOR BUCK SEMEN

Sperm from each species have specific characteristics that depend on their biological structure. Therefore, cryopreservation protocols should be optimized for each particular species.

As previously stated, one of the biggest differences between protocols for buck sperm and other species is the necessity of seminal plasma elimination, which is closely related to the type of extender chosen for cryopreservation. With respect to the washing procedure, its efficiency will depend on the dilution ratio of the semen and on the number of washings (Leboeuf et. al., 2000). Dilution ratios of 1:5 or 1:10 are recommended and double washing may be more effective than single washing (Leboeuf et. al., 2000).

With respect to the cooling, freezing and thawing of the sperm, several protocols have been published. Cooling to 4-5 ºC is usually performed slowly over 1-4 hours and after that sperm can be frozen in pellets or straws (Leboeuf et al., 2000; Purdy, 2006). Straws (0.25 or 0.50 mL) are frozen in liquid nitrogen vapor by suspending the straws at different heights over the liquid nitrogen level (from 3 to 5 cm; Leboeuf et al., 2000; Purdy, 2006) during a specific time (from 4 to 8 min, Leboeuf et al., 2000; Purdy, 2006) or in controlled freezing rate machines (Holt, 2000b).

Several protocols have been also described to thaw the straws. Straws are thawed in water baths at different temperatures and for different times. In some of the protocols, straws were thawed at high temperatures (60-70 ºC) for a short period of time (2-5 seconds). However, straws are usually thawed by placing them in a water bath at 37 ºC for 12–30 seconds (Deka and Rao, 1987).

1.6. EFFECTS OF CHOLESTEROL-LOADED CYCLODEXTRINS (CLC) ON SPERM CRYOPRESERVATION

Cooling and freezing-thawing of sperm provoke several damages as mentioned above. When sperm of most farm animals are rapidly cooled to 5 ^{o}C, an irreversible loss of motility and a significant reduction in respiratory activity and glycolysis are observed (Pickett and Komarek, 1966). This phenomenon is commonly known as cold shock. Cryopreservation leads to an alteration of the membrane structure, therefore protein and lipid associations are disrupted and, as a result, a loss of membrane selectivity and changes in membrane fluidity can be observed. After cryopreservation sperm are in a state similar to capacitation (phenomenon known as cryocapacitation), which is partially due to a loss in membrane cholesterol that takes place during the freezing-thawing process (Cormier and Bailey, 2003). In order to prevent these damages, a primary important goal should be the understanding of cryopreservation mechanism and its components. With this knowledge, optimal additives could be included in the extenders and a suitable cryopreservation process could be established.

In terms of cryopreservation success, important differences exist between species. Differences in sperm membrane composition cause a variable response to the cooling process between species (Medeiros et al., 2002). The extent of lateral phase separation depends on a combination of membrane elements such as cholesterol/phospholipid ratio, content of nonbilayer- preferring lipids, degree of hydrocarbon chain saturation, and protein/phospholipid ratio in the membrane (Medeiros et al., 2002). Sperm sensitivity to cold shock damage is determined by the membrane phospholipid composition (for a review, see Holt, 2000b), and species which possess sperm with high cholesterol:phospholipid ratio (such as human and rabbit) are more resistant to cold shock than species presenting membranes with low cholesterol:phospholipid ratio such as stallion, ram and bull (Darin-Bennett and White, 1977; Watson, 1981). While the cholesterol:phosholipid molar ratio observed in rabbit and human sperm is 0.88 and 0.99, respectively, this ratio is lower for ram and bull sperm (0.38 and 0.45, respectively; Darin-Bennett and White, 1977). These higher cholesterol levels of rabbit and human sperm result in a more cohesive, rigid and impermeable structure. Sperm plasma membrane lipids respond to temperature changes by alterations in their physical phase state (Holt, 2000a). Thus, when temperature decreases transitions from liquid to gel state are favored, although the presence of sterols is thought to inhibit these phase changes (Holt, 2000a).

Cholesterol is a necessary component to build and maintain cell membranes and it regulates membrane fluidity over the range of physiological temperatures. It is known mammalian cells require cholesterol for normal function and free cholesterol is an important structural component of the cell plasma membrane, where it regulates lipid bilayer dynamics and structure (Koronkiewicz and Kalinowski, 2004). Pettitt and Buhr (1998) have shown the importance of modulation of the lipid environment of the plasma membranes during cooling, implicating the lipid component in mechanisms of injury. Human sperm membranes that are classified as freezing- resistant have unusually high cholesterol content and these high levels stabilize membranes during cooling (Drobnis et al., 2004).

According to this information, the enrichment of sperm membranes with cholesterol before freezing might reduce damages produced during cryopreservation.

Cholesterol can be added to plasma membrane of several cell types, including sperm, to prevent phospholipid rearrangement and to increase membrane fluidity at low temperatures (Moore et al., 2005)

Cholesterol is not soluble in aqueous solutions, but it can be easily loaded in cyclodextrins, so cyclodextrins can be successfully used as carriers for altering cholesterol content of membranes in several cell types (Navratil et al., 2003; Belmonte et al., 2005). Cyclodextrins are cyclic oligosaccharides consisting of (1–4)-linked D-glycopyranose units, which are primary degradation products of starch and they have an external hydrophilic face and an internal hydrophobic core (Christian et al., 1997; Zidovetzki and Levitan, 2007). Cyclodextrins are classified according to the number of glucose units as γ- (8 units), β- (7 units) and α (6 units) cyclodextrins. From these types, β-cyclodextrins showed the highest affinity for inclusion of cholesterol from erythrocytes and model membranes (Zidovetzki and Levitan, 2007) and methyl-β-cyclodextrin (MBCD) is very efficient in stimulating the efflux of membrane cholesterol from a variety of cells in vitro (Zeng and Terada, 2001).

Therefore, the use of cholesterol-loaded cyclodextrins (CLC) on cryopreservation of sperm presents a remarkable potential to minimize cryoinjury effects. Up to date, several papers have been published where CLC were used as an strategy to improve quality of frozen-thawed sperm in several species.

Treating bull sperm with 1.5 to 2 mg of CLC/ 120×10^6 sperm before freezing increased percentages of motile and viable sperm after thawing (Purdy and Graham, 2004; Mocé and Graham, 2006). In addition, this effect is also observed when cyclodextrins preloaded with some cholesteryl conjugates (pelargonate) are used (Amorim et al., 2009).

As for the bull sperm, treatment of stallion sperm with CLC improved sperm quality after thawing (Combes et al., 2000; Moore et al., 2005). In addition, this treatment is also beneficial for cooled semen (Torres et al., 2006). Addition of CLC to sperm increased the permeabilityof stallion sperm to the cryoprotectants and reduced osmotic stress endured by stallion sperm during cryopreservation (Glazar et al., 2009). On the contrary, some authors did not observe any effect of CLC treatment on sperm motility after thawing and reported that fertilizing ability was lower for CLC-treated than for control

sperm, although CLC treatment improved post-thaw membrane integrity (Zahn et al., 2002).

Similarly to the other species, treatment of donkey sperm with cholesterol-loaded cyclodextrins prior to cryopreservation resulted in higher percentages of motile and viable sperm after thawing (Álvarez et al., 2006).

Boar sperm are very sensitive to cooling and osmotic stress, and both phenomena are observed during the cryopreservation process (Amann, 1998). For this reason, and according to the reports in other species, CLC treatment could be beneficial in this species. However, reports have been contradictory. While some authors observed that treatment of boar sperm with methyl-β-cyclodextrin or 2-hydroxypropyl-β-cyclodextrin (which presumably eliminates cholesterol) improved acrosome integrity and motion parameters (Zeng and Terada, 2000a; 2000b; 2001), other studies reported that the benefit was observed after CLC treatment (Bailey et al. 2008; Torres et al., 2009), and other authors did not observe any difference between treatments in terms of sperm motility and viability after thawing (Tomás et al., 2009). Nevertheless, boar sperm treated with CLC present higher penetrating ability of immature oocytes in vitro than control or sperm treated with cyclodextrin (Blanch et al., 2009; Tomás et al., 2009). In addition, the use of 2-hydroxypropyl-β-cyclodextrin with cholesterol protects porcine spermatozoa against cold shock by improving viability without promoting premature capacitation (Galantino-Hommer et al., 2006).

With respect to ram sperm, the addition of CLC prior to cryopreservation increased post-thaw sperm motility, plasma membrane integrity and sperm longevity after thawing (Morrier et al., 2004; Purdy et al., 2009; Mocé et al., 2009). Additionally, treating ram sperm with CLC increased the cholesterol content, osmotic tolerance, and zona-binding capabilities of ram sperm (Mocé et al., 2009).

There are not many studies about the effect that CLC treatment has on buck sperm cryosurvival. Some authors observed that CLC treatment improved post-thaw sperm motility, although it did not affect plasma membrane and acrosomal membrane integrity (Barrera-Compean et al., 2005). In a preliminary study, Tomás et al (2008) observed that

treatment of buck sperm with 1 mg CLC/120 x 10^6 sperm after seminal plasma elimination improved both sperm motility and membrane integrity after thawing.

1.7. OBJECTIVE

The aim of this study is to determine if treatment of buck sperm with cholesterol-loaded cyclodextrins improves sperm quality after thawing and to study how this treatment affects sperm quality when different freezing extenders are used.

2. MATERIAL AND METHODS

2.1. MATERIALS

All chemicals used in this study were reagent grade and purchased from Sigma-Aldrich (Madrid, Spain), except for Propidium iodide (PI) and SYBR-14, which were purchased from Invitrogen (Barcelona, Spain).

2.2. PREPARATION OF EXTENDERS

In this study, Tris extender (Trizma® Base- Sigma-T1503; D-(+)-Glucose- Sigma-G8270, Citric Acid- Sigma-C1857) was used as a basic solution to dilute and make egg-yolk freezing extenders. The composition of this extender is detailed in Table 2.2.1. Chemicals were dissolved in ultra-pure water in laboratory conditions at ambient temperature (24ºC), and osmolality and pH were adjusted to 300 mOsm and 7.0, respectively. This extender was kept frozen until use.

Egg yolk (20% or 2%), skim milk and glycerolated Tris buffered extenders were used for sperm freezing. Their composition is detailed in Table 2.2.1.

Fresh eggs were used to make the egg yolk extender. Before use, egg shells were washed with bi-distilled water and they were dried with a paper towel. Egg yolk was prepared by separating the egg yolks from the egg whites, and removing excess white from the yolk using a paper towel. The perivitelline membrane was ruptured and the yolk was recovered in a beaker, leaving the perivitelline membrane into the paper towel. Egg yolk (20% or 2% depending on the experiment) was added to the Tris extender and this was clarified by centrifuging at 5 ºC and 12,000 g for 20 minutes. The supernatant was recovered and filtered through 5, 3 and 1.2 μm filters. This extender was kept frozen until use.

Skim milk extender was made by diluting skim milk (Skimmed Milk Powder; Central Lechera Austriana, Oviedo, Spain) and glucose with ultrapure water and heating the

extender at 95 °C for 10 minutes. This extender was cooled down to room temperature, before adding the glycerol (in the glycerolated extender) and they were stored at 5 °C until use for a maximum of two weeks.

Table 2.2.1 Composition of extenders used.

Ingredients	Tris Buffered Diluent		20% Egg Yolk		Skim Milk		2% Egg Yolk	
	Diluent 1	*Diluent 2*	*Diluent 1*	*Diluent 2*	*Diluent 1*	*Diluent 2*	*Diluent 1*	*Diluent 2*
Tris (g)	3.027	3.027	3.027	3.027	-	-	3.027	3.027
Citric Acid (g)	1.594	1.594	1.594	1.594	-	-	1.594	1.594
Glucose (g)	1.243	1.243	1.243	1.243	0.2	-	1.243	1.243
Glycerol	-	10mL (8%)	-	10mL (8%)	-	14mL (14%) -	-	8.2mL (8%)
Diluent 1 Skim milk	-	-	-	-	-	86mL		
Ultrapure water	Up to 100mL	Up to 100mL	Up to 100mL	Up to 100mL	Up to 100mL	-	Up to 100mL	Up to 100mL
Egg Yolk (20%)	-	-	25mL	25mL	-	-	-	-
Egg Yolk (2%)	-	-	-	-	-	-	2.5mL	2.5mL
Skim Milk (10%)	-	-	-	-	10 g	-	-	-

2.3. PREPARATION OF CHOLESTEROL-LOADED CYCLODEXTRINS

Methyl-β-cyclodextrin was preloaded with cholesterol as described by Purdy and Graham (2004). Briefly, a 0.45 mL aliquot of cholesterol dissolved in chloroform (200 mg cholesterol per 1 mL chloroform) was added to 2 mL of methanol containing 1 g of methyl-β-cyclodextrin and the mixture was stirred until the combined solution was clear. The solution was poured into a glass Petri dish and was left to dry during 1 or 2 days at 37-39 ^{o}C. After that the crystals were removed from the Petri dish and stored in cap glass tubes at 22 ^{o}C until use.

A working solution of CLC was prepared by adding 50 mg of CLC to 1mL of Tris diluent (Purdy and Graham, 2004) and mixing the solution using a vortex mixer. Aliquots (0.5 mL) of this working solution were split in eppendorf tubes and they were stored frozen until use.

2.4. ANIMALS

Eight adult bucks (2-7 years of age) of the Murciano-Granadina breed housed in the Centro de Tecnología Animal, Instituto Valenciano de Investigaciones Agrarias (CITA-IVIA; Segorbe, Castellón) were used in the experiments.

Bucks were housed in a common pen and they were fed daily with a diet containing hay *ad-libitum* and 1 kg complemantary feed (crude protein 16.5%, crude oils and fat 4.5%) per male. Fresh water was provided *ad libitum*.

2.5.   SEMEN COLLECTION

Ejaculates were collected early in the morning with an artificial vagina from October to May. Two ejaculates were collected per week from each male in two separate days.

Semen was collected according to the protocol described by Silvestre et al., (2004). Briefly, before collecting the ejaculates, artificial vaginas were warmed in a heater chamber at 39-40 ºC. A female attached and partially immobilized to a stanchion was used as a teaser, and when the buck mounted the female, his penis was gently guided inside the artificial vagina which had a temperature of approximately 40-42°C. Immediately after collection ejaculates were kept in a water bath at 35-37 ^{o}C until their assessment.

