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EFFECT OF THE PRE-FREEZING TREATMENT OF BOAR SPERM WITH CHOLESTEROL-LOADED CYCLODEXTRINS ON THE FUNCTIONALITY AND THE FERTILIZING ABILITY OF THE SPERM AFTER CRYOPRESERVATION

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Para que pueda surgir lo posible

es preciso intentar una y otra vez lo imposible

Hermann Hesse

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Abbreviation key

ADM	adrenomedullin
AI	artificial insemination
ANOVA	analysis of variance
AO	acridine orange
BP	band pass
BSA	bovine serum albumin
BTS	Beltsville thawing solution
CASA	computer-assisted sperm analysis
CIAR	Centro de Inseminación, Peñarroya de Tastavins, Teruel, Spain
CITA-IVIA	Centro de Tecnología Animal-Instituto Valenciano de Investigaciones Agrarias
CLC	cholesterol-loaded cyclodextrins
COCs	cumulus oocyte complexes
CPAs	cryoprotective agents
DFI	DNA fragmentation index
DMEM/F-12	Dulbecco's modified Eagle's Medium/Ham's F-12 Nutrient Mixture
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DUI	deep intrauterine insemination
eCG	equine chorionic gonadotropin

EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FAO	Food and Agriculture Organization of the United Nations
FBS	foetal bovine serum
Fluo-3-AM	Fluo-3-AcetoMethoxy
FT	frozen-thawed
hCG	human chorionic gonadotropin
HDS	high DNA stainability
hIVP	homologous in vitro penetration assay
HSPA8	Heat-shock 70 k-Da protein 8
IVF-TCM199	in vitro fertilization medium
LEY	lactose-egg yolk
LIA	live sperm with intact acrosomes
LIN	linearity coefficient
LP	long pass
LS	live sperm (sperm with an intact plasma membrane)
LSM	least-square means
M540	merocyanine-540
MAGRAMA	Ministerio de Agricultura, Alimentación y Medio Ambiente
mDPBS	modified Dulbecco's phosphate-buffered saline
mTALP	Tyrode's albumin lactate pyruvate medium
mTBM	modified Tris-buffered medium
mTCM-199	modified M-199
MβCD	methyl-β-cyclodextrin
OEP	Orvus Es Paste

OPEC	oviductal porcine epithelial cell
PBS	phosphate-buffered saline
PGES	prostaglandin E synthase
PI	propidium iodide
PMS	progressively motile sperm
PMT	photomultiplier
PVA	polyvinyl alcohol
PVP	polyvinylpyrrolidone
R-PE-PNA	peanut agglutinin-conjugated phycoerytrin
RPMS	rapid progressively motile sperm
SCSA	sperm chromatin structure assay
SD	standard deviation
SD-DFI	standard deviation of DNA fragmentation index
sDMEM/F-12	Dulbecco's modified Eagle's Medium/Ham's F-12 Nutrient Mixture, supplemented
SEM	standard error of the mean
SR	sperm reservoir
STR	straightness coefficient
TERT-OPEC	telomerase-immortalised oviductal epithelial cell line
TMS	total motile sperm
TNE	Tris-NaCl-EDTA
VAP	average path velocity
WOB	wobble coefficient

Abstract

The treatment of sperm with cholesterol-loaded cyclodextrins (CLC) prior to cryopreservation enhances the percentage of sperm surviving the process in cold-shock sensitive species. It was recently reported that CLC improved also the quality of boar sperm (species highly sensitive to cold and osmotic shocks) when used at a concentration of 1 mg CLC/120 x 10^6 sperm. Nevertheless, it is unknown how this treatment affects the sperm functionality in vitro and the sperm fertilizing ability.

The aim of this Thesis was to determine the effect of the treatment of boar sperm with CLC prior to cryopreservation, on different aspects of the sperm functionality in vitro (osmotic resistance, motility and integrity of the sperm plasma membrane, capacitation status and capacitation dynamics, ability to adhere to oviductal epithelial cells and chromatin integrity dynamics), and on the in vitro (ability to penetrate immature oocytes) and in vivo fertilizing abilities (in weaned sows hormonally treated with eCG-hCG and cervically inseminated 37 or 30 h after hCG injection).

Treating boar sperm with CLC widened ($P < 0.05$) the osmotic resistance limits of the fresh sperm in both hypo- (50, 75 y 150 mOsm/kg) and hyper-osmotic sides (600 y 800 mOsm/kg). At least of 45% of the CLC-treated sperm maintained the sperm plasma membrane integrity (%LS) and the percentages of progressively motile (%PMS) and total motile sperm (%TMS) were maintained between 40% and 50% in these anisosmotic conditions. After freezing-thawing, the CLC-treated sperm exhibited similar quality (percentage of live sperm with intact acrosomes, %LIA; %TMS; %RMS) than control samples, but maintained their quality for longer (%LS, %TMS and %RMS, +1.5 h; $P <$

0.05) than the control sperm. In addition, the quality of the sperm stored at 16 ºC remained in acceptable values for 6 h (43% TMS and 30% PMS) and after 26 h the samples maintain 55% LS and 28% TMS. This aspect could be useful in the handling of frozen-thawed semen in the farms.

The CLC-treated sperm showed a similar capacitation status and dynamics than control sperm after freezing-thawing. Moreover, this treatment did not affect to the chromatin integrity dynamics. However, the CLC-treated sperm showed a higher ability to adhere to oviductal epithelial cells in vitro (+5; $P < 0.0001$) than control sperm.

Respect to the fertility, the CLC-treated sperm penetrated more effectively immature oocytes in vitro (+17%; $P < 0.0001$) and presented a higher number of sperm per oocyte (+2; $P < 0.0001$) than control sperm. Nevertheless, this treatment did not improve the fertilizing ability of the sperm in vivo (neither at 37 nor at 30 h of asynchrony), although when the timing of insemination with respect to the ovulation induction was reduced (from 37 to 30 h), an improvement in the farrowing rate was observed for the CLC-treated samples that matched the percentage obtained for the control samples ($P > 0.05$). Surprisingly, the control sperm fertilized equally well at both asynchronies.

Although the data obtained are not conclusive, it is likely that this treatment modifies the sperm plasma membrane structure and its organization, and as a result its functionality. It is unknown how the membrane is exactly modified, but it seems to have some influence in the stages which take part in the fertilization process, and the CLC-treated sperm need different asynchronies (between ovulation and insemination) to those commonly used for frozen-thawed sperm.

Resumen

El tratamiento de los espermatozoides de especies sensibles al choque térmico con ciclodextrinas saturadas de colesterol (CLC) previamente a la congelación mejora el porcentaje de espermatozoides que sobreviven tras la descongelación. Recientemente se demostró que este tratamiento mejoraba la calidad del semen de verracos (especie muy sensible a los choques térmicos y osmóticos) cuando se utilizaba a una concentración de 1 mg/120 x 10^6 espermatozoides. No obstante, se desconoce cómo afecta este tratamiento a la funcionalidad de los espermatozoides in vitro y a su fertilidad.