Semen volume was measured in a conical tube and the sperm concentration was determined by a spectrophotometer (Accucell, IMV Technologies, France) calibrated for goat species. For determination of sperm concentration semen was diluted (1/400; v/v) with a sodium chloride solution (0.9%;v:v). An aliquot was diluted to 15x10^{6} sperm/mL with Tris buffered extender supplemented with BSA (6 mg/mL; Tris-BSA) and incubated for 10 min at 37°C temperature for motility analysis.

2.6.   FREEZING PROTOCOL

When required, semen was washed twice by centrifugation to eliminate seminal plasma. After collection, ejaculates were cooled to 22 ºC, diluted (dilution 1:10; v:v) with Tris diluent and centrifuged (Eppendorf Centrifuge 5810; 22331 Hamburg, Germany) at 500 g, for 15 min. After centrifugation, supernatant was removed, the pellet was resuspended in 10 mL Tris-buffered diluent and samples were centrifuged for a second time. Then, supernatant was removed, the pellets were resuspended with 500 μL of Tris-buffered diluent and the sperm concentration of each sample was determined with an spectrophotometer as previously described. Sperm concentration in each sample was

adjusted to 200×10^6 sperm/mL with *diluent 1* (Table 2.2.1) at 22 ^{0}C. Later, these samples were placed in 100 mL beakers filled with 80 mL of water at 22 ºC and they were cooled to 5 ºC over 2 h in a cooler chamber. When the cooling phase was completed, samples were diluted 1:1 (v:v) with their corresponding Diluent 2 pre-cooled to 5ºC (containing glycerol; Table 2.2.1) and they were kept at 5ºC for 15 additional minutes. After equilibration, sperm were packed into 0.5 mL straws (IMV Technologies, L'Aigle, France), sealed with polyvinyl alcohol powder (PVA; IMV Technologies, L'Aigle, France) and frozen in liquid nitrogen vapor, with the straws horizontally suspended 3 cm above the liquid nitrogen level for 10 minutes, before being plunged into liquid nitrogen for storage. Straws were thawed in a water bath at 38 ºC for 30 s, prior to analyses.

2.7.   ANALYSIS OF SPERM MOTILITY

The percentages of total motile and progressively motile sperm in each sample were determined using a computer-assisted sperm analysis system (CASA; ISAS, version 1.0.17, Proiser, Valencia, Spain) with settings of 3-70 μM particular area. Sperm were considered progressively motile when they presented a linearity indexs > 80%. Sperm motility was assessed at 39 ºC using a 10x negative phase contrast objective and a Nikon Eclipse 90i microscope (Nikon Corporation Instruments Company; IZASA, Barcelona, Spain) connected to the computer through a monochrome video camera Basler A312f (Basler Vision Technologies, Proiser, Paterna, Valencia Spain). For each sample, sperm concentration was adjusted to 15×10^6 sperm/mL with Tris-BSA and samples were incubated in a water bath at 38 ºC for 10 min before motility evaluation. Then, 5 μL sub-samples were placed on a Makler chamber (Counting Chamber Makler, Sefi-Medical Instruments) pre-warmed at 39 ^{0}C in a thermal plate and a minimum of 200 sperm from three different fields were captured to be analysed.

Motility characteristics were evaluated for fresh, cooled to 5ºC (after equilibration period), and frozen-thawed sperm. Fresh sperm motility was evaluated within 30-40 min of semen collection. The longevity of sperm motility was determined for cryopreserved sperm

incubated after thawing for up to 150 min in a waterbath at 38 ºC (Figure 2.7.1). After thawing, one aliquot from each sample was diluted immediately after thawing to 15×10^6 sperm/mL with Tris-BSA while another aliquot was kept undiluted, and the samples were incubated in a water bath at 38 ºC for up to 150 min. Motility was evaluated at 0, 10, 30 and 150 min after incubation. For the undiluted aliquots, sperm concentration was adjusted to 15×10^6 sperm/mL with Tris-BSA immediately before each motility evaluation (except for the evaluation performed at time 0).
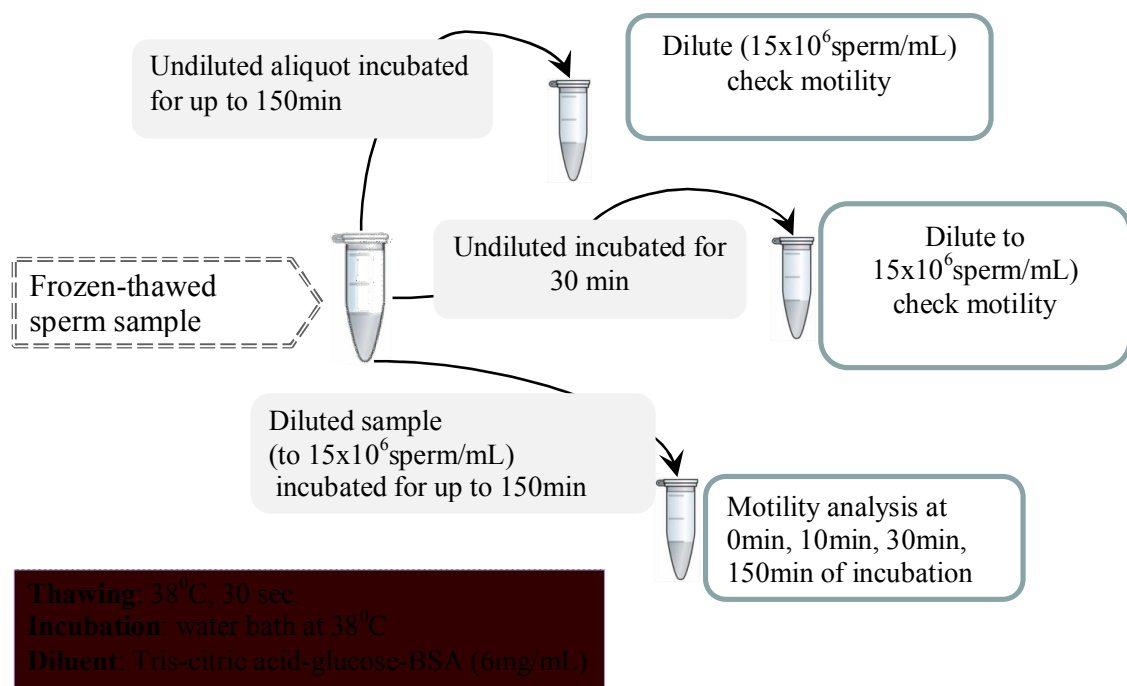
Figure 2.7.1. Analysis of Sperm Motility over 150 min

2.8.   ANALYSIS OF PLASMA MEMBRANE INTEGRITY

The percentage of viable (plasma membrane intact) sperm in each frozen-thawed sample was determined using flow cytometry (Figure 2.8.1), as described by Purdy and Graham (2004). Briefly, straws were thawed and each sample was diluted with Tris-BSA to 30×10^6 sperm/mL. Then, each sample was stained for flow cytometric analysis by transferring a 0.1 mL aliquot into a tube containing 0.45 mL Tris-BSA (6 mg BSA/mL) diluent, 2.5 μL SYBR-14 (10 μM solution in DMSO) and 2.5 μL PI (1.5 mM solution in distilled water). The samples were incubated for 10 min at 38ºC and filtered through a 40μm nylon mesh before being analyzed using an Epics XL-MCL flow cytometer (Beckman Coulter, IZASA, Barcelona, Spain) equipped with an argon laser tuned to 488 nm at 100 mW power. Fluorescence from 10,000 cells was measured using a 550 long pass filter, a 525 nm band pass filter to detect SYBR-14, a 600 nm long pass filter and a 620 nm band pass filter to detect PI. Using this protocol, all cells stain with SYBR-14, permitting cells to be distinguished from egg yolk particles, but only non-viable cells stain with PI.
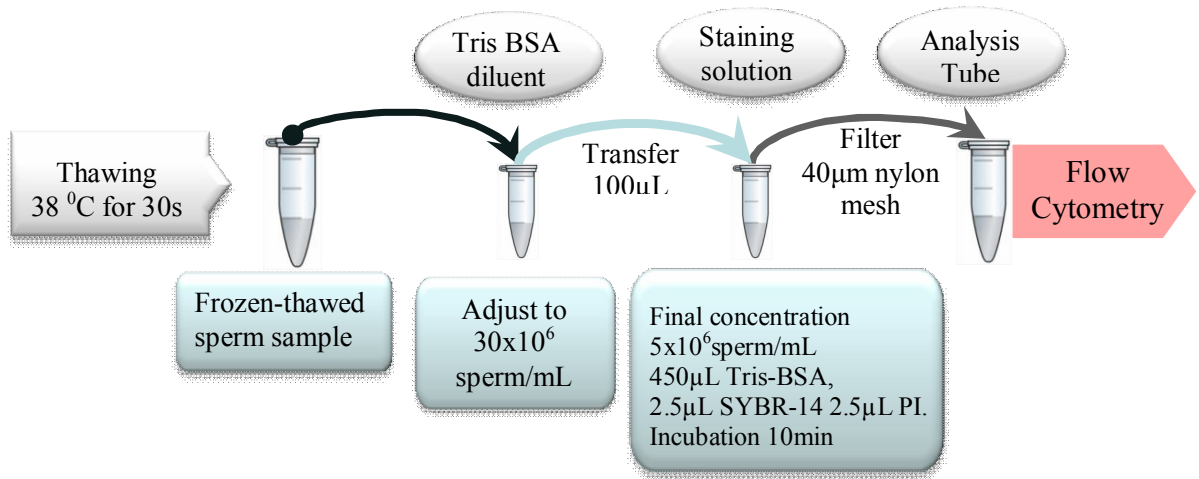
Figure 2.8.1. Analysis of Plasma Membrane Integrity (Viability): Flow cytometry protocol

2.9.   EXPERIMENTS

2.9.1   EXPERIMENT 1: DETERMINING THE OPTIMAL MOMENT FOR CLC TREATMENT

In this experiment, the optimal moment for CLC treatment of goat sperm was determined (Figure 2.9.1.1). Ejaculates from 3 bucks collected from October, 28th, 2008 through December, 04th, 2008 were used in this study. Immediately after collection, semen volume and sperm concentration were determined as described above and each ejaculate was split into three aliquots. One of the aliquots served as control (no-CLC treatment) and the other aliquots were treated with CLC either before (CLC-before) or after (CLC-after) the centrifugation step. Both control and CLC-after were diluted with Tris extender, and CLC-before was treated with CLC at a concentration of 1 mg CLC/120 x 10^6 sperm (Tomás, 2008), and incubated at room temperature for 15 min before dilution with Tris extender and centrifugation. Samples were washed and pellets resuspended as described above. After centrifugation, sperm concentration was determined, CLC were added to the treatment CLC-after (1 mg CLC/120 x 10^6 sperm), they were incubated for 15 min at room temperature and later sperm concentration was adjusted in all the treatments to 200 x 10^6 sperm/mL with Tris- citric acid- glucose extender supplemented with 20% egg yolk. Each sample was cooled, diluted with glycerolated extender, packed, frozen, thawed and evaluated as described above.
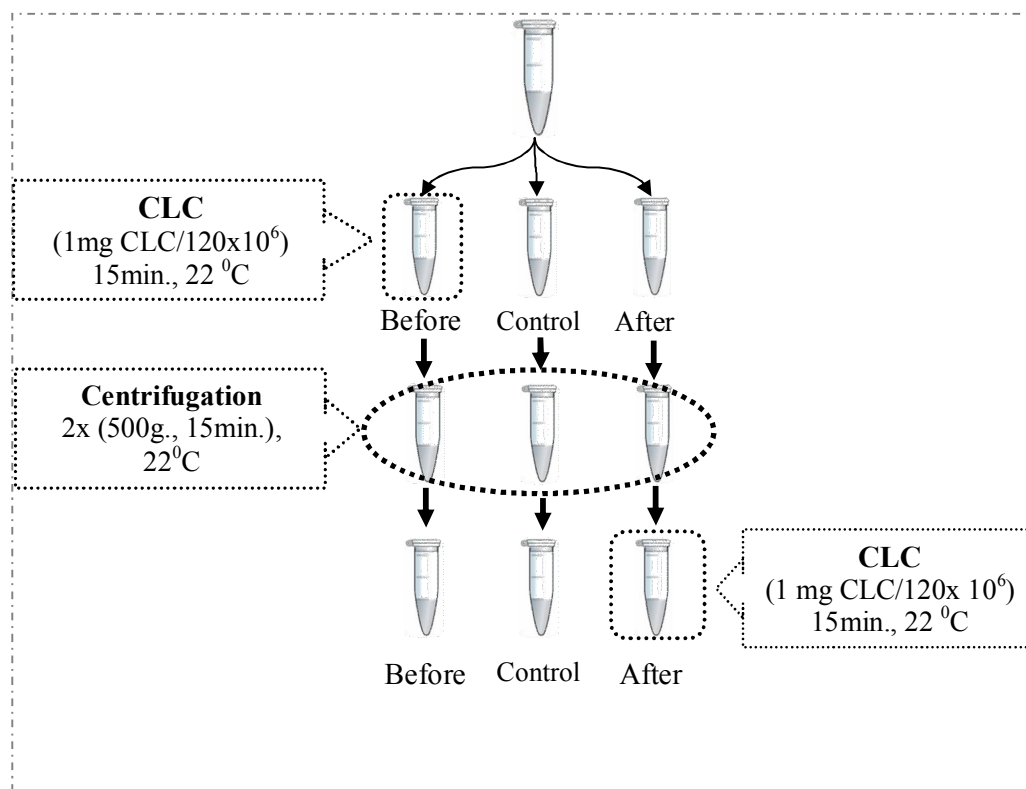
Figure 2.9.1.1. Experiment 1. Determination of the optimal moment for CLC addition.

2.9.2   EXPERIMENT 2: EFFECT OF CLC TREATMENT ON THE QUALITY OF GOAT SPERM FROZEN WITH DIFFERENT DILUENTS

In this experiment, control and CLC-treated sperm were frozen with three extenders habitually used for buck sperm cryopreservation: Tris-citric acid-glucose- egg yolk (20 %), Tris-citric acid-glucose- egg yolk (2 %) and skim milk-glucose extender (Table 2.2.1). Both Tris-20 % egg yolk and skim milk diluents require the elimination of seminal plasma, while Tris- 2% egg yolk avoids the washing step.