El objetivo de esta Tesis ha sido determinar el efecto del tratamiento de los espermatozoides de verracos con CLC previamente a la congelación en distintos aspectos de funcionalidad in vitro (resistencia osmótica, movilidad e integridad de la membrana plasmática, estado y dinámica de capacitación, capacidad de adherencia a células epiteliales del oviducto y dinámica de la integridad de la cromatina) y en la fertilidad de los espermatozoides in vitro (capacidad de penetrar ovocitos inmaduros) e in vivo (en cerdas destetadas y tratadas hormonalmente con eCG-hCG e inseminadas cervicalmente a las 37 ó 30 h tras la inyección de hCG).

El tratamiento de los espermatozoides de porcino con CLC amplió ($P < 0,05$) los límites de resistencia osmótica de los espermatozoides refrigerados tanto en el lado hipo- (50, 75 y 150 mOsm/kg) como hiper-osmolar (600 y 800 mOsm/kg). Así, como mínimo el 45% de los espermatozoides tratados con CLC mantuvieron la integridad de su membrana (%LS) y entre el 40 y el 50% mantuvieron el porcentaje de espermatozoides móviles

progresivos (%PMS) y móviles totales (%TMS) respectivamente. Tras la descongelación, los espermatozoides tratados con CLC mostraron valores similares al control en cuanto a calidad espermática (porcentaje de espermatozoides vivos con acrosoma intacto, %LIA; %TMS; %PMS), pero fueron capaces de mantener su calidad durante más tiempo (%LS, %TMS y %PMS, +1,5 h; $P < 0,05$) que las muestras control. Además, se observó que los espermatozoides conservados a 16 ºC mantienen una calidad aceptable durante al menos 6 h (43% TMS y 30% PMS) y tras 26 h las muestras mantienen 55% de LS y 28% de TMS. Esto podría resultar útil para el manejo de este tipo de semen en las granjas.

Los espermatozoides tratados con CLC presentaron tras la descongelación un estado de capacitación y una dinámica de capacitación similares a las muestras control. Además, este tratamiento no afectó a la dinámica de la integridad de la cromatina. Sin embargo, los espermatozoides tratados con CLC presentaron mayor capacidad de adherencia (+5; $P < 0,0001$) a las células epiteliales del oviducto in vitro que las muestras control.

Con respecto a la capacidad fecundante, los espermatozoides tratados con CLC penetraron más eficazmente los ovocitos inmaduros (+17%; $P < 0,0001$) y se observó mayor número de espermatozoides penetrados por ovocito (+2; $P < 0,0001$) in vitro que en las muestras control. No obstante, este tratamiento no mejoró la capacidad fecundante de los espermatozoides in vivo (ni a 37 ni a 30 h de asincronía), aunque cuando se redujo la asincronía entre la inducción de la ovulación y la inseminación (de 37 a 30 h), se observó una mejora en el porcentaje de fertilidad a parto que se igualó a los valores obtenidos con las muestras control ($P > 0,05$). Por otra parte, las muestras control presentaron una fertilidad similar en ambas asincronías.

Aunque los datos obtenidos no resultan concluyentes, es posible que este tratamiento modifique la estructura y organización de la membrana plasmática y como consecuencia su funcionalidad. Desconocemos en qué consiste exactamente esta modificación, pero parece que influye en los procesos que intervienen en la fecundación, y estos espermatozoides tratados con CLC necesitan asincronías (entre ovulación e inseminación) diferentes a las utilizadas para las dosis de semen congelado-descongelado.

Resum

El tractament dels espermatozoides d'espècies sensibles al xoc tèrmic amb ciclodextrinas saturades de colesterol (CLC) prèviament a la congelació millora el percentatge d'espermatozoides que sobreviuen després de la descongelació. Recentment es va demostrar que este tractament millorava la qualitat del semen de porc (espècie molt sensible als xocs tèrmics i osmòtics) quan s'utilitzava a una concentració d'1 mg/120 x 10^6 espermatozoides. No obstant, es desconeix com afecta este tractament a la funcionalitat dels espermatozoides in vitro i a la seua fertilitat.

L'objectiu d'esta Tesi ha sigut determinar l'efecte del tractament dels espermatozoides de porc amb CLC prèviament a la congelació en distints aspectes de la funcionalitat in vitro (resistència osmòtica, mobilitat i integritat de la membrana plàsmica, estat i dinàmica de capacitació, capacitat d'adherència a cèl·lules epitelials de l'oviducte i dinàmica de la integritat de la cromatina) i en la fertilitat dels espermatozoides in vitro (capacitat de penetrar ovòcits immadurs) i in vivo (en porques deslletades i tractades hormonalment amb eCG-hCG i inseminades cervicalment a les 37 ò 30 h després de la injecció de hCG).

El tractament dels espermatozoides de porcí amb CLC va ampliar ($P < 0,05$) els límits de resistència osmòtica dels espermatozoides refrigerats tant en el costat hipo- (50, 75 i 150 mOsm/kg) com hiper-osmolar (600 i 800 mOsm/kg). Així, com a mínim el 45% dels espermatozoides tractats amb CLC van mantindre la integritat de la seua membrana (%LS) i entre el 40 i el 50% van mantindre el percentatge d'espermatozoides mòbils progressius (%PMS) i mòbils totals (%TMS) respectivament. Després de la descongelació,

els espermatozoides tractats amb CLC van mostrar valors semblants al control quant a qualitat espermàtica (percentatge d'espermatozoides vius amb acrosoma intacte, %LIA; %TMS; %PMS), però van ser capaços de mantindre la seua qualitat durant més temps (%LS, %TMS i %PMS, +1,5 h; $P < 0,05$) que les mostres control. A més, es va observar que els espermatozoides conservats a 16 ºC mantenen una qualitat acceptable durant almenys 6 h (43% TMS i 30% PMS) i després de 26 h les mostres mantenen 55% de LS i 28% de TMS. Açò podria resultar útil per al maneig d'este tipus de semen en les granges.

Els espermatozoides tractats amb CLC després de la descongelació van presentar un estat de capacitació i una dinàmica de capacitació semblants a les mostres control. A més, este tractament no va afectar la dinàmica de la integritat de la cromatina. No obstant, els espermatozoides tractats amb CLC van presentar major capacitat d'adherència (+5; $P < 0,0001$) a les cèl·lules epitelials de l'oviducte in vitro que les mostres control.