Ejaculates from 6 bucks collected from January, 23rd , 2009 through March, 31st, 2009 were used in this study. Immediately after collection, semen volume and sperm concentration were determined as described above and each ejaculate was split into two

aliquots (Figure 2.9.2.1). One of the aliquots served as control (no-CLC treatment) and the other one was treated with CLC. For samples diluted with Tris- 20% egg yolk and skim milk extender, aliquots were treated with CLC after the centrifugation step, while samples diluted with Tris- 2% egg yolk were treated with CLC immediately after collection of the ejaculates. Samples were treated with CLC and processed as previously described.
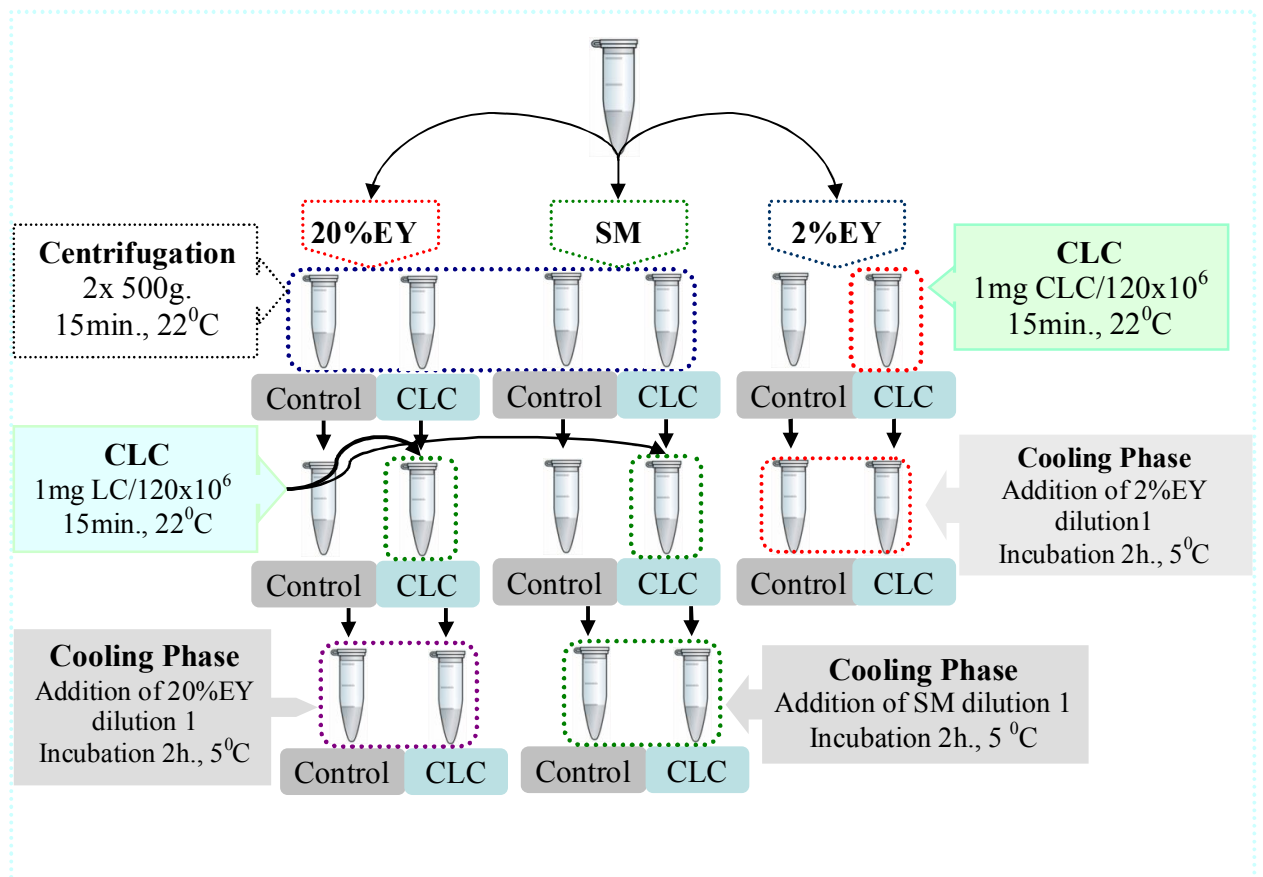
Figure 2.9.2.1. Experiment 2. Effect of Cholesterol on the Quality of the Goat Sperm Frozen with Different Diluents

2.9.3 EXPERIMENT 3: EFFECT OF CLC TREATMENT ON THE QUALITY OF GOAT SPERM FROZEN IN DILUENTS DEPRIVED OF ANIMAL PRODUCTS

In this experiment, control and CLC-treated sperm were frozen with two extenders: one of them contained egg yolk (Tris-citric acid- glucose- 20% egg yolk) while the other one was deprived of animal products (Tris-citric acid-glucose extender). For the samples diluted in the egg yolk extender, seminal plasma elimination was required.

Ejaculates from 7 bucks collected from Abril, 14th, 2009 through May, 26th, 2009 were used in this study. Immediately after collection, semen volume and sperm concentration were determined as described above and each ejaculate was split into two aliquots (Figure 2.9.3.1). One of the aliquots served as control (no-CLC treatment) and the other one was treated with CLC. For samples diluted with Tris- 20% egg yolk, aliquots were treated with CLC after the centrifugation step, while samples diluted with Tris extender were treated with CLC immediately after collection of the ejaculates. Samples were treated with CLC and processed as previously described.

Unlike in the other experiments, motility in frozen-thawed semen samples was determined 10 and 150 min after thawing.
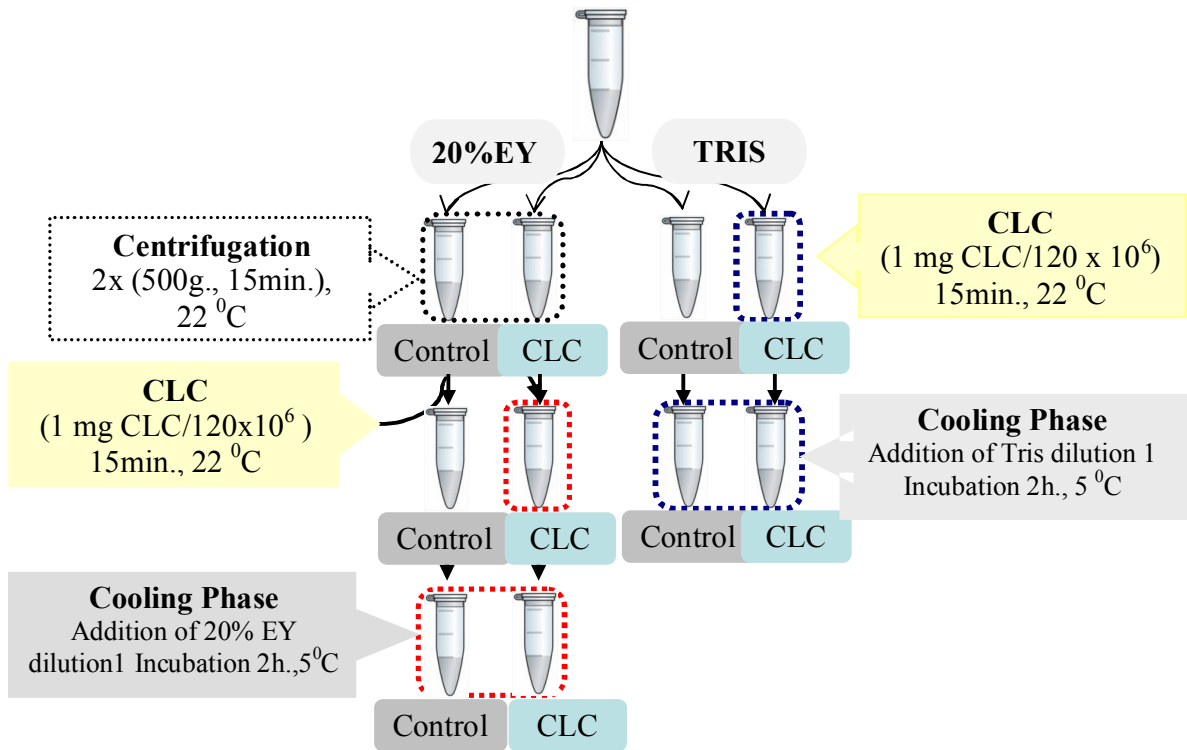
Figure 2.9.3.1. Experiment 3: Effect of CLC treatment on the quality of goat sperm frozen in diluents deprived of animal products

2.10. STATISTICAL ANALYSES

All statistical analyses were performed using SAS Programm (The SAS System for Windows 9.0). Data were analysed with a mixed model including ejaculate within male as random effect. The Tukey-Kramer adjustment was used to test the differences of least square means at a fixed 5% error level.

For the analyses of total motile, progressive motile and plasma membrane integrity treatment, extender and time and their interaction were used as fixed effects, depending on the experiment.

3 RESULTS

3.1. EXPERIMENT 1: DETERMINING THE OPTIMAL MOMENT FOR CLC TREATMENT

The characteristics of the fresh ejaculates used in the first experiment are presented in Table 3.1.1.

Table 3.1.1. Mean values, standard deviation, maximum and minimum values observed for motility (%), volume (mL) and concentration ($x10^6$/mL) in fresh ejaculates used in Experiment 1.

	N	Mean	Std. Dev.	Max.	Min.
Total motile sperm (%)	18	86.44	8.60	94	62
Progressively motile sperm (%)	18	53.78	8.30	62	33
Volume (ml)	18	1.13	0.38	1.9	0.5
Concentration ($x10^6$/ml)	18	3210.67	707.37	5067	1897

After refrigeration during 2 hours at 5 $^{\circ}$C, CLC-treated samples presented higher percentages of total motile sperm than control samples (Table 3.1.2). Nevertheless, the percentages of total motile sperm remained high (> 78%) for all the treatments after this step of the protocol.

Samples treated with CLC after seminal plasma elimination exhibited greater percentages of total motile sperm ($P < 0.05$) 10 min after thawing (68.65 %) than control or samples treated with CLC before seminal plasma elimination (55.59% and 60.92%, respectively). However, percentages of progressively motile sperm were similar for all the treatments (between 35.49% and 40.71 %). In addition, control samples presented lower

percentages of plasma membrane integrity (live sperm) after thawing (36.37 %; $P < 0.05$) than CLC-treated samples. On the other hand, when CLC were added to the sperm after seminal plasma elimination, the percentage of live sperm was higher (51.61%; $P < 0.05$) than when sperm were treated with CLC before the centrifugation step (45.42%). Therefore, in this species sperm should be treated with CLC after seminal plasma elimination.

Table 3.1.2 Percentages of total motile and progressively motile sperm after cooling to 5ºC when buck sperm were treated with 0 (control) or 1 mg of cholesterol loaded cyclodextrins (CLC; per 120 x 10^6 sperm) before or after seminal plasma removal

	Total motile sperm (%)		Progressively motile sperm (%)		Total motile sperm (*P*-value)	Progressively motile sperm (*P*-value)
Treatments	**L.S.M.**	**S.E.**	**L.S.M.**	**S.E.**		
CLC, *Before*	84.32[b]	2.69	57.91	3.25		
Control	78.18[a]	2.61	59.12	2.96	0.0026	N.S
CLC, *After*	83.67[b]	2.64	58.03	3.04		

L.S.M.: Least squares means; S.E.: standard error; N.S.: non significant; [a,b]Different superscripts within the same column indicate differences between treatments

Sperm treated with cholesterol loaded cyclodextrins after seminal plasma elimination maintained higher ($P < 0.05$) percentages of total motile sperm (59.05 %; Table 3.1.4.) and progressively motile sperm after incubation at 38ºC for 150 min (38.21%) than sperm treated with CLC before centrifugation (49.78% and 31.97%, respectively), and both of them presented higher longevity to incubation at 38ºC than control samples (40% and 27.6%, respectively). On the other hand, motility remained high in all the treatments for 30

min after thawing and incubation at 38ºC (61.92 % and 57.33 % total motile sperm for times 0 and 30 min, respectively) but it decreased abruptly when samples were incubated for 150 min (39.75%). This decrease in the percentage of total motile sperm was more pronounced when samples were kept undiluted during the incubation period (21.43 %), although this difference was not observed between diluted and non diluted samples at 30 min.

Table 3.1.3. Percentages of motile and live sperm 10 min after thawing when buck sperm were treated with 0 (control) or 1 mg of cholesterol loaded cyclodextrins (CLC; per 120 x 10^6 sperm) before or after seminal plasma removal

	Total motile sperm (%)		Progressively motile sperm (%)		Live sperm (%)	
Treatments	**L.S.M.**	**S.E.**	**L.S.M.**	**S.E.**	**L.S.M.**	**S.E.**
CLC, *Before*	60.92[a]	2.64	35.49	3.02	45.42[b]	2.29
Control	55.59[a]	2.60	38.82	2.96	36.37[a]	2.25
CLC, *After*	68.65[b]	2.60	40.71	2.96	51.61[c]	2.24
***P*-value**	< 0.0001		N.S.		< 0.0001	

L.S.M.: least squares means; S.E.:standard error; N.S.: non significant; [a,b,c]Different superscripts within the same column indicate differences between treatments.

Although the interaction between treatment and incubation time was not statistically significant, it is remarkable that the percentage of total motile sperm for CLC-treated samples remained above 40% after 150 min of incubation in diluted samples (43.07 and 47.76% for samples treated with CLC before or after centrifugation, respectively; data not shown in tables) while control samples presented 28.41% of total motile sperm for the same incubation time. With respect to the percentages of progressively motile sperm, this parameter was maximum after 30 min of incubation at 38 ºC (44.24% versus 38.17% and

38.24% for 30 min, 0 and 10 min after thawing, respectively; Table 3.1.4) and decreased in samples incubated during 150 min diluted (23.62%) or undiluted (12.08%).

Table 3.1.4. Percentages of total motile and progressively motile sperm for up to 150 min of incubation at 38 ºC after cryopreservation, when buck sperm were treated prior to cryopreservation with 0 (control) or 1 mg of cholesterol loaded cyclodextrins (CLC; per 120 x 106 sperm) before or after seminal plasma removal.