Respecte a la capacitat fecundant, els espermatozoides tractats amb CLC van penetrar més eficaçment els ovòcits immadurs (+17%; $P < 0,0001$) i es va observar un major nombre d'espermatozoides penetrats per ovòcit (+2; $P < 0,0001$) in vitro que en les mostres control. No obstant, este tractament no va millorar la capacitat fecundant dels espermatozoides in vivo (ni a 37 ni a 30 h d'asincronia), encara que quan es va reduir la asincronia entre la inducció de l'ovulació i la inseminació (de 37 a 30 h), es va observar una millora en el percentatge de fertilitat i es va igualar als valors obtinguts amb les mostres control ($P > 0,05$). D'altra banda, les mostres control van ser igualment eficaços a les dos asincronies.

Encara que les dades obtinguts no resulten concloents, és possible que este tractament modifique l'estructura i organització de la membrana plàsmica i com a conseqüència la seua funcionalitat. Desconeixem en què consistix exactament esta modificació, però pareix que influïx en els processos que intervenen en la fecundació, i estos espermatozoides tractats amb CLC necessiten asincronies (entre ovulació i inseminació) diferents de les utilitzades per a les dosis de semen congelat-descongelat.

Chapter 1

General Introduction

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1. Importance of pig production

Pork is the most highly consumed meat in the world today and continues to be an important part of the human diet throughout the world. The main production areas for pork are East Asia, North America and Europe. China is the first producer and contributes with nearly 50% of the world pig production, and UE-27 occupies the second position and produces 21% of the total. Of this 21%, Spain produces 15% of the pork meat (3,369 miles of Tn) behind Germany which is the first producer with a 24% of the European pig production (Figure 1; MAGRAMA, Ministerio de Agricultura, Alimentación y Medio Ambiente, 2011; FAO, Food and Agriculture Organization, 2010).

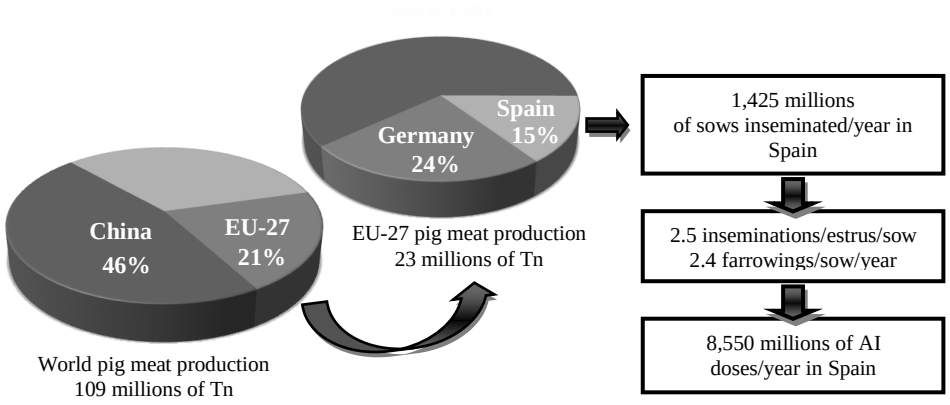

Figure 1. World and EU-27 pig meat production (millions of Tn), and number of sows inseminated and of artificial inseminations (AI) performed per year in Spain.

The improvement in the efficiency of pork production, especially in recent years, is the result of the implementation of several novel biotechnologies and production practices. Major research advances have been made in genetics, nutrition and control of diseases (Gerris et al., 2005). The use of artificial insemination (AI) in the pig has had a major

impact on the improvement of pig breeding, because biotechnological and genomic advances have the potential to significantly improve global swine populations when implemented as part of an AI program.

The AI of sows is widely practiced in countries with intensive pig production. Thus, between 80 and 95% of the sows are artificially inseminated in Europe, and in Spain AI is performed in 95% of the sows (Riesenbeck, 2011). The census of sows in Spain is of 1,500 million (MAGRAMA, 2011) which means that a total of 1,425 millions of sows are inseminated per year and that 8,550 millions of insemination doses are produced per year (Figure 1).

In practice, fresh-refrigerated (15-17 ºC) semen is mostly used in pigs and semen is obtained from boars located in the own farm or from specialized AI centres. Usually, the type of AI in this species is intracervical, AIs are performed with 80 mL AI doses containing 3 billions of sperm and each sow is inseminated an average of 2.5 times per estrus (Rozeboom et al., 2004). The extenders available for boar semen guarantee shelf life of the sperm between three to five days (Riesenbeck, 2011). However, the relatively short life of fresh-refrigerated diluted semen represents a barrier when trading over long distances.

2. Applications and limitations of frozen-thawed sperm doses

Cryopreservation of sperm offers an effective means for long-term storage of important genetic material. This method eliminates the difficulty associated with transporting animals or fresh-semen over long distances or extended periods of time. Moreover the use of frozen-thawed (FT) sperm could reduce the risk of disease associated

with boar and liquid semen entry, this means that the storage of FT sperm would provide additional time for routine and supplementary tests (needed for the diagnosis of diseases), and extended observation of boars before shipment (Knox, 2011).

Since the discovery of the potential of the glycerol as a cryoprotective agent (Polge et al., 1949), sperm cryopreservation protocols have been developed considerably for the majority of the domestic species. The first farrowing coming from FT boar sperm inseminated cervically took place in the 70's (for a review, see Johnson et al., 2000).

From this moment, specific protocols for boar sperm, packaging systems, adequate cryopreservation extenders and optimal strategies for insemination were developed. Despite all the advances made in FT boar sperm research during the past 40 years, at the present time, FT sperm provides an average of 50 to 60% farrowing rate and about 9 to 10 pigs born per litter (Table 1).

When these results are compared with those obtained with fresh-semen (stored at 15 to 16 ºC), farrowing rates are around 25% lower and 2 piglets less are born per litter (Bolarín et al., 2006; Casas et al., 2010; Garcia et al., 2010). However, promising fertility results with cervical artificial insemination and FT sperm have been recently reported obtaining results very similar to those of fresh semen (85.6% farrowing rate and a litter size of 12.6 piglets; Roca et al., 2011). Nevertheless, in order to get these results between 2 and 3 inseminations with 6 x 10^9 FT sperm per insemination were necessary, which means that the cost of the AI with FT sperm is between 1.3 to 2.4 fold higher than with fresh semen.

Table 1. Farrowing rates and litter size obtained with frozen-thawed boar sperm in recent studies

	Weaned sows treated hormonally	Type of insemination	Number of sperm (x 10^6)/doses	Number of inseminations	Farrowing rate (%)	Litter size (total born piglets)
Roca et al., 2003	yes	cervical	6,000	1	75.8	9.6
	yes	intrauterine	1,000	1	77.5	9.3
	no	intrauterine	1,000	2	70	9.3
Bolarín et al., 2006	no	intrauterine	1,000-2,000	2	51.2-70.1	9.2*
Bolarín et al., 2009	no	intrauterine	1,500	2	50-77.6	9-9.9
	yes	intrauterine	1,500	1	63.6	10.3
Yamaguchi et al., 2009	no	intrauterine	2,500	2	28.6-61.9	7.28.2
Casas et al., 2010	yes	post-cervical	7,500	2	26.3-53.9	9.4-10.6
Garcia et al., 2010	yes	cervical	5,000	2	65.4-69.2	9.8-12.5
Roca et al., 2011	-	cervical	6,000	2-3	85.6	12.6
Yamaguchi and Funahashi, 2012	yes	intrauterine	2,500	1	20-21	5.7-10

*Litter size as live born piglets.