		Total motile sperm (%)		Progressively motile sperm (%)	
		L.S.M	S.E	L.S.M	S.E
Treatment	**CLC,** *Before*	49.78[b]	2.74	31.97[b]	2.33
	Control	40.00[a]	2.73	27.60[a]	2.31
	CLC, *After*	59.05[c]	2.73	38.21[c]	2.31
	P-value	< 0.0001		< 0.0001	
Incubation Time (min)	**0min.**	61.92[D]	2.87	38.17[C]	2.51
	10min.	61.60[D]	2.87	38.24[C]	2.51
	30min.	57.33[CD]	2.87	44.24[D]	2.51
	30min. diluted	55.63[C]	2.87	39.20[CD]	2.51
	150min.	21.43[A]	2.87	12.08[A]	2.52
	150min. diluted	39.75[B]	2.87	23.62[B]	2.51
	P-value	< 0.0001		< 0.0001	

L.S.M.: least squares means; S.E.:standard error; [a,b,c]Different superscripts within the same column indicate differences between semen treatments; [A,B,C,D]Different superscripts within the same column indicate differences between incubation times.

3.2. EXPERIMENT 2: EFFECT OF CLC TREATMENT ON THE QUALITY OF GOAT SPERM FROZEN WITH DIFFERENT DILUENTS

The characteristics of the fresh ejaculates used in experiment 2 are summarized in Table 3.2.1.

Table 3.2.1. Mean values, standard deviation, maximum and minimum values observed for motility (%), volume (mL) and concentration ($x10^6$/mL) in fresh ejaculates used in Experiment 2

	N	Mean	Std. Dev.	Max.	Min.
Total motile sperm (%)	22	86.23	6.41	97	77
Progressively motile sperm (%)	22	57.18	7.63	71	42
Volume (ml)	22	0.88	0.30	1.50	0.30
Concentration ($x10^6$/ml)	22	4251	1019.92	6140	2869

After refrigeration to 5ºC, the percentages of total motile sperm remained high and were similar for all the extenders used (Table 3.2.2). Similarly to the previous experiment, CLC-treated sperm presented greater percentages of total motile sperm (86.61%; $P < 0.05$; Table 3.2.2) than control samples (83.40%). The interaction between these factors was not significant, but it should be emphasized that the control group diluted with 2 % egg yolk presented the lowest motility after cooling to 5ºC (80.91%), and this motility was significantly different from the motility exhibited by the fresh sample.

Table 3.2.2. Percentages of total motile and progressively motile sperm after cooling to 5ºC when buck sperm were treated with 0 (control) or 1 mg of cholesterol loaded cyclodextrins (CLC; per 120 x 10^6 sperm) and were diluted with different extenders

			Total motile sperm (%)		Progressively motile sperm (%)	
			L.S.M.	S.E.	L.S.M.	S.E.
Treatments		**Control**	83.40[a]	1.60	58.67[a]	1.73
		CLC	86.61[b]	1.60	62.30[b]	1.74
		***P*-value**	0.0029		0.0120	
Extenders		**20% EY**	85.41	1.69	61.95[B]	1.88
		Skim milk	85.63	1.69	62.59[B]	1.88
		2% EY	83.98	1.68	59.91[A]	1.87
		***P*-value**	N.S		0.0027	

EY: egg yolk; L.S.M.: least squares means; S.E.: standard error; N.S.: non significant; [a,b]Different superscripts within the same column indicate differences between semen treatments; [A, B]Different superscripts within the same column indicate differences between extenders.

Similarly to total sperm motility, the addition of CLC had a beneficial effect on the percentage of progressively motile sperm after cooling to 5ºC (58.67 vs 62.30% for control and CLC-treated sperm; $P < 0.05$; Table 3.2.2). On the other hand, samples diluted with diluents containing 20% egg yolk or skim milk maintained greater percentages of progressively motile sperm (61.95 and 62.59% for 20% egg yolk and skim milk, respectively; Table 3.2.2) than samples diluted with extenders containing 2% egg yolk (59.91%). The interaction between these factors was not significant.

Table 3.2.3. Percentages of motile and live sperm 10 min after thawing when buck sperm were treated with 0 (control) or 1 mg of cholesterol loaded cyclodextrins (CLC; per 120 x 10^6 sperm) prior to cryopreservation and were frozen with different extenders

		Total motile sperm (%)		Progressively motile sperm (%)		Live Sperm (%)	
		L.S.M	S.E.	L.S.M	S.E.	L.S.M	S.E.
Treatments	**Control**	59.34[a]	2.43	41.00[a]	2.33	43.72[a]	2.18
	CLC	72.92[b]	2.43	50.65[b]	2.33	60.77[b]	2.18
	_P_-value	< 0.0001		< 0.0001		0.0009	
Extenders	**20% EY**	70.05[B]	2.56	50.11[B]	2.45	56.34[B]	2.40
	Skim Milk	69.83[B]	2.59	47.01[B]	2.48	52.95[B]	2.38
	2% EY	58.50[A]	2.56	40.34[A]	2.45	47.45[A]	2.36
	_P_-value	< 0.0001		< 0.0001		< 0.0001	

EY: egg yolk; L.S.M.: least squares means; S.E. standard error; [a,b]Different superscripts within the same column indicate differences between semen treatments; [A,B]Different superscripts within the same column indicate differences between extenders.

Sperm quality values observed 10 min after thawing are presented in Table 3.2.3. Samples frozen in extenders containing high egg yolk concentrations (20%) or skim milk presented greater percentages of total motile (70.05% and 69.83%, respectively), progressively motile (50.11 and 47.01, respectively) and live sperm (56.34 and 52.95%, respectively) than sperm frozen in extenders with 2% egg yolk (58.50 %, 40.34% and

47.45% total motile, progressively motile and live sperm, respectively). In addition, CLC-treated sperm exhibited higher quality after thawing (72.92 %, 50.65% and 60.77% total motile, progressively motile and live sperm, respectively) than control sperm (59.34 %, 41.00% and 43.72% total motile, progressively motile and live sperm, respectively).

The interaction extender*semen treatment was significant for the percentages of total motile sperm and membrane integrity (Table 3.2.4), but this interaction was not significant for the percentage of progressively motile sperm. Control samples frozen in the extender containing 2% of egg yolk presented the lowest percentages of total sperm motility and sperm viability (49.36 and 36.89%, respectively), while the other two extenders offered similar results for control samples.

However, when CLC-treated sperm were frozen in 2% egg yolk extender, the percentage of total motile sperm increased (67.64%) to the level observed for the control samples frozen in the other extenders (62.41% and 66.24%, for 20% egg yolk and skim milk, respectively), but this value was significantly lower than the values observed for CLC-treated sperm diluted with 20% egg yolk or skim milk extender. With respect to the sperm viability after thawing, values observed for CLC-treated sperm were similar for the extenders containing 2% egg yolk or skim milk (58.01 and 58.12%, respectively) and, although these results were higher than for the control samples, they did not reach the percentage of viable sperm observed for the CLC-treated samples and frozen in 20% egg yolk extender (66.19%). It is remarkable that the when the skim milk extender was used for cryopreservation, the increase in the percentages of total motile (7 percentage points) and live sperm (10 percentage points) were lower than those observed for the Tris extenders independently of the percentage of egg yolk used (15-17 percentage points for total motile and 20-22 percentage points for live sperm).

Table 3.2.4. Percentages of motile and live sperm belonging to extender*treatment interaction 10 min after thawing when buck sperm were treated with 0 (control) or 1 mg of cholesterol loaded cyclodextrins (CLC; per 120 x 10^6 sperm) prior to cryopreservation and were frozen with different extenders

		Total motile sperm (%)		Progressively motile sperm (%)		Live sperm(%)	
		L.S.M.	S.E.	L.S.M.	S.E.	L.S.M.	S.E.
Extender * Treatment	20%EY-Control	62.41[b]	2.93	45.82	2.81	46.49[b]	2.94
	20%EY-CLC	77.68[d]	2.93	54.41	2.81	66.19[c]	2.88
	Skim Milk-Control	66.24[bc]	2.97	42.73	2.85	47.77[b]	2.88
	Skim Milk-CLC	73.43[cd]	2.97	51.30	2.85	58.12[c]	2.88
	2%EY-Control	49.36[a]	2.93	34.46	2.81	36.89[a]	2.83
	2%EY-CLC	67.64[bc]	2.93	46.23	2.81	58.01[c]	2.88
	P-value	0.0233		N.S.		0.0432	

EY: egg yolk; L.S.M.: least squares means; S.E. standard error; N.S.: non significant; [a,b,c,d]Different superscripts within the same column indicate differences between semen treatments.

Sperm treated with CLC prior to cryopreservation maintained higher percentages of total motile and progressively motile sperm than control samples during incubation at 38ºC for 150 min (Table 3.2.5). In addition, when sperm were frozen in 2% egg yolk extender, motility parameters were lower than when sperm were frozen in 20% egg yolk or skim milk extender. With respect to the incubation time, motility remained high during 30

min of incubation at 38 ºC, but it decreased significantly when samples were incubated for a longer time (150 min).

Interactions were observed for both, total and progressively motile sperm between the extender and the semen treatment (Table 3.2.6). Differences were observed for control sperm between all the extenders used, presenting the samples frozen with 2% egg yolk the lowest values for total sperm motility (42.07%) and the samples frozen with skim milk exhibited the highest values for this parameter (53.41%), while samples frozen qith 20% egg yolk extender presented middle values (48.80%). With respect to the CLC-treated samples, differences were observed in the percentage of total motile sperm between the samples frozen with 2% or 20% egg yolk (61.57% vs 66.05%).

Interactions between the extender and the incubation time were observed. It is remarkable that samples frozen in skim milk extender (control or CLC-treated) and diluted with Tris-BSA extender after thawing maintained the percentages of total (72.10 and 60.46% for time 0 and 150 min, respectively) and progressively motile sperm (50.42 and 36.10% for time 0 and 150, respectively) trough all the incubation period (data not show in tables). However, samples frozen with egg-yolk Tris extenders lost sperm motility after 30 min of incubation at 38 ºC, irrespective of the amount of egg yolk used (70.07% and 59.60 % total sperm motility at time 0 vs 47.39 and 43.11% total sperm motility at 150 min for samples frozen in 20% and 2% egg yolk, respectively). In addition, the longevity of the undiluted samples after incubation at 38 ºC was lower for the samples frozen in skim milk or in 20% egg yolk extenders than for the samples frozen with low egg yolk levels (2%).

Table 3.2.5. Percentages of total motile and progressively motile sperm for up to 150 min of incubation at 38 ºC after cryopreservation, when buck sperm were treated prior to cryopreservation with 0 (control) or 1 mg of cholesterol loaded cyclodextrins (CLC; per 120 x 10^6 sperm) and were frozen with different extenders

		Total motile sperm (%)		Progressively motile sperm (%)	
		L.S.M.	S.E.	L.S.M	S.E.
Extenders	**2% Egg yolk**	51.82[a]	2.39	33.71[a]	1.94
	20% Egg yolk	57.42[b]	2.39	40.21[b]	1.94
	Skim milk	57.56[b]	2.40	38.15[b]	1.95
	P-value	< 0.0001		< 0.0001	
Treatments	**Control**	48.09[A]	2.35	32.16[A]	1.90
	CLC	63.11[B]	2.35	42.56[B]	1.90
	***P*-value**	< 0.0001		< 0.0001	
Incubation time (min)	**0 min**	67.25[w]	2.52	45.33[x]	2.06
	10 min	66.10[w]	2.52	45.88[w]	2.06
	30 min	58.65[x]	2.52	42.94[x]	2.06
	30min diluted	63.09[w]	2.52	44.53[x]	2.06
	150min	28.19[z]	2.52	15.73[z]	2.06
	150min diluted	50.32[y]	2.52	29.75[y]	2.06
	***P*-value**	< 0.0001		< 0.0001	

L.S.M.: least squares means; S.E.:standard error; [a,b]Different superscripts within the same column indicate differences between semen treatments; [A,B]Different superscripts within the same column indicate differences between extenders; [w,x,y,z]Different superscripts within the same column indicate differences between incubation times.

Table 3.2.6. Percentages of total motile and progressively motile sperm belonging to extender*treatment interaction for up to 150 min of incubation at 38 ºC after cryopreservation, when buck sperm were treated prior to cryopreservation with 0 (control) or 1 mg of cholesterol loaded cyclodextrins (CLC; per 120 x 106 sperm) and were frozen with different extenders.