These results, added to the cost of production of FT doses, are a handicap for the use of FT sperm commercially. To date the use of FT boar sperm is limited to a few specific situations, associated with the introduction of genetic material of high value in selection farms (Almlid et al., 1996; Tribout et al., 2010), conservation of genetic resources of endangered breeds or of genetically valuable boars (Labroue et al., 2000) or research. However, with an appropriate distribution of boar frozen doses, and producing frozen doses from males with excellent genetic merits and of sufficient sperm quality, the additional cost of this process would be easily offset by productive improvements as a result of the

introduction in the farm of high genetics, and not directly by the results of fertility with the use of FT sperm.

Several fronts have been tackled to try to include most of the factors that may have an impact on the fertilizing ability of FT sperm (male, female and artificial insemination techniques). On the male, the efforts are centralized in the identification of differences between 'good' and 'bad' boar freezers (Watson, 1995; Thurston et al., 2001; Hernández et al., 2006) and in the optimization of the cryopreservation protocols including new packaging systems (Eriksson and Rodriguez-Martinez, 2000), optimization of freezing and thawing velocities (Thurston et al., 2003; Hernández et al., 2007) or modifications of freezing extenders (He et al., 2001, Roca et al., 2003, de Mercado et al., 2009). On the female, the effect of the timing of insemination with respect to the ovulation on fertilization rate has been studied (Bolarin et al., 2006; Larsson, 1976; Waberski et al., 1994). In addition, new strategies of insemination (Deep Uterine Insemination; DUI) have been developed to deposit the FT sperm near the utero-tubal junction (Roca et al., 2002; Bolarin et al., 2006). Nevertheless, despite all the progress made nowadays the use of FT sperm commercially is not a reality in this species.

3. Sperm cryopreservation

Millions of spermatozoa are released into the female genital tract at artificial insemination, but only a small number of them reach the oviductal ampulla to participate in the fertilization process. Fertilizing potential is directly related to the functional capacity of the sperm. The aim of the sperm freezing process is to disrupt the sperm cell metabolism and to keep this cellular state indefinitely, without altering the fertilizing ability of sperm after thawing (Watson, 1995).

Unfortunately, the cryopreservation process causes also different changes in the sperm, as a result of the different stresses (thermal and osmotic) that they are exposed to throughout the process. Induction of premature acrosomal reaction, altered mitochondrial function, reduction of motility and failure of chromatin decondensation, all of which influencing the viability and fertility of the sperm cells have been reported by different authors (for a review, see Watson, 2000). Nevertheless, the damage to the cellular membranes is of most significance because it has a carry-over effect on other cellular structures and functions.

As a result of all these changes about 50% of the population of the sperm does not survive the freezing-thawing process and the survivors remain with sublethal dysfunctions (Watson, 2000) which reduce the longevity of sperm and their fertilizing ability. After cryopreservation a 'premature aging' of the sperm is observed, which is characterized by alterations of the plasma membrane and loss of motility (Watson, 1995; 2000).

3.1. Cold shock

During the freezing process, the sperm are subjected to an initial cooling phase where the temperature drops from physiological values to a temperature slightly above 0 °C. This cooling cause a state of hypothermia in the sperm and it is a step potencially harmful for them. The severity of the damage depends on the speed of cooling, the length of this phase and the final temperature reached (Amann and Pickett, 1987). In this regard, the boar sperm are extremely sensitive to drops below 15 °C (Pursel et al., 1973). One of the most common damages caused by thermal shock is the irreversible loss of motility (Watson, 1995), followed by injury in acrosomal, plasma and mitochondrial membranes. In addition, changes in the plasma membrane have been also described, such as an increase in

its permeability (Ortman and Rodriguez-Martinez, 1994) and a loss of lipids (Plummer and Watson, 1985; White, 1993). Some of these changes are similar to those occurring during the physiological process of capacitation and they have been named as 'capacitation-like changes' (Green and Watson, 2001). Thus, the cooling process causes an increase in the number of capacitated sperm in boars (Green and Watson, 2001; Kaneto et al., 2002; Vadnais et al., 2005).

The susceptibility of the sperm from different species to the damage provoked by the temperature decrease is attributed to the temperature range in which the plasmatic membrane transitions from a liquid-crystalline to a gel phase (Parks and Lynch, 1992; Drobnis et al., 1993). The biological membranes are in a liquid-crystalline phase at physiological temperatures, where the presence of cholesterol significantly increases the orientational order of the phospholipid hydrocarbon chains and decreases the cross-sectional area occupied by the phospholipid molecules, while only moderately restricting the rates of phospholipid lateral diffusion or hydrocarbon chain motion (Amann and Pickett, 1987; Johnson et al., 2000). In addition, the presence of cholesterol increases both the thickness and mechanical strength and decreases the permeability of the phospholipid bilayer in the physiologically relevant liquid crystalline phase. A decrease in temperature is accompanied by an increase in the order in the hydrocarbon chains, resulting in a more rigid membrane structure, to form a gel phase (McMullen et al., 2004). Temperatures to which the transition from the liquid-crystalline to the gel phase takes place in the membrane have been associated with the amount of cholesterol. Thus, as the temperature is lowered, the cholesterol prevents molecular packing required to form a gel phase through its steric interaction with the fatty acyl chains of the phospholipids (Amann and Pickett, 1987; Barenholz, 2002). Therefore, the susceptibility of the plasma membrane to undergo lipid

phase transitions during cooling is inversely related to the amount of cholesterol present (for review, see Bailey et al., 2000). From the domestic species, boar sperm possess membranes with the lowest cholesterol: phospholipid molar ratio (0.26) and this has been correlated with a lower resistance to cold shock (Darin-Bennett and White, 1977; Parks and Lynch, 1992).

Precisely because of the composition of the boar sperm plasma membrane, the boar membrane transitions from the liquid to the gel phase occur at higher temperatures during cooling (Parks and Lynch, 1992; Drobnis et al., 1993; White, 1993; Brouwers et al., 2005), compared to other species. However, the membrane damage provoked by thermal shock during the cooling phase from 16 ºC to 5 ºC can be diminished if the sperm are incubated with seminal plasma prior to cryopreservation (Pursel et al., 1973; Maxwell and Johnson, 1999; Vadnais et al., 2005) and by adding some saccharides (such as the lactose) and lipids (with the inclusion of egg yolk; White, 1993; Buhr et al., 2000) to the freezing extender.