		Total motile sperm (%)		Progressively motile sperm (%)	
		L.S.M.	**S.E.**	**L.S.M**	**S.E.**
Extender * Treatment	**2%EY-Control**	42.07^z	2.51	27.53^z	2.05
	2%EY-CLC	61.57^w	2.51	39.89^x	2.05
	20%EY-Control	48.80^y	2.51	34.49^y	2.05
	20% EY-CLC	66.05^v	2.51	45.92^w	2.05
	Skim milk-Control	53.41^x	2.53	34.45^y	2.07
	Skim milk-CLC	61.70^{vw}	2.53	41.86^x	2.07
	***P*-value**	< 0.0001		0.0255	

EY: egg yolk; L.S.M.: least squares means; S.E.:standard error; ^{v,w,x,y,z}Different superscripts within the same column indicate differences between between the interaction extender*treatment.
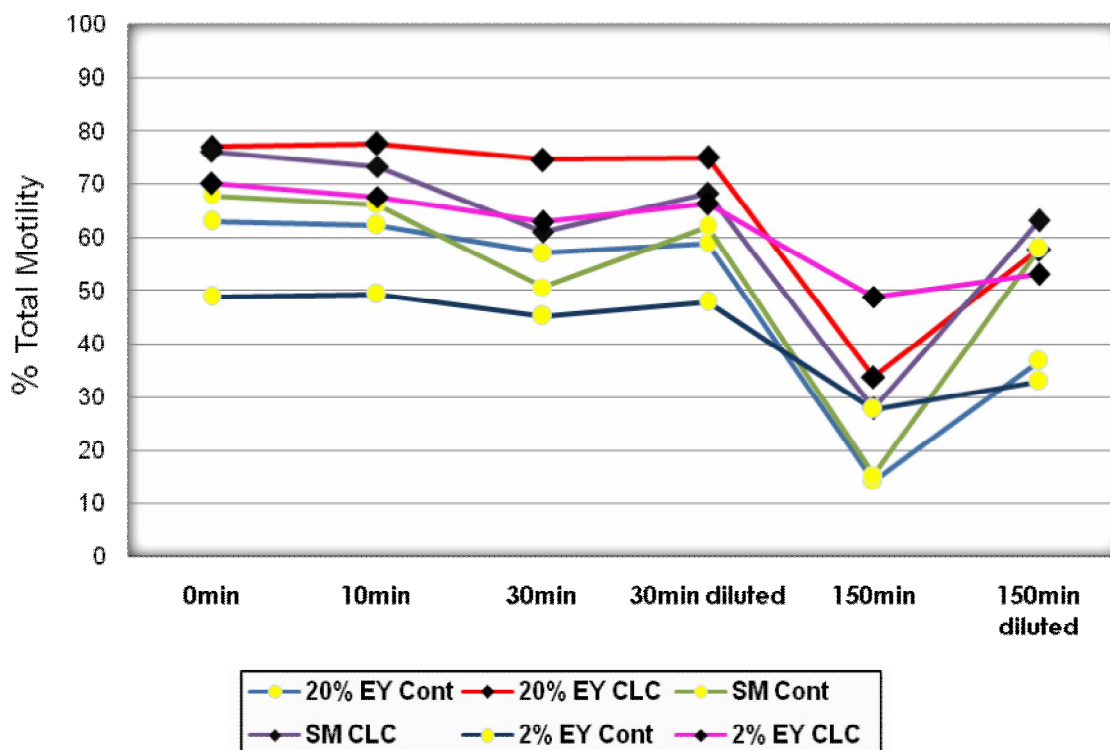
Figure 3.2.1. Percentages of total motile sperm for up to 150 min of incubation at 38 ºC after cryopreservation when buck sperm were treated prior to cryopreservation with 0 (control; Cont) or 1 mg of cholesterol-loaded cyclodextrins (CLC; per 120 x 10^6 sperm) and were frozen with different extenders (20% EY: Tris diluent containing 20% egg yolk; 2% EY: Tris diluent containing 2% egg yolk; SM: skim milk extender).

The evolution of both the percentage of total motile and the percentage of progressively motile sperm for all the groups can be observed in Figures 3.2.1 and 3.2.2, respectively. Sperm motility decreased after 30 min of incubation at 38 ºC, although CLC treated samples maintained higher motility during incubation for 150 min. It is remarkable that skim milk - control group maintained high motility values during incubation for 150 min when diluted in Tris-BSA diluent. Soon after thawing the percentages of total motile sperm after 150 min incubation for CLC treated groups in each extender (20%EY, SM and

2%EY) and skim milk control group diluted in Tris-BSA immediately after thawing were 57.91%, 63.28%, 53.18% and 58.09%, respectively (*P*>0.05).
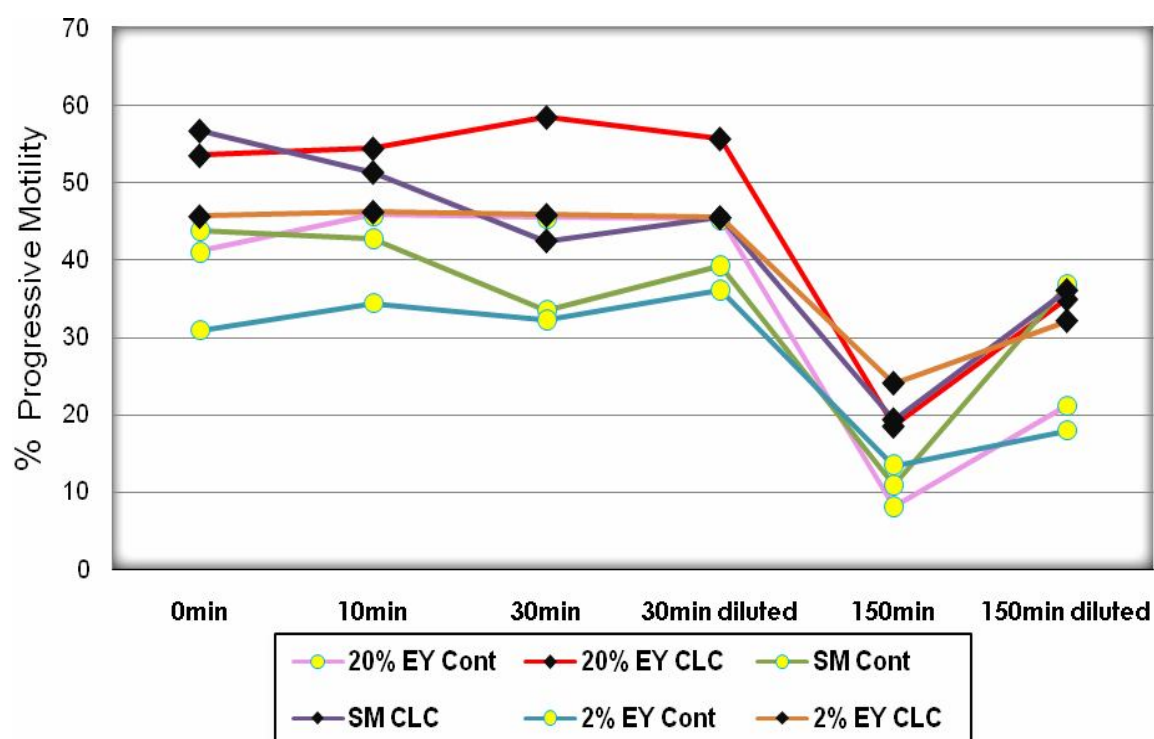
Figure 3.2.2. Percentages of progressively motile sperm for up to 150 min of incubation at 38 °C after cryopreservation when buck sperm were treated prior to cryopreservation with 0 (control; Cont) or 1 mg of cholesterol-loaded cyclodextrins (CLC; per 120 x 10^6 sperm) and were frozen with different extenders (20% EY: Tris diluent containing 20% egg yolk; 2% EY: Tris diluent containing 2% egg yolk; SM: skim milk extender).

On the other hand, 2% egg yolk-CLC group exhibited highest percentages of total and progressive motile sperm at 150 min (48.98% and 27.73%) when sperm samples were kept undiluted during incubation (Figures 3.2.1 and 3.2.2).

3.3. EXPERIMENT 3: EFFECT OF CLC TREATMENT ON THE QUALITY OF GOAT SPERM FROZEN IN DILUENTS DEPRIVED OF ANIMAL PRODUCTS

The parameters obtained for fresh ejaculates used in experiment 3 are summarized in Table 3.3.1. The percentages of total and progressively motile sperm observed in fresh samples were 90.43% and 58.71%, respectively.

Table 3.3.1. Mean values, standard deviation, maximum and minimum values observed for motility (%), volume (mL) and concentration ($x10^6$/mL) in fresh ejaculates used in Experiment 3.

	N	Mean	Std. Dev.	Max.	Min.
Total Motile sperm (%)	21	90.43	3.90	90	80
Progressively Motile sperm (%)	21	58.71	7.96	72	41
Volume (mL)	21	1.02	0.33	0.60	1.70
Concentration ($x10^6$/mL)	21	4222.53	1541.59	1705	6675

The percentages of total and progressively motile sperm remained high after cooling to 5 ºC and glycerol equilibration (Table 3.3.2). Similarly to the other experiments, samples treated with CLC presented higher values of total motile (87.86%) and progressively motile sperm (65.52 %) than control samples (85.08% and 57.97% for total and progressively motile sperm, respectively), although this difference is physiologically irrelevant. In addition, samples diluted in egg yolk extender presented greater percentages

of total motile sperm (88.87%) than samples diluted with egg yolk-free extender (84.07%), but the percentage of progressively motile sperm was similar for both diluents. It is remarkable that samples diluted with Tris extender maintained high motility levels after cooling to 5 ^{o}C, considering that they were cooled in absence of cryoprotectants.

Table 3.3.2. Percentages of total motile and progressively motile sperm after cooling to 5ºC when buck sperm were treated with 0 (control) or 1 mg of cholesterol loaded cyclodextrins (CLC; per 120 x 10^6 sperm) and were diluted with 20% egg yolk or egg yolk-free extenders (Tris)

		Total Motile sperm (%)		Progressively Motile sperm (%)	
		L.S.M.	**S.E.**	**L.S.M**	**S.E.**
Treatments	*Control*	85.08[a]	1.64	57.97[a]	1.59
	CLC	87.86[b]	1.63	65.52[b]	1.58
	P-value	0.0128		< 0.0001	
Extenders	*20% EY*	88.87[B]	1.64	62.92	1.61
	TRIS	84.07[A]	1.62	60.57	1.56
	P-value	<.0001		N.S.	
Treatment* Extender	*20% EY, Control*	89.16[y]	1.83	59.43	2.08
	20% EY, CLC	88.58[y]	1.81	65.89	2.04
	Tris, Control	81.00[z]	1.79	56.00	1.99
	TRIS, CLC	87.14[y]	1.79	65.14	1.99
	P-value	0.0029		N.S.	

EY: egg yolk; L.S.M.: Least squares means; S.E.: standard error; N.S.: non significant; [a,b]Different superscripts within the same column indicate differences between treatments; [A,B]Different superscripts within the same column indicate differences between diluents; [y,z] Different superscripts within the same column indicate differences between the interaction treatment * extender.

Table 3.3.3. Percentages of motile and live sperm 10 min after thawing when buck sperm were treated with 0 (control) or 1 mg of cholesterol loaded cyclodextrins (CLC; per 120 x 10^6 sperm) and were diluted with 20% egg yolk or egg yolk-free extenders (Tris)

		Total motile sperm (%)		Progressively motile sperm (%)		Live sperm (%)	
		L.S.M	S.E	L.S.M	S.E	L.S.M	S.E
Treatment	*Control*	34.12^a	2.64	25.05^a	2.34	24.70^a	2.56
	CLC	52.69^b	2.64	39.19^b	2.34	39.18^b	2.56
	P-value	< 0.0001		< 0.0001		< 0.0001	
Extender	*20%EY*	64.93^B	2.64	48.12^B	2.34	46.81^B	2.56
	Tris	21.88^A	2.64	16.12^A	2.34	17.06^A	2.56
	P-value	< 0.0001		< 0.0001		< 0.0001	
Treatment *Extender	*20%EY, control*	57.33	3.32	42.67	2.83	39.41	3.16
	20%EY, CLC	72.52	3.32	53.57	2.83	54.21	3.16
	Tris, control	10.95	3.32	7.43	2.83	9.98	3.16
	Tris, CLC	32.86	3.32	24.81	2.83	24.14	3.16
	P-value	N.S		N.S		N.S	

EY: egg yolk; L.S.M.: least squares means; S.E.:standard error; N.S.: non significant; [a,b]Different superscripts within the same column indicate differences between semen treatments; [A,B]Different superscripts within the same column indicate differences between extenders.

Interaction was observed between the semen treatment and the extender for the percentage of total motile sperm after cooling to 5 °C. Thus, when control sperm were

diluted with egg yolk-free extender a significant decrease in the percentage of total motile sperm was observed (81.00%) compared to the values observed for the other treatments (between 87.14 and 89.16%) and for fresh sperm (90.43%).

Similarly to experiments 1 and 2, CLC-treated sperm presented higher quality after thawing (52.69% total motile, 39.19% progressively motile and 39.18 % live sperm; Table 3.3.3) than control sperm (34.12% total motile, 25.05% progressively motile and 24.70 % live sperm; Table 3.3.3). In addition, egg yolk extender maintained sperm quality better (64.93%, 48.12% and 46.81% total motile, progressively motile, and live sperm, respectively) than the egg yolk-free extender (21.88%, 16.12% and 17.06% total motile, progressively motile, and live sperm, respectively).

Samples treated with CLC and samples frozen with egg yolk extender showed greater motility during incubation at 38ºC for 150 min than control samples and samples frozen with tris extender (Table 3.3.4). In addition, sperm motility decreased during the incubation at 38ºC (43.41% vs 30.29% total motile sperm 10 min or 150 after incubation for samples diluted with Tris-BSA after thawing, respectively). Similarly to previous observations, motility remained higher when samples were diluted in tris-BSA after thawing than when they were incubated undiluted.

Interaction was observed between the extender and the incubation time. Thus, the percentage of total motile sperm decreased significantly during incubation only when samples were frozen with egg yolk extender, but this was not observed for samples frozen in tris extender. Nevertheless the percentage of progressively motile sperm decreased during the incubation for both extenders.

Table 3.3.4. Percentages of total motile and progressively motile sperm for up to 150 min of incubation at 38°C after cryopreservation, when buck sperm were treated prior to cryopreservation with 0 (control) or 1 mg of cholesterol loaded cyclodextrins (CLC; per 120 x 10^6 sperm) and were diluted with 20% egg yolk or egg yolk-free extenders (Tris)

		Total motile sperm (%)		Progressively motile sperm (%)	
		L.S.M.	S.E.	L.S.M.	S.E.
Extender	**20% Egg yolk**	43.08^b	2.31	27.25^b	1.69
	Tris	17.46^a	2.32	10.83^a	1.69
	P-value	< 0.0001		< 0.0001	
Treatment	**Control**	21.24^A	2.32	12.80^A	1.69
	CLC	39.30^B	2.31	25.29^B	1.69
	P-value	< 0.0001		< 0.0001	
Incubation time (min)	**10min**	43.41$^\gamma$	2.46	32.12$^\gamma$	1.79
	150min	17.12$^\alpha$	2.48	8.33$^\alpha$	1.81
	150min diluted	30.29$^\beta$	2.46	16.68$^\beta$	1.79
	P-value	< 0.0001		< 0.0001	
Extender * incubation time	**20% EY, 10min**	64.93^x	2.84	48.12^w	2.09
	20% EY, 150min	19.35^z	2.86	8.94^z	2.10
	20%EY, 150min diluted	44.98^y	2.84	24.71^x	2.09
	Tris, 10min	21.88^z	2.84	16.12^y	2.09
	Tris, 150min	14.89^z	2.88	7.74^z	2.12
	Tris, 150min diluted	15.60^z	2.84	8.64^z	2.09
	P-value	< 0.0001		< 0.0001	

EY: egg yolk; L.S.M.: least squares means; S.E.:standard error; [a,b]Different superscripts within the same column indicate differences between semen extenders; [A, B]Different superscripts within the same column indicate differences between treatments; [.α,β,γ]Different superscripts within the same column indicate differences between incubation times; [w,x,y,z]Different superscripts within the same column indicate differences between the interaction extender*incubation time.

4 DISCUSSION

Artificial insemination (AI) plays an important role in animal breeding and the impact reproductive technologies have in commercial farms is constantly increasing. However, artificial insemination in goat breeding is not as widely used as in cattle industry. The use of this technology will certainly increase in the forthcoming years associated to the development and introduction of breeding programs in this species.

The Murciano-Granadina is one of the most important dairy goat breeds in the Mediterranean area and it is well adapted to the southeast of Spain. This breed produces milk with high cheese yield and it gathers most of the goat population of Spain. ACRIMUR (Spanish Association of Murciano-Granadina Breeders) is an organization responsible for maintenance, promotion, standardization and official testing of Murciano-Granadina goats (Anonym 1, 2009). The potential milk production of Murciano-Granadina goats is being enhanced through genetic improvement programs, therefore AI is an important tool for this purpose.

Although the artificial insemination and the use of frozen semen are very important for breeding programs, artificial insemination with frozen semen is still not used at a large scale in goat production due to the variable results obtained in both *in vivo* conditions and *in vitro* studies. These variable results are due to the negative effect that cryopreservation has on sperm function. Sperm quality after the freezing-thawing process will depend on the freezing method used, equilibration periods and cooling rate (Maxwell et al., 1993), as well as on the extenders used, and season where the semen is collected.

Several authors indicated that freezing and thawing cause both loss of viability and functionality of sperm cells (Curry, 2000; Holt, 2000a; Watson, 2000; Medeiros et. al., 2002; O'Connel et. al., 2002; Purdy, 2006; Hidalgo et. al., 2007). Some of the alterations in the structure and physiology of the sperm induced by cryopreservation are due to the temperature decrease. When temperature decreases from body temperature to $0-5^{\circ}C$ membrane lipids undergo phase transitions which lead to an increase of membrane permeability to ions and promote membrane fusion, causing an irreversible loss of motility

and distruption of acrosomes (Parks and Lynch, 1992; Drobnis et al., 1993; Watson, 2000). Membrane damage occurs when the membrane lipids undergo a phase change from fluid to gel state (Graham, 1998). This phase transition is affected by membrane constituents such as membrane cholesterol, which is an important determinant of membrane fluidity and permeability (Hartel et al., 1998; McGrath, 1988 reviwed by Zeng and Terada, 2001).

Membrane composition is an important factor in the freezability of sperm. In particular, both cholesterol:phospholipid ratio, and the ratio of unsaturated: saturated phospholipid-bound fatty acids are related with the susceptibility of the sperm to cold shock and sperm possessing membranes with high cholesterol:phospholipid ratio are resistant to cold-shock (Darin-Bennett and White, 1977; Combes et al., 2000; Mocé and Graham, 2006; Graham et al., 2006).

Cholesterol plays an important role in controlling the fluidity of the hydrocarbon chains of the phospholipids from the membranes, providing a coherent structure stable over a wide temperature range and allowing variation in the fatty acid composition, which itself influences the membrane fluidity (Darin-Bennett and White, 1977). Cholesterol can be added to plasma membrane of several cell types to prevent phospholipid rearrangement and to increase membrane fluidity at low temperatures (Ladbrooke, et al., 1968; Rottem et al., 1973; Klein et al., 1995 reviwed by Moore et al., 2005). Therefore, enrichment of sperm plasma membrane with cholesterol could be used as a strategy to minimize cryopreservation damages and to increase the sperm quality after freezing-thawing. The insolubility of cholesterol in aqueous solutions has been solved by means of using cyclodextrins, which can be used to transfer cholesterol to the membranes (Purdy and Graham, 2004).

According to results obtained in this study, treating buck sperm with cholesterol-loaded cyclodextrins prior to freezing is an effective strategy to improve sperm quality after thawing. The percentage of total motile sperm increased 7 - 22 percentage points and the percentage of membrane integrity increased 10 - 20 percentage points. Although differences exist between studies in the concentration of CLC used or the freezing extender used, as well as in the response obtained, these results are in agreement with previous reports in this species (Barrera-Compean et al., 2005; Tomás et al., 2008) as well as in

other cold-shock sensitive species such as bulls (Purdy and Graham, 2004; Mocé and Graham, 2006), rams (Morrier et al., 2004; Mocé et al., 2009), stallions (Combes et al., 1998; Graham, 1998; Moore et al., 2005; Oliveira et al., 2009), donkeys (Álvarez et al., 2006) or mice (Loomis and Graham, 2008). In these species, total motile sperm increased between 8 and 24 percentage points and membrane integrity increased 9- 21 with respect to the control. However, controversy exists about the effect of CLC on boar sperm quality after cryopreservation: while most of the authors did not observe differences between control and CLC-treated sperm in sperm motility or sperm membrane integrity (Zeng and Terada, 2001; Blanch et al., 2008), some authors reported an increase in the percentage of total motile sperm for CLC-treated sperm (Torres et al., 2009)

Although the exact mechanism by which cholesterol improves sperm cryosurvival is still not known, it has been observed that CLC-treated sperm presented higher cholesterol content in the membrane (between 1.93 and 2.7 fold) than control samples in bulls, stallions and rams (Purdy and Graham, 2004; Moore et al., 2005; Mocé et al., 2009). By increasing the cholesterol:phospholipid ratio, sperm membranes may exhibit increased resistance to cold shock, reduced membrane phase separations and therefore reduced leakage of cellular components (such as potassium) or it may inhibit calcium entry into the sperm, which is a prerequisite for capacitation and/or senescence (Mocé et al., 2009). On the other hand, CLC-treated sperm exhibited wider osmotic tolerance limits than untreated sperm in bulls, stallions, rams and boars (Walters et al., 2008; Amorim et al., 2009; Glazar et al., 2009; Mocé et al., 2009). This could provide a mechanism to increase post-thaw survival of sperm, taking into account that sperm suffer from osmotic stress during the cryopreservation process. In addition, CLC-treatment increased the permeability of sperm plasma membrane in stallions and boars (Walters et al., 2009; Glazar et al., 2009), which could reduce the amount of osmotic stress endured by sperm during cryopreservation.

On the other hand, CLC treatment improved also sperm quality of cooled stallion sperm stored at 5ºC for up to 72 h (Torres et al., 2006). In our study we observed very little differences between CLC-treated and control sperm after refrigeration to 5ºC (3-4 points of difference). This lack of response after cooling to 5ºC could be due to male effects (it is possible that the males used in our study were good coolers), but it is likely that the

beneficial effect of CLC is only observed after long storage at this temperature (and we evaluated sperm motility 2 h after refrigeration).

Some studies proposed that longevity during incubation is a more reliable parameter as an indicator of in-vitro fertilization (Dorado et al., 2007), since the maintenance of a higher motility during incubation reflects a greater likelihood to survive in the female genital tract, undergo capacitation and fertilize the ovum (Fiser et al., 1991). In the present study, we observed that treatment of sperm with CLC not only improved sperm motility after thawing but sperm motility was maintained longer during incubation at 38°C for 150 min. These results corroborate previous reports with ram sperm (Mocé et al., 2009).

Egg yolk and skim milk protect sperm during the refrigeration and the freezing process, therefore both components are usually present in semen freezing extenders (Purdy, 2006). However, centrifugation is a mandatory step in protocols for buck sperm freezing when high egg yolk levels or skim milk are used in the extenders. This is due to the enzymes present in the seminal plasma of this species that interact with some components of egg yolk and skim milk, producing toxic components for sperm (for a review see Leboeuf et al., 2000; Purdy, 2006). Nevertheless, elimination of seminal plasma could have a negative impact on the sperm since some authors indicated that cholesterol loss begins soon after sperm are removed from seminal plasma and it is a required alteration for sperm capacitation (Belmonte et al., 2005). Therefore, cholesterol level in the membrane could be decreased as a consequence of seminal plasma elimination. Buck sperm can be treated with CLC before or after the centrifugation step of the protocol and the optimal moment for CLC treatment should be determined. According to our results, treating buck sperm with CLC after washing resulted in the highest percentages of motile and viable sperm and these results are in agreement with previous reports in this species (Tomás et al., 2008). The reason why sperm treated with CLC after centrifugation presented higher sperm quality than sperm treated before washing is unknown. Perhaps the transferred cholesterol could be lost during the centrifugation process or perhaps the transference was not very efficient in the presence of seminal plasma. Nevertheless, this last hypothesis is not very likely since

CLC treatment was beneficial when buck sperm were frozen in diluents containing 0 or 2% egg yolk (and in these cases seminal plasma was not removed).

When modifications are made in a freezing protocol and the objective is to succeed in introducing these modifications in the industry, it is necessary to demonstrate that the modification works with all the freezing extenders used in a particular species. Concerning to buck sperm freezing, Tris-egg yolk (20%) and skim milk extenders are the diluents most widely used (Purdy, 2006), but both of them require the elimination of seminal plasma. Although the removal of seminal plasma improves semen quality, it is a time-consuming process, it may cause loss of the sperm and the efficiency of washing may vary depending on the season (Leboeuf et al., 2000). In addition, some beneficial components of seminal plasma could be also lost during washing, therefore an alternative Tris extender with low egg yolk level (2%) can be used to avoid the centrifugation step (Ritar and Salamon, 1982). In this study we observed that freezing extender used significantly affects post-thaw sperm motility and viability parameters. Thus, samples frozen in the diluent with low egg yolk level presented lower sperm quality than samples frozen with Tris-egg yolk (20%) and skim milk diluents. This observation is similar to previous studies where Tris extenders with high (20%) or low (1.5%) egg yolk levels were compared (Kozdrowski et al., 2007). The protective mechanism of egg yolk seems to rely on the phospholipids and low density lipoprotein components and in case of skim milk seems to reside on the protein fraction (Medeiros et al., 2002). It is likely that at these low egg yolk levels the cryoprotection is not fully accomplished and this could explain the difference between the diluents. However, negative effects of seminal plasma cannot be discarded, taking into account that seminal plasma composition may vary throughout the year and this will cause changes in freezeability depending on the season (Cabrera et al., 2005). Seminal plasma may contain some detrimental factors apart from enzymes that hydrolyze to egg yolk (EY) or skim milk components that are also removed during washing (reviewed by Cabrera et al., 2005). With this respect, some autors observed that removal of seminal plasma improved sperm cryosurvival even when the freezing media did not contain egg yolk at all (Corteel, 1974; Ritar and Salamon, 1982).

Skim milk and Tris-egg yolk (20%) extenders provided similar results in the present study. However, sperm frozen in skim milk extender maintained sperm motility throughout the incubation period at 38ºC, compared to the egg yolk extenders which lost sperm motility after 30 min of incubation. Therefore, skim milk extender seems to provide better results than egg yolk extender. Comparisons with other studies are difficult, due to the differences between extender composition and incubation time. In a recent study comparisons were made between Tris-20%EY and Milk-20%EY-Tris extenders and it was reported that the percentage of motile sperm remained higher after 1 h of incubation at 37ºC when sperm were frozen in Tris-20%EY (56.19%) than in Milk-20%EY-Tris extender (52.43%; Dorado et al., 2007). Whether the difference we observed between extenders is due to the glycerol level (4% vs 7% in the egg yolk and skim milk diluents, respectively) or to specific components of egg yolk and skim milk should be tested in later studies. With this respect, some authors observed that heat treatment increased the antioxidant activity of skim milk and milk proteins, principally caseins, responsible for this antioxidant activity (Taylor and Richardson, 1980), and egg yolk-glycerol extender presented very limited antioxidant capacity based on its capacity to neutralize H_2O_2 (Bilodeau et al., 2002). Therefore, it is likely that the oxidative damage that sperm suffered was different depending on the extender, and this latent damage was observed after incubation.

In correlation with these reports, cryopreservation of sperm is associated with an oxidative stress induced by free radicals (Salvador et al 2006), and washed frozen-thawed sperm is more vulnerable to ROS stress than freshly diluted semen (Neild et al., 2005). In addition, sperm membranes are characterized by relatively greater concentrations of polyunsaturated fatty acids (Parks and Lynch, 1992) that are succeptible to peroxidative damage (Aitken, 1995 reviewed by Ball 2008). Some studies concluded that lipid peroxidation of sperm membrane lipids is a major cause for the loss of motility and fertilizing ability of human and mammalian spermatozoa (Aitken et al., 1989; Jones and Mann, 1977 reviwed by Ceylan and Serin, 2007), and the loss of motility in human sperm exposed to extracellular reactive oxygen species is correlated to the level of lipid peroxidation (Gomez et al., 1998). This difference in the antioxidant capacity of the heated

skim milk and the egg yolk could explain, at least in part, the differences observed in longevity between extenders.

In addition, CLC treatment was effective for all the diluents tested, although the increase in sperm quality was higher for the diluents containing egg yolk (+15-17 % total motile and +20% live sperm) than for the skim milk diluent (+7% total motile and +10% live sperm). The reason for this phenomenon is unknown, although some authors reported that incubation of sperm in an egg yolk medium can decrease the cholesterol/phospholipid ratio of the sperm plasma membrane (Moubasher et al., 1985). This could partly explain the higher response observed when sperm were frozen in egg yolk diluents.

Elimination of animal products from the freezing extenders is desirable for sanitary reasons. Therefore, we studied if CLC treatment could provide enough protection in Tris extender. Our results showed that, although CLC treatment increased the percentages of total motile sperm in the egg yolk-free diluent (32.86% total motile sperm), the results were clearly lower to those obtained with egg yolk extenders (72.52%). Results observed for control sperm in our study are similar to previous reports (Aboagla and Terada, 2004). Obviously, some of the components that egg yolk contains are important for cryoprotection and CLC cannot equalize this protection. With this respect, lipoproteins from egg yolk are proven to prevent sperm cells from cold shock by solubilizing the lipid and binding to cell membrane (Watson, 1976; Cheng et al., 2004). On the other hand, it is possible that egg yolk exerts its protection through its phospholipids either by merging with sperm membrane and replacing some of the sperm phospholipids (Graham and Foote, 1987) or by forming a protective film at the surface of the sperm membranes after disruption of the lipoprotein (Quinn et al., 1980). Whether these results could be increased or not by increasing the glycerol level in the extender should be tested in following studies.