Following the cooling stage to 5 ºC the temperature is lowered further during the freezing phase of the protocol, where the sperm are frozen in liquid nitrogen vapour. This stage is also potentially harmful for the sperm, especially when they traverse the critical temperature range between -15 ºC and -60 ºC (Eriksson et al., 2000). Cryoprotective agents (CPAs) are included in the freezing extenders and help the sperm to survive the freezing-thawing process by modifying the colligative properties of water to lower the freezing point (Hammerstedt et al., 1990).

Albeit part of the damage inflicted to the sperm during the freezing stage was classically attributed to intracellular ice formation (associated to the use of fast freezing rates), a recent study demonstrated that the intracellular ice was not formed after rapid

freezing and the sperm frozen this way were instead damaged during thawing due to the osmotic imbalance they encountered (Morris et al., 2012).

3.2. Osmotic stress

Both the addition and elimination of CPAs as well as the freezing and the thawing of the extracellular water provoke osmotic stress in the sperm (Hammerstedt et al., 1990). Glycerol is the CPA commonly used in boar sperm cryopreservation. Albeit glycerol is essential for successful freezing of semen, it induces osmotic stress (Gao et al., 1993) and it is also toxic to the sperm if used at concentrations higher than 6% (v:v; for a review, see Johnson et al., 2000). The tolerance of the sperm to glycerol is different between species, and freezing extenders used for boar sperm typically contain 3% glycerol (Johnson et al., 2000). During the process, the sperm suffer extensive volume excursions that are harmful for the membranes, since the sperm may swell and shrink above their osmotic tolerance limits, which are species specific (Meyers, 2005). Thus, boar sperm do not tolerate well the volume changes due to their osmotic tolerance limits (both to hypo and hyperosmotic conditions), which are very narrow (Gilmore et al., 1998).

Although the sperm capacity to respond with cell volume adjustments when subjected to anisosmotic conditions is determined by several factors, the cholesterol content in the membrane plays an important role (Hoffmann et al., 2009). When the membrane is subjected to anisosmotic conditions, the cholesterol prevents or minimizes local membrane ruptures that are produced by local fluctuations in its tensile strength (Muldrew and McGann, 1994).

3.3. Membrane status after cryopreservation

The cryopreservation process alters the membrane state, which is functionally similar to that observed in capacitated and/or acrosome reacted sperm (Watson, 1995; Maxwell and Johnson, 1997). Several studies have demonstrated similarities between the changes associated with capacitation and the alterations the sperm present after cryopreservation (Collin et al., 2000; Green and Watson, 2001; Cormier and Bailey, 2003) and for this reason they have been named as 'cryocapacitation' (Bailey et al., 2000). Capacitated spermatozoa do not form a functional sperm reservoir (Fazeli et al., 1999; Tienthai et al., 2004) and similarly, cryopreserved sperm do not form a normal sperm reservoir (Abad et al., 2007). Albeit the molecular mechanisms of capacitation are not completely elucidated, various reports have described a loss of cholesterol in the process of capacitation (Cross, 1998; Visconti et al., 1999), phenomenon also observed after cryopreservation (Cerolini et al., 2001; Kadirvel et al., 2009).

4. Recent advances in boar sperm cryopreservation

During the last decade many studies have been conducted with the aim of optimizing the number of sperm surviving the cryopreservation process and the quality of the survivors (for a review, see Medeiros et al., 2002; Holt et al., 2005; Bailey et al., 2008; Großfeld et al., 2008; Mocé et al., 2010).

In the last two years the efforts have been focused on four fundamental aspects: the improvement of the freezing extenders, the optimization of the cryopreservation process, the determination of the physical and biochemical characteristics affected during the process and finally the modification of the sperm.

The studies conducted to improve the freezing extenders have been directed towards the discovery of new cryoprotective substances (Kaeoket et al., 2012; Chanapiwat et al., 2012; Gómez-Fernández et al., 2012b), the optimization of the use of glycerol as CPA (Parrilla et al., 2012; Malo et al., 2012a), the use of other CPAs alternative to the glycerol (Buranaamnuay et al., 2011; Kim et al., 2011) or the addition of substances to the freezing extenders, such as seminal plasma (Gómez-Fernández et al., 2012a) or antioxidants (Malo et al., 2011; Malo et al., 2012b), in an attempt to protect the sperm through the cryopreservation process.

The studies dealing with the optimization of the cryopreservation process channeled their efforts in reducing the length of the cooling phase at 5 ºC. Thus, it has been recently demonstrated that boar sperm can tolerate rapid cooling rates prior to freezing and the cooling phase at 5 ºC could be completed in just 8 min, instead of 90 or 120 min (Juarez et al., 2011). A large number of works have focused on understanding how the cryopreservation process affects the physical and biochemical sperm characteristics. Recently, it has been observed that the cryopreservation cause alterations in the histones (they are the responsible for the DNA condensation; Flores et al., 2011) or in the protein tyrosine phosphorilation (Kumaresan et al., 2011; 2012)

Finally, some of the strategies tried to act on the sperm themselves and not only on the environment surrounding them with the aim of increasing their resistance to the cryopreservation process. Thus, it has been demonstrated that the treatment with hydrostatic pressure prior to cryopreservation increases the cryotolerance of the sperm (for a review, see Pribenszky et al., 2011). In addition, the pre-freezing treatment of boar sperm with cholesterol-loaded cyclodextrins enhances the quality of FT sperm (Blanch et al., 2012).

5. Cholesterol addition to boar sperm plasma membrane as a strategy against the damage caused by cryopreservation

According to the data exposed previously, increasing the cholesterol content in the plasma membrane would render sperm with a higher resistance to cold shock and to osmotic stress. Additionally, a higher concentration of cholesterol would decrease the fluidity of the acrosomal region which would slow down the membrane fusion during the capacitation and the acrosome reaction (for a review, see Ladha, 1998), and therefore would delay the premature capacitation of FT sperm.

The cholesterol content in the plasma membranes can be reversibly modified by the use of cyclodextrins or cyclodextrins pre-loaded with cholesterol. Cyclodextrins are cyclic oligosaccharides that can encapsulate hydrophobic compounds, such as cholesterol. These molecules have a high affinity for sterols in vitro, and are very efficient in stimulating the removal of cholesterol from the membranes of many types of cells (Christian et al., 1997), including sperm (Visconti et al., 1999; among others). In addition, if they are pre-loaded with cholesterol they can insert cholesterol into cell membranes (Navratil et al., 2003). Several studies have showed that incubation of sperm with cholesterol-loaded cyclodextrins (CLC), increases the sperm cholesterol content 2 to 3 fold in bull, trout, ram and stallion (for a review, see Mocé et al., 2010), and this additional cholesterol would raise the cholesterol: phospholipid ratios of these sperm to cholesterol: phospholipid ratios (> 0.8) that are similar to sperm that are not sensitive to cold-shock. In addition to the increase in the cholesterol content in sperm immediately after treatment, sperm treated with CLC prior to freezing maintained higher cholesterol levels after cryopreservation as well, for stallion, boar and ram sperm (for a review, see Mocé et al., 2010; Tomás et al., 2012).