In general, samples diluted immediately after thawing with Tris-BSA extender maintained better motility during incubation than samples undiluted, which lost sperm motility after 30 min of incubation. This results were expected and can be due to several factors, such as the glycerol level and the egg yolk and skim milk levels. Cryoprotectants are necessary for cryopreservation processes, but they can be toxic for the sperm (Holt, 2000a; Watson, 2000), and this toxicity increases as the temperature of addition increases

(in rams, Colas et al., 1975). On the other hand, both egg yolk and skim milk extenders are efficient for storing at 4-5ºC (Leboeuf et al., 2003), but they are not recommended for storing the sperm at higher temperatures. On the other hand, the presence of BSA in the incubation extender could exert a beneficial effect on sperm motility, since some authors reported that BSA enhances the motility and viability of ram sperm following the freezing-thawing process (Matsuoka, 2006, reviewed by Uysal et al. 2007) and it eliminates free radicals generated by oxidative reactions and protects the membrane integrity of sperm from lipid peroxidation (Lewis et al. 1997, reviewed by Uysal et al. 2007).

The differences observed between the percentages of total motile sperm and the percentages of plasma membrane integrity in our experiments are in agreement with previous studies (Gil et al. 1999). Several authors reported that the freezing-thawing process reduces motility to a lesser degree than structural integrity and it indicates that the plasma and acrosome membranes are more vulnerable than parts of spermatozoa involved in locomotion (Salamon and Visser, 1972; Salamon and Maxwell, 1995; Dorado et al., 2007).

In conclusion, treating buck sperm with CLC prior to cryopreservation benefits sperm cryosurvival. In addition, the CLCs were effective with all the freezing extenders tested, making this technology practical for application in the industry, using current cryopreservation techniques. Nevertheless, additional studies should be conducted to determine how added cholesterol affects sperm functionality and sperm fertilizing ability both in vitro and in vivo.

5 REFERENCES

Aamdal, J., Lyngset, O., Fossum, K., 1965. Toxic effect of lysolecithin on sperm. A preliminary report. Nord. Vet. Med. 17, 318–319.

Aboagla, E.M.-E., Terada, T., 2004. Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. Theriogenology 62, 1160–1172.

Aitken, R.J., Clarkson, J.S., Fishel, S., 1989. Generation of reactive oxygen species, lipid peroxidation and human sperm function. Biol. Reprod. 41, 183-187.

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

Álvarez, A.L. Serres, C., Torres, P., Crespo, F., Mateos, E., Gómez-Cuétara, C., 2006. Effect of cholesterol-loaded cyclodextrin on the cryopreservation of donkey spermatozoa. Abstract. Animal Reproduction Science 94, 89–91.

Amann, R.P., 1998. Cryopreservation of sperm. In Encyclopedia of Reproduction Vol. 1 (Eds, Knobil, E. and Neill, J.) Academic Press, London, pp. 773-783.

Amorim, E.A.M., Graham, J.K., Spizziri, B., Meyers, M., Torres, C.A.A., 2009. Effect of cholesterol or cholesteryl conjugates on the cryosurvival of bull sperm. Cryobiology Vol.58, Issue 2: 210-214.

Anonym 1, 2009. http://www.ansi.okstate.edu/breeds/goats/murciagranada/index.htm

Anonym 2, 2000. Introduction to Flow Cytometry: A Learning Guide. BD Biosciences 2350 Qume Drive San Jose, CA 95131-1807-1-800-448-2347.

Bailey, J.L., Lessard, C., Jacques, J., Bréque, C., Dobrinski, I., Zeng, W., Galantino-Homer, H.L., 2008. Cryopreservation of boar semen and its future importance to the industry. Theriogenology Vol. 70, Issue 8, 1251-1259.

Ball, B.A., 2008. Oxidative stress, osmotic stress and apoptosis: Impacts on sperm function and preservation in the horse. An. Rep. Sc. Vol. 107, Issue.3-4, 257-267.

Barbas, J., P., Mascarenhas, R., D., 2008. Cryopreservation of domestic animal sperm cells. Review Paper. Springer Science Business Media, Cell Tissue Banking.

Barker, C.A., 1957. Some aspects of artificial insemination in swine and goats. In:Proc.10th Ann. Conv.National Assoc. Artif. Breeders, Toronto, pp. 127–132.

Barrera-Compean, M.H., Purdy, P.H., Dzakuma, J.M., Newton, G.R., Nuti, L.C., 2005. Cholesterol-loaded cyclodextrin improves post-thaw goat sperm motility. J. Anim. Sci. Vol. 83.

Batista, M., Niňo T., Alamo D., Castro N., Santana M., González F., Cabrera F., Gracia A., 2009. Successful artificial insemination using semen frozen and stored by an ultrafreezer in the Majorera goat breed. Theriogenology, Vol.71, Issue 8, 1307-1315.

Belmonte, S.A., López, C.I., Roggero, C.M., De Blas, G.A, Tomes, C.N., Mayorga, L.S., 2005. Cholesterol content regulates acrosomal exocytosis by enhancing Rab3A plasma membrane association. Developmental Biology 285, 393- 408.

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 Vol. 57, Issue 3, 1105-1122.

Blanch, E., Tomás, C., Mocé, M.L., de Castro, M.P.V., Vicente, J.S., Mocé, E., 2008. Effect of cholesterol-loaded cyclodextrins and two diluents on boar sperm cryosurvival. 1st Joint International Meeting of the Spanish-Association-for-Animal-Reproduction/British-Andrology-Society Gijon, Spain, Oct 22-25, 2008. Rep.in Dom. An., Vol.43,.71-72, suppl.4.

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 Dom Anim 40, 191–195.

Ceylan, A., Serin, I., 2007. Influence of ascorbic acid addition to the extender on dog sperm motility, viability and acrosomal integrity during cooled storage. Revue Méd. Vét. 158, 7, 384-387

Cheng, F., Wu, T., Chan, P.J., Wang, J., Fung, H., Colenbrander, B., Tung K., 2004. The effect of different extenders on post-thaw sperm survival, acrosomal integrity and longevity in cryopreserved semen of Formosan Sika deer and Formosan Sambar deer. Theriogenology 61, 1605–1616 .

Christian, A.E., Haynes, M.P., Phillips, M.C., Rothblat, G.H., 1997. Use of cyclodextrins for manipulating cellular cholesterol content. J. Lipid. Res. 38, 2264-2272.

Colas, G., 1975. Effect of initial freezing temperature, addition of glycerol and dilution on the survival and fertilizing ability of deep-frozen ram semen. J. Reprod. Fertil. 42, 277–285.

Combes, G.B., Varner, D.D., Schroeder, F., Burghardt, R.C., Blanchard, T.L., 1998. Effect of supplemental cholesterol on postthaw motility and plasma membrane integrity of equine sperm, in: Proceedings of the Seventh International Symposium on Equine Reproduction, Pretoria, South Africa, , pp. 35–36.

Combes, G.B., Varner, D.D., Schroeder, F., Burghardt, R.C., Blanchard, T.L., 2000. Effect of cholesterol on the motility and plasma membrane integrity of frozen equine spermatozoa after thawing. J. Reprod. Fertil. Suppl. 56, 127–132.

Cordelli, E., Eleuteri, P., Leter, G., Rescia, M., Spanó, M., 2005. Flow cytometry applications in the evaluation of sperm quality: semen analysis, sperm function and DNA integrity. Contraception 72, 273– 279

Cormier, N., Bailey, J.L., 2003. A differential mechanism is involved during heparinand cryopreservation-induced capacitation, Biol. Reprod. 69, 177–185.

Corteel, J.M., 1973. L'insemination artificielle caprine: Bases physiologiques, état actuel et perspectives d'avenir. Word Rev. Anim. Prod. 9, 73–99.

Corteel, J.M., 1974. Viabilité des spermatozoides de bouc conservés et congelés avec ou sans leur plasma seminal: effet du glucose. Ann. Biol. Anim., Biochim., Biophys. Vol.14 Issue 4-B., 741–745.

Corteel, J.M., 1975. Effet du 'lavage' sur la conservation des spermatozoides de bouc a basse temperature. Ann. Biol. Anim., Biochim., Biophys. 15, 525–528.

Corteel, J.M., 1980. Effets du plasma séminal sur la survie et la fertilité des spermatozoídes conservés in vitro. Reprod. Nutr. Develop. 20, 1111–1123.

Corteel, J.M., 1981. Collection, proceeding and artificial insemination of goat semen. In: Gall, C. _Ed.., Goat Production. Academic Press, London, pp. 171–191.

Curry, M.R., 2000. Cryopreservation of semen from domestic livestock. Rev. Reprod. 5, 46–52.

Darin-Bennett, A., White, I.G., 1977. Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold shock. Cryobiology 14, 466-470.

Deka, B.B., Rao, A.R., 1987. Effect of extenders and thawing methods on post-thawing preservation of goat semen. Ind. Vet. J. 64, 591–594.

Dorado, J., Rodrígez, I., Hidalgo, M., 2007. Cryopreservation of goat spermatozoa: Comparison of two freezing extenders based on post-thaw sperm quality and fertility rates after artificial insemination. Theriogenology 68, 168–177.

Dorado, J., Hidalgo, M., Muñoz, A., Rodrígez, I., 2009. Assesment of goat semen freezability acording to the spermatozoa characteristics from fresh and frozen samples. Short communication. Animal Reproduction Science 112, 150–157.

Drobnis, E.Z., Nelson, E.A., Burrill, M.J., 1980. Effect of several processing variables on motility and glutamic oxalacetic transaminase levels for frozen goat semen. I. Diluent. J. Anim. Sci. Suppl. 51, 439 (Abstract).

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; Vol. 265, Issue 4, 432–7.

Dubeuf, J.P., Boyazoglu, J., 2009. An international panorama of goat selection and breeds. Livestock Science 120, 225–231.

Evans, G., Maxwell, W.M.C., 1987. Frozen storage of semen. In: Purdy, P., 2006. A review on goat sperm cryopreservation. Small Ruminant Research.

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.

Fukuhara, R., Nishikawa, Y., 1973. Effects of pH, sperm concentration, washing and substrate concentration on respiration and motility of goat spermatozoa. Jpn. J. Zootech. Sci. 44, 266–270.

Gacitua, H., Arav, A., 2005. Successful pregnancies with directional freezing of large volume buck semen. Theriogenology 63, 931–938.

Galantino-Homer, H.L., Zeng., W., Megee, S.O., Dallmeyer, M., Voelkl, D., Dobrinski, I., 2006. Efects of 2-hydroxypropyl-β -cyclodextrin and cholesterol on porcine sperm viability and capacitation status following cold shock or incubation. Molecular reproduction and development 73, 638-650.

Gao, G.Y., Ashworth, E., Watson, P.F., Kleinhans, F.W., Mazur, P., Critser, J.K., 1993. Hyperosmotic tolerance of human spermatozoa: separate effects of glycerol, sodium chloride and sucrose on spermolysis.Biol. Reprod. 49, 112–123.

Gil, J., Sijderquist, L., Rodriguez-Martinez, H., 1999. Influence of centrifugation and different extenders on post-thaw sperm quality of ram sperm. Theriogenology 54, 93-108.

Gillan, L., Kroetsch, T., Chis Maxwell, W.M., Evans, G., 2008. Assessment of in vitro sperm characteristics in relation to fertility in dairy bulls. Animal Reproduction Science 103, 201–214.

Glazar, A.I., Mullen, S.F., Liu, J., Benson, D.B., Critser, J.K., Squires, E.L., Graham, J.K., 2009. Osmotic tolerance limits and membrane permeability characteristics 3 of stallion spermatozoa treated with cholesterol. Cryobiology 2009.

Gomez, E., Irvine, D.S., Aitken, R.J., 1998. Evaluation of a spectrophotometric assay for the measurement of malondialdehyde and 4-hydroxyalkenals in human spermatozoa: relationships with semen quality and sperm function, Int. J. Androl. 21, 81–94.

Graham, E.F., Schmehl, M.K.L., Nelson, D.S., 1980. Problems with laboratory assays. In: Proc. Eighth Natl. Assoc. Anim Breeders Tech. Conf. A.I. Reprod., pp. 59–66.

Graham, J.K., Foote, R.H., 1987. Effect of several lipids, fatty acyl chain length and degree of unsaturation on the motility of bull spermatozoaafter cold shock and freezing. Cryobiology;24:42–52.

Graham, J.K., 1998. An update on semen extenders and cryoprotectants, in: Proceedings of the 17th Technical Conference on A.I. and Reprod., Madison, WI, pp. 32–36.

Graham, J.K., 2001. Assessment of sperm quality: a flow cytometric approach. Animal Reproduction Science 68, 239–247.

Graham, J.K., Purdy, P.H., Moore, A.I., Mocé, E., 2006. Mechanisms of cryopreservation: The effect of membrane cholesterol. Proceedings of 21. Technical conference on artificial insemination and reproduction.

Hafez, E.S.E., 1993. Reproduction in farm animals. 6. Edition. Lea&Febiger, Philadelphia.

Hartel, S., Diehl, H.A., Ojeda, S.F., 1998. Methyl-beta-cyclodextrins and liposomes as water-soluble carriers for cholesterol incorporation into membranes and its evaluation by a microenzymatic fluorescence assay and membrane fluidity-sensitive dyes. Anal Biochem. 258, 277–284.

Herman, H., A., Mitchell, J. R., Doak,G., A., 1994. The Artificial Insemination and Embryo Transfer of Dairy and Beef Cattle. 7. Edition. Interstate Publishers, Inc.

Hidalgo, M., Rodrigez, I., Dorado, J.M., 2007. The effect of cryopreservation on sperm head morphometry in Florida male goat related to sperm freezability. Animal Reproduction. Science 100, 61-72

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

Holt, W.V., 2000a. Basic aspects of frozen storage of semen. Animal Reproduction Science 62, 3–22.

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

Iritani, A., Nishikawa, 1961. Studies on the egg yolk coagulating factors in goat semen: II properties of the coagulating factor and influential conditions for coagulation. In: Proceedings of Silver Jubilee Laboratory of Animal Husbandry, Kyoto University, pp. 97–104.