Apart from an increase in the amount of cholesterol after cryopreservation, treating sperm with CLC prior to cryopreservation, benefit sperm from several species, whose sperm are sensitive to cold-shock. This treatment increased the sperm quality and the sperm fertilizing ability in vitro in several cold-shock sensitive species (for a review, see Mocé et al., 2010).

This treatment has also been used in boar sperm, with non-conclusive results. Some authors observed that it was the pre-treatment with cyclodextrin (non-loaded with cholesterol) rather than the pre-treatment with CLC responsible of the improvement in the acrosome integrity and motility, after inducing a cold-shock (Zeng and Terada, 2001a) and post-thawing (Zeng y Terada, 2000; 2001b). Nevertheless, these results were not corroborated in subsequent studies. Thus, other authors observed that treating boar sperm with CLC prior to cold shock or freezing improved the percentage the sperm surviving these processes (Galantino-Homer et al., 2005; Blanch et al., 2012). Therefore, these studies show that the pre-freezing treatment of boar sperm with CLC could be useful in this species, although it is still to be determined how this treatment affects the sperm functionality and the sperm fertilizing ability.

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Chapter 2

Objectives

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General aim and specific objectives

The general aim of this Thesis was to determine if the treatment of boar sperm with cholesterol-loaded cyclodextrins (CLC) prior to cryopreservation affects the structure, and therefore the functionality and the fertilizing ability of frozen-thawed sperm. The aim was itemized into the following specific objectives:

1. To determine the effect of pre-treatment with CLC and cyclodextrin on the osmotic resistance of fresh boar sperm by means of incubation in diluents of different osmolality.

2. To determine the effect of pre-freezing treatment with CLC and cyclodextrin on the boar sperm motility and on the membrane integrity after cryopreservation and in long term incubation.

3. To evaluate the effect of pre-freezing treatment of boar sperm with CLC on the functionality of frozen-thawed sperm in vitro. For this, the capacitation status and capacitation dynamics, the ability to adhere to oviductal epithelial cells and the chromatin integrity dynamics were studied.

4. To study the effect of pre-freezing treatment with CLC on the fertilizing ability of frozen-thawed boar sperm in vitro

5. To study the effect of pre-freezing treatment with CLC on the fertilizing ability of frozen-thawed boar sperm in vivo inseminated at two different timings with respect to the ovulation induction.

Organization of the Thesis

The scientific work carried out resulted in three studies, whose contents are presented in the different chapters of this dissertation. The references for these studies and the chapters where they have been included in the dissertation are:

Chapter 3, Study 1: Tomás, C., Hernández, M., Gil, M. A., Roca, J., Vázquez, J. M., Martínez, E. A., Mocé, E. 2011. Treating boar sperm with cholesterol-loaded cyclodextrins widens the sperm osmotic tolerance limits and enhances the in vitro sperm fertilizing ability. Anim. Reprod. Sci. 129: 209-220.

Chapter 4, Study 2: Tomás, C., Blanch, E., Fazeli, A., Mocé, E. 2012. Effect of a pre-freezing treatment with cholesterol-loaded cyclodextrins on boar sperm longevity, capacitation dynamics, ability to adhere to porcine oviductal epithelial cells in vitro and DNA fragmentation dynamics. Reprod. Fertil. Devel. Doi: 10.1071/RD12079.

Chapter 5, Study 3: Tomás, C., Blanch, E., Cebrián, B., Mocé, E. 2012. In vivo fertilizing ability of frozen-thawed boar sperm treated with cholesterol-loaded cyclodextrins prior to cryopreservation. Submitted to Anim. Reprod. Sci.

In Chapter 3 (Study1) we determine the effect of CLC and cyclodextrin on the fresh boar sperm osmotic resistance and on the structure and functionality and the in vitro fertilizing ability of boar sperm after thawing. In this study, the sperm motility, acrosome integrity and capacitation status are evaluated. In addition, a homologous in vitro penetration assay is performed to study the fertilizing ability of frozen-thawed boar sperm in vitro.

In Chapter 4 (Study 2) we study further the effect of the pre-freezing treatment of boar sperm with CLC on the dynamics of different parameters (motility, membrane integrity, capacitation and chromatin integrity) after long term incubation (24 to 26 h). In addition, the ability of the sperm to adhere to oviductal epithelial cells in vitro is also evaluated.

In Chapter 5 (Study 3) we study how the CLC treatment affects the fertilizing ability of frozen-thawed boar sperm in vivo when they are inseminated at two different timings with respect to the ovulation induction with hCG. In the first experiment the inseminations are performed 37 h after hCG injection, and in the second experiment the timing of insemination is reduced to 30 h after hCG injection.

Chapter 3: Study 1

Treating boar sperm with cholesterol-loaded cyclodextrins widens the sperm osmotic tolerance limits and enhances the in vitro sperm fertilizing ability

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Abstract

Treating sperm with cholesterol-loaded cyclodextrins (CLC) improves the cryosurvival of the sperm of different cold-shock sensitive species. However, the response of boar sperm to this treatment is not fully understood. The aim of this study was to determine how CLC and methyl-β-cyclodextrin (MβCD, not loaded with cholesterol) affect the parameters for boar sperm functionality, including sperm osmotic resistance, and the ability of the sperm to capacitate and to penetrate sow's immature oocytes in vitro. Samples treated with CLC or MβCD prior to freezing exhibited similar percentages of motile sperm, live sperm and sperm with intact acrosomes as the control samples ($P > 0.05$). In addition, these treatments did not alter the response of the boar sperm to capacitating conditions. However, when compared to the controls and the MβCD-treated samples, the CLC-treated sperm maintained greater percentages of motile sperm and live sperm in a wide range of osmotic solutions including hypo- (50, 75 and 150 mOsm/kg) and hyper-osmotic (600, 800 mOsm/kg) conditions ($P < 0.05$). In addition, the CLC-treated sperm exhibited greater oocyte penetration ability than the control and the MβCD-treated sperm ($P < 0.0001$). In conclusion, the pre-freezing treatment of boar sperm with CLC does not alter the ability of the sperm to respond to capacitating conditions. Despite not increasing the cryosurvival of the sperm, this treatment widens the sperm osmotic tolerance limits and enhances the in vitro sperm fertilizing ability.

1. Introduction

During the cryopreservation process, sperm membranes must withstand a variety of stresses including thermal, mechanical, chemical and osmotic stresses (Parks and Graham, 1992). As result of these stresses, approximately half of the sperm population does not survive the cryopreservation process, and the membranes of the surviving sperm are more labile than the membranes of fresh sperm (for a review, see Peña, 2007). Consequently, research conducted to optimize the sperm freezing-thawing process should be directed towards enhancing the sperm cryosurvival rates and guaranteeing the membrane functionality in the sperm population that survives the cryopreservation process.

The sensitivity of sperm membranes to cryopreservation stresses is determined in part by the membrane composition (for a review, see Meyers, 2005). Boar sperm are extremely sensitive to cryopreservation stresses, and this sensitivity is related to the particular lipid composition of their membranes, which is characterised by a comparatively higher ratio of unsaturated/saturated fatty acids and a lower cholesterol content than other species (Parks and Lynch, 1992). Therefore, the membrane phase transition of sperm occurs at a relatively high temperature during cooling in this species, resulting in the boar sperm suffering the harmful effects of cryopreservation more strongly than other species (Drobnis et al., 1993).

The role of cholesterol in modulating sperm membrane fluidity and stability is well established (Travis and Kopf, 2002). The relatively high cholesterol content in the sperm membranes reduces the temperature at which the phase transition occurs, maintaining the fluidity of the sperm membranes at low temperatures (Ohvo-Rekilä et al., 2002). A loss of cholesterol leads to membrane destabilisation; therefore, a reduction in the membrane

cholesterol content represents one of the first steps in the capacitation process (Witte and Schäfer-Somi, 2007). Boar sperm membranes not only exhibit a low cholesterol content, but they also lose some of their cholesterol throughout the cryopreservation process (Cerolini et al., 2001). Therefore, some studies suggest that cryopreservation induces capacitation-like changes in the boar sperm population that survives the freezing-thawing process (Watson, 1995; Maxwell and Johnson, 1997), leading to the impaired fertilizing ability of thawed sperm. Because of the importance of cholesterol in sperm cryoresistance and functionality, increasing the cholesterol content of sperm membranes could be a strategy to improve the resistance of sperm to the cryopreservation process, which will increase the number of sperm surviving the process and the membrane stability of the survivors.

Cholesterol is incorporated into the sperm membranes using cyclodextrins (Zidovetzki and Levitan, 2007). Thus, when sperm from several cold-shock-sensitive species were incubated with cyclodextrins loaded with cholesterol (CLC) prior to freezing, the percentages of motile and viable sperm that remained after thawing increased (for a review, see Mocé et al., 2010b). No conclusive studies have been published concerning the effectiveness of CLC-treatment for boar sperm cryopreservation (Zeng and Terada, 2000; 2001). However, promising results have been published showing that these compounds minimise cold shock and freezing damage (Galantino-Hommer et al., 2006; Torres et al., 2009).

The objective of this study was to determine the effect of CLC and cyclodextrin on the functionality and the in vitro fertilizing ability of boar sperm.

2. Materials and methods

2.1. Reagents and media

All the chemicals were of analytical grade. Unless otherwise stated, all the media components were purchased from Sigma-Aldrich Quimica, S.A. (Madrid, Spain), and the media were prepared under sterile conditions (HH48, Holten LaminAir, Allerod, Denmark) using purified water (18.2 megohm-cm, Milli-Q water purification system, Millipore Co, Billerica, MA). The fluorochromes were purchased from Invitrogen S.A. (Barcelona, Spain), with the exception of peanut agglutinin-conjugated phycoerythrin (Phycoprobe R-PE-PNA, P44), which was purchased from Biomeda Corp. (Foster City, CA, USA).

The basic medium used for the sperm extension was Beltsville thawing solution (BTS), which was composed of 205 mM glucose, 20.39 mM NaCl, 5.4 mM KCl, 15.01 mM $NaHCO_3$, and 3.35 mM EDTA (pH = 7.2 and 290 ± 5 mOsm/kg, Johnson et al., 1988) and contained kanamycin sulphate (50 µg/mL). The basic medium used for the sperm freezing was lactose-egg yolk extender (LEY), which was composed of 80% (v:v) β-lactose solution (310 mM in water) and 20% (v:v) egg yolk (pH = 6.2 and 330 ± 5 mOsm/kg; Westendorf et al., 1975).

To evaluate the post-thaw sperm membrane functionality and the intracellular calcium levels, the sperm samples were incubated in non-capacitating [phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 0.86 mM Na_2HPO_4 and 6.4 mM $Na_2HPO_4 \cdot 7H_20$; pH = 6.8 and 292 ± 2.2 mOsm/kg] or in capacitating medium [modified

Tris-buffered medium (mTBM) composed of 131.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 1 mM caffeine and 0.2% BSA (fraction V, Cat nr A 7888, initial fractionation performed using cold alcohol precipitation); pH = 7.4 and 299 ± 1.6 mOsm/kg; Caballero et al., 2009]. The capacitating medium was equilibrated in the dark at 39 ºC under a modified atmosphere containing 5% CO_2 and 100% humidity for 15 min before use.

To wash the sperm, a saline medium and a sucrose medium were used. The saline medium was composed of 137 mM NaCl, 10 mM glucose, 2.5 mM KOH and 20 mM Hepes buffered to pH 7.5 using NaOH, to which 1 mg/mL polyvinyl alcohol (PVA; average M_r 30,000-70,000) and 1 mg/mL polyvinylpyrrolidone (PVP; average M_r 40,000) were added. The sucrose medium was composed of 220 mM sucrose, 10 mM NaCl, 10 mM glucose, 2.5 mM KOH and 20 mM Hepes, buffered to pH 7.5 using NaOH, to which 0.5 mg/mL PVA and 0.5 mg/mL PVP were added.

To evaluate the in vitro fertilizing ability of the sperm, a homologous in vitro penetration assay (hIVP) was performed according to the method described by Martínez et al. (1993), which requires that the ovaries are stored and washed with an NaCl solution [0.9% NaCl (w:v) supplemented with 70 µg/mL kanamycin sulphate (pH = 7.2-7.4 and 280-300 mOsm/kg)]. The cumulus oocyte complexes were recovered, selected and washed with modified Dulbecco's phosphate-buffered saline [mDPBS, DPBS supplemented with 4 mg/mL BSA (fraction V, Cat nr A 7888, initial fractionation by cold alcohol precipitation), 0.34 mM sodium pyruvate, 5.5 mM D-glucose and 70 µg/mL kanamycin sulphate (pH = 7.2-7.4 and 280-300 mOsm/kg)]. The sperm were diluted using modified M-199 with Earle`s salts and 26.19 mM sodium hydrogen carbonate [mTCM-199, M199 supplemented with 12% heated foetal calf serum (inactivated at 56 ºC for 30 min), 0.91 mM sodium

pyruvate, 3.05 mM D-glucose, 2.92 mM calcium lactate, 50 UI/mL penicillin G and 30 µg/mL streptomycin sulphate; pH = 7.8 and 280-300 mOsm/kg]. The sperm-oocyte co-incubation was carried out in IVF-TCM199 medium (mTCM-199 supplemented with 2 mM caffeine and 5.84 mM calcium lactate; pH = 7.4 and 280-300 mOsm/kg).