Iritani, A., Nishikawa, 1963. Studies on the egg-coagulating enzyme in goat semen; IV. On the position of yolk consitituents attacked by the coagulating enzyme. Jpn. J. Anim. Reprod. 8.

Januskauskas, A., Johannisson, A., Soderquist, L., Rodriguez-Martinez, H., 2000. Assessment of sperm characteristics post-thaw and response to calcium ionophore in relation to fertility in Swedish dairy AI bulls. Theriogenology 53, 859–875.

Jones, R., Mann, T., 1977. Damage to ram spermatozoa by peroxidation of endogenous phospholipids. J. Reprod. Fertil. 50, 261-268

Karatzas, G., Karagiannidis, A., Varsakeli., S., Brikas, P., 1997. Fertility of fresh and frozen-thawed goat semen during the nonbreeding season. Theriogenelogy 53, 47-58.

Klein, U., Gimpi, G., Fahrenholz, F., 1995. Alteration of the myometrial plasma membrane cholesterol content with β-cyclodextrin modulates the binding affinity of the oxytocin receptor, Biochemistry 34, 13784–13793.

Koronkiewicz, S., Kalinowski, S., 2004. Influence of cholesterol on electroporation of bilayer lipid membranes: chronopotentiometric studies. Biochimica et Biophysica Acta 1661, 196– 203.

Kozdrowski, R., Dubiel, A., Bielas, W., Dzięcioł, M., 2007. Two Protocols of Cryopreservation of Goat Semen with the Use of Computer-Assisted Semen Analysis System. Acta Vet. Brno 76, 601-604.

La Falci, V.S.N., Tortorella, H., Rodrigues, J.L., Brandelli, A., 2002. Seasonal variation of goat seminal plasma proteins. Theriogenology 57, 1035-1048.

Ladbrooke, B.D., Williams, R.M., Chapman, D., 1968. Studies on lecithin–cholesterol-water interactions by diVerential scanning calorimetry and X-ray diVraction, Biochim. Biophys. Acta 150, 333–340.

Leboeuf, B., Restall, B., Salamon, S., 2000. Production and storage of goat semen for artificial insemination. Animal Reproduction Science 62, 113–141.

Leboeuf, B., Guillouet, P., Batellier, F., Bernelas, D., Bonne, J.L., Forgerit, Y., Renaud, G., Magistrini, M., 2003. Effect of native phosphocaseinate on the in vitro preservation of fresh semen. Theriogenology 60, 867–877.

Lewis, S.E., Sterling, E.S., Young, I.S., Thompson, W., 1997. Comparison of invidual antioxidants of sperm and seminal plasma in fertile and infertile men. Fer. Steril., 67, 142-147.

Loomis, P.R., Graham, J.K., 2008. Commercial semen freezing: Individual male variations in cryosurvival and the response of stallion sperm to customized freezing protocols. Anim. Rep. Sci.105, 199-128.

Matsuoka, T., Imai, H., Kohno, H., Fukui, Y., 2006. Effects of bovine serum albumine and trehalose in semen diluents for improvement of frozen thawed ram spermatozoa. J. Rep. Dev., 52, 675-683.

Maxwell W.M.C., Salamon, S., 1993. Liquid storage of ram semen: a review. Reprod Fertil Dev;14:83–9.

Maxwell, W.M.,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.

McGrath, J.J., 1988. Membrane transport properties. In: McGrath JJ, Diller KR, eds. Low Temperature Biotechnology: Emerging Applications and Engineering Contribution. New York, Ny: Asme Press, Bed-Vol 10, Htd-Vol 98; 273–330.

Medeiros, C., M., O., Forell, F., Oliveira, A., T., D., Rodrigues, J., L., 2002. Current status of sperm cryopreservation: Why isn't it better? Theriogenology 57, 327-344.

Memon, M.A., Bretzlaff, K.N., Ott, R.S., 1985. Effect of washing on motility and acrosome morphology of frozen-thawed goat spermatozoa. Am. J. Vet. Res. 46, 473–475

Mocé, E., Graham, J. K., 2006. Cholesterol-loaded cyclodextrins added to fresh bull ejaculates improve sperm cryosurvival J. Anim. Sci: 84, 826–833

Mocé, E., Graham, J. K., 2008. In vitro evaluation of sperm quality. Animal Reproduction Science 105, 104–118.

Mocé, E., Purdy, P.H., Graham, J.K., 2009. Treating ram sperm with cholesterol-loaded cyclodextrins improves cryosurvival. Animal Reproduction Science. Article in press.

Moore, A.I., Squires, E.L., Graham, J.K., 2005. Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. Cryobiology 51, 241–249.

Morrier, A., Thériault, M., Castonguay, F., Bailey, J., 2004. Effect of cholesterol loaded methyl-β-cyclodextrin on ram sperm during cryopreservation, cold-shock and

artificial insemination. Proceedings of the Society for the Study of Reproduction Meeting. Vancouver, Canada. P 239 Abstract 636.

Moubasher, A.E.D., Prentice, D.A., Wolf, D.P., 1985. Membrane lipid changes in human sperm during capacitation. J. Androl; 6:135.

Navratil, A.M., Bliss, S.P., Berghorn, K.A., Haughian, J.M., Farmerie, T.A., Graham, J.K., Clay, C.M., Roberson, M.S., 2003. Constitutive localization of the gonadotropin-releasing hormone (GnRH) receptor to low density membrane microdomains is necessary for GnRH signaling to ERK. J. Biol. Chem. 278, 31593–31602.

Neild, D.M., Brouwers, J.F., Colenbrander, B., Aguero, A., Gadella, B.M., 2005. Lipid peroxide formation in relation to membrane stability of fresh and frozen-thawed stallion spermatozoa, Mol Reprod Dev 72, pp. 230–238.

Nunes, J., 1982. Etude des effets du plasma séminal sur la survie in vitro des spermatozoides de bouc. Thése de Doctorat, Université Pierre et Marie Curie, Paris, 33.

O'Connel, M., McClure, N., Lewis, S.E.M., 2002. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Human Rep., Vol.17, No.3, 704-709.

Oliveira, C.H., Vasconcelos, A.B., Souza, F.A., Martins Filho, O.A., Silva, M.X., Varago, F.C., Lagares, M.A., 2009. Cholesterol addition protects membrane intactness during cryopreservation of stallion sperm. Article in Press. Animal Reproduction Science

Parks, J.E., Lynch, D.V., 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion and rooster sperm membranes. Cryobiology, 29, 255-266.

Pellicer-Rubio, M.T., Magallon, T., Combarnous, Y., 1997. Deterioration of goat sperm viability in milk extenders is due to a bulbourethral 60-kilodaltonglycoprotein with triglyceride lipase activity. Biol. Reprod. 57, 1023–1031

Peterson, K., Kappen, M.A.P.M., Ursem, P.J.F , Nöthling , 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, 863–871

Pettitt, M.J., Buhr, M.M., 1998. Extender components and surfactants affect boar sperm function and membrane behaviour during cryopreservation. J.Androl.19, 736–746.

Pickett, B.W., Komarek, R.J., 1966. Effect of cold shock and freezing on loss of lipid from spermatozoa. J.Dairy Science Vol. 50, No.5.

Polge, C., Smith, A., Parkes, A., 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature London. 164-166.

Purdy, P.H., Graham, J.K., 2004. Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. Cryobiology 48, 36-45.

Purdy, P., 2006. A review on goat sperm cryopreservation. Small Ruminant Research 63, 215–225.

Purdy, P.H., Mocé, E., Stobart, R., Murdoch, W.J., Moss, G.E., Larson, B., Ramsey, S., Graham, J.K., Blackburn, H.D., 2009. The fertility of ram sperm held for 24 h at 5 C prior to cryopreservation. Animal Reproduction Science. Article in press.

Quinn, P.J., Chow, P.Y.W., White, I.G., 1980. Evidence that phospholipid protects ram spermatozoa from cold shock at plasma membrane site. J Reprod Fertil 1980;60:403–7.

Ritar, A.J., Salamon, S., 1982. Effects of seminal plasma and its removal and of egg yolk in the diluent on the survival of fresh and frozen-thawed spermatozoa of the Angora goat. Aust. J. Biol. Sci. 35, 305–312.

Ritar, A.J., Salamon S., 1991. Effect of month of collection, method of processing, concentration of egg yolk and duration of frozen storage on viability of Angora goat spermatozoa. Small Ruminant Res 4, 29–37.

Rottem, S., Yashouv, J., Ne'eman, A., Razin, A., 1973. Composition, ultra-structure and biological properties of membrane from Mycoplasma mycoides var. capri cells adapted to grow with low cholesterol concentrations, Biochim. Biophys. Acta 323, 495–508.

Rouge, M., 2003. Sperm motility. http://www.vivo.colostate.edu/hbooks.

Roy, A., 1957. Egg-yolk coagulating enzyme in the semen and Cowper's gland of the goat. Nature London.179, 318.

Salamon S, Visser, D., 1972. Effect of the composition of Tris-based diluent and of thawing solution on survival of ram spermatozoa frozen by the pellet method. Aust J Biol Sci. 25, 605–18.

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.

Salvador, I., Yániz, J., Viudes-de-Castro, M.P., Gómez, E.A., Silvestre, M.A., 2006. Effect of solid storage on caprine semen conservation at 5 °C. Theriogenology 66, 974–981.

Sawyer, D.E., Brown, D.B., 1995. The use on an in vitro sperm activation assay to detect chemically induced damage of human sperm nuclei. Reprod. Toxicol. 9, 351–357.

Silvestre, M.A., Salvador, I., Sanchez, J.P., Gómez, E.A., 2004. Effect of changing female stimulus on intensive semen collection in young Murciano-Granadina male goats. Jour. Anim. Sci., 82, 1641-1645

Smith, A.H., Polge, C., 1950. Survival of spermatozoa at low temperatures. Nature London. 166, 668–671.

Taylor, M.J., Richardson, T., 1980. Antioxidant activity of skim milk: effect of heat and resultant sulfhydryl groups.Journal of Dairy Science Vol. 63, No. 11.

Tomás, C., Blanch, E., Moce, E., 2008. Cholesterol addition after centrifugation improves goat sperm quality after cryopreservation. 1st Joint International Meeting of the Spanish-Association-for-Animal-Reproduction/British-Andrology-Society Gijon, Spain. Reproductio In Domestic Animals Vol:43,53-53, Suppl. 4.

Tomás, C., Blanch, E., Mocé, E., 2009. Evaluación de la motilidad durante el proceso de crioconservación tras la adición o eliminación de colesterol de las membranas plasmáticas de espermatozoides de verraco. XIII Jornadas de Producción Animal (AIDA). AIDA, Tomo II, pp 732-734. Zaragoza, España. 12-13 Mayo, 2009.

Torres, P., Serres, C., Gómez-Cuétara, C., Santiago, I., Mateos, E., Álvarez, A.L., 2006. Effect of cholesterol-loaded cyclodextrin on motility and plasma membrane integrity of cooled stallion sperm. Abstract. Animal Reproduction Science 94, 148–151.

Torres, C.A.A., Moraes, E.A., Graham, J.K., Romualdo, P.L., 2009. The Effect of Adding Cholesterol Loaded Cyclodextrin on Boar Sperm Cryosurvival. Abstract. Biology of Reproduction 81, 455.

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.

Upreti, G.C., Hall, E.L., Koppens, D., Oliver, J.E., Vishwanath, R., 1999. Studies on the measurement of phospholipase A2 (PLA2) and PLA2 inhibitor activities in ram semen. Anim. Reprod. Sci. 56, 107–121.

Uysal, O., Bucak, M.N., Yavaş, İ., Varışlı, Ö., 2007. Effect of variou antioxidants on the quality of frozen-thawed bull semen. Jour. of An. and Vet. Ad. Vol. 6, Issue 12, 1362-1366

Verstegen, J., Iguer-Ouada, M., Onclin K., 2002. Computer assisted semen analyzers in andrology research and veterınary practice. Theriogenology 57, 149-l 79.

Varela Junior, A.,S., Corcini, C., D., Ulguim, R., R., Alvarenga, M.V., Bianchi, F.I., Corrêa, M.N., Lucia Jr., T., Deschamps, J.C., 2009. Effect of low density lipoprotein on the quality of cryopreserved dog semen. Short communication. Animal Reproduction Science 115, 323–327.

Walters, E.M., Rieke, A., Graham, J.K., Critser, J.K, 2008. Improved osmotic tolerance limits of boar spermatozoa treated with cholesterol-loaded methyl-β-cyclodextrin. Abstract. Theriogenology Vol.70, Issue 8,

Watson, P.F., 1976. Electroejaculation, semen characteristic and semen preservation of the brindled gnu. J Reprod Fertil;47, 123–6.

Watson, P.F., 1976. The protection of ram and bull spermatozoa by the low-density lipoprotein fraction of egg yolk during storage at 5 8C and deep freezing. J Thermal Biol 1976;1:37–141.

Watson, P.F., 1981. The effects of cold shock on sperm cell membranes. In: Watson, P., F., 2000. The causes of reduced fertility with cryopreserved semen. Animal Reproduction Science 60-61, 481-492.

Watson PF, 1990. Artificial insemination and the preservation of semen. In: 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 Dom Anim 40, 191–195.

Watson, P., F., 2000. The causes of reduced fertility with cryopreserved semen. Animal Reproduction Science 60-61, 481-492.

Zahn, F.S., Papa, F.O., Dell'Aqua, J.A., 2002. Cholesterol incorporation on equine sperm membrane: effects on post-thaw sperm parameters and fertility. Theriogenology 58, 273-240. Abstract.

Zeng, W., Terada, T., 2000a. Freezability of boar spermatozoa is improved by exposure to 2-hydroxypropyl-beta-cyclodextrin. Reprod. Fertil. Dev., 12, 223-228.

Zeng, W., Terada, T., 2000b. Protection of boar spermatozoa from cold shock damage by 2-hydroxypropyl-beta-cyclodextrin. Theriogenology 55, 615-627.

Zeng, W., Terada, T., 2001. Effects of Methyl-Beta-Cyclodextrin on Cryosurvival of Boar Spermatozoa. Journal of Andrology, Vol. 22, No. 1.

Zidovetzki, R., Levitan, I., 2007. Use of cyclodextrins to manipulate plasma membrane cholesterol content: Evidence, misconceptions and control strategies (Review). Biochimica et Biophysica Acta 1768, 1311–1324