2.2. Preparation of cholesterol-loaded cyclodextrins

Cholesterol-loaded cyclodextrins (CLC) were prepared as described by Purdy and Graham (2004). Briefly, 1 g methyl-β-cyclodextrin (MβCD) was dissolved into 2 mL methanol (solution A), and 200 mg cholesterol was dissolved in 1 mL of chloroform (solution B). Next, 0.45 mL solution B was added to solution A, and the combined solutions were vigorously mixed until the solution was clear. The solution was poured into a glass Petri dish and was dried at 39 ºC. The resulting crystals were recovered and stored in a glass vial at room temperature.

The working solutions of the CLC crystals or the MβCD (50 mg/mL BTS) were freshly prepared on the day of the experiment.

2.3. Sourcing, collecting and handling of the ejaculates

Eight healthy mature (2–4 years of age) crossbred boars of proven fertility housed at a commercial insemination station (Hypor España, La Coruña, Spain) and four fertile Pietrain boars housed at the experimental farm at the "Centro de Tecnología Animal-Instituto Valenciano de Investigaciones Agrarias" (CITA-IVIA, Segorbe, Spain) were used as ejaculate donors. The boars were housed in climate-controlled individual pens (15 to 25 °C), were fed a commercial diet in accordance with the guidelines for the nutritional requirements for adult boars and received water *ad libitum*. The protocols that were used

fulfilled the European regulations for the care and use of animals for scientific purposes (EC Directive 2010/63/EU).

The sperm-rich ejaculate fractions were collected using the gloved-hand method, and the fractions were extended (1:1, v:v) in BTS and evaluated for conventional semen characteristics.

The extracted semen from Hypor España was transferred to 50 mL plastic tubes, cooled to 17 °C, packaged in insulated containers and sent by courier service to the sperm cryopreservation laboratory at the Faculty of Veterinary Medicine, University of Murcia. The extended semen arrived at the laboratory between 14 and 15 h after the ejaculate collection. When the ejaculates came from the CITA-IVIA, the extended semen was transported to the laboratory at 22 ºC in less than 30 min after collection, and it was maintained at 16 ºC for a minimum of 2 h before cryopreservation.

2.4. Sperm cryopreservation

Immediately after reception, the concentration and motility of the samples were evaluated. Only ejaculates containing greater than 200 x 10^6 sperm/mL, greater than 75-80% motile sperm and sperm with an intact plasma membrane were selected for cryopreservation. The sperm samples were cryopreserved according to the protocol described by Westendorf et al. (1975). Briefly, the extended sperm-rich fractions were centrifuged at 800 x g for 10 min at 16 °C, and the supernatant, containing mostly seminal plasma, was removed by aspiration. The sperm pellet was suspended in the LEY extender to yield a concentration of 225 x 10^6 sperm/mL. After cooling to 5 °C within 120 min, the extended sperm were diluted (2:1; v:v) in LEY-Glycerol-Orvus Es Paste extender [89.5% LEY, 9% glycerol (v:v) and 1.5% OEP (Equex STM; Nova Chemical Sales Inc., Scituate,

MA, USA)] to a final concentration of 150 x 10^6 sperm/mL, 3% glycerol and 0.5% OEP. The cooled sperm was equilibrated using the glycerated extender for 15 min and packaged into 0.5 mL straws. The straws were frozen in static nitrogen vapour (4 cm above the liquid nitrogen for 20 min) and were plunged into liquid nitrogen for storage. The straws remained in the liquid nitrogen tank for a minimum of two weeks before thawing. The straws were thawed in a water bath at 37 ºC for 30 sec.

2.5. Sperm assessments

The sperm concentration in each ejaculate was evaluated using an improved Neubauer chamber (Marienfield, Germany) in aliquots that were fixed with a solution of 0.25% glutaraldehyde in Dulbecco's phosphate-buffered saline (Pursel and Johnson, 1974).

The thawed sperm were diluted in BTS-BSA (6 mg/mL) to a final concentration of 30 x 10^6 sperm/mL. Before the motility analysis, the samples were incubated in a water bath at 37 ºC for 10 min. The sperm motility was evaluated objectively using a computer-assisted sperm analysis system (ISAS®, version 1.0.17, Proiser R+D, Paterna, Spain) following the procedure described by Cremades et al. (2005). For each evaluation, a 5 µL aliquot of the sperm sample was placed in a pre-warmed (39 °C) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and 3-5 fields were analysed at 39 °C; a minimum of 200 sperm/sample was assessed. The percentages of total motile sperm (VAP > 10 µm/sec) and the rapid progressively motile sperm (VAP > 50 µm/sec and STR > 75%) were recorded.

The flow cytometry analyses were performed at room temperature under a dimmed light in an EPICS XL flow cytometer (Coulter Corporation Inc., Miami, FL, USA) equipped with standard optics and an argon ion laser (Cyonics, Coherent, Santa Clara, CA,

USA) with 15-mW laser power at 488 nm and EXPO 2000 software (Coulter Corporation). Forward and side scatter were recorded in a linear mode for a total of 10,000–25,000 events per sample, and only sperm-specific events, which appeared in a typically L-shape scatter profile, were positively gated for the analysis.

2.6. Experimental design

Experiment 1. The effect of CLC and MβCD treatments on the osmotic tolerance limits of fresh boar sperm

For this experiment, fresh ejaculates (n = 10) from the boars from the CITA-IVIA were used. The ejaculates (at 22 ºC) were centrifuged at 800 x g for 10 min at 22 ºC, and the pellets were resuspended to a final concentration of 850 x 10^6 sperm/mL (the seminal plasma was previously removed). The samples were divided into three aliquots; one served as a control (non-treated), the second aliquot was treated with 1 mg MβCD/120 x 10^6 sperm (negative control), and the third aliquot was treated with 1 mg CLC/120 x 10^6 sperm. The aliquots were incubated with MβCD or CLC for 15 min at 22 ºC.

The osmotic tolerance assay was performed in accordance with a previously described protocol (Mocé et al., 2010a). Briefly, 10 µL aliquots from each sample were added to 150 µL anisosmotic BTS solutions (supplemented with 6 mg of BSA/mL) with the following osmolalities: 0, 50, 75, 150, 225, 270, 300, 350, 370, 425, 600, 800, 1200 and 2400 mOsm/kg. The sperm were incubated in each of the anisosmotic solutions for 5 min at 22 ºC. The sperm were returned to near isosmolality (290-331 mOsm/kg) by transferring a 100 µL aliquot from each anisosmotic sample into BTS-BSA solutions (Table 1) to restore the sperm to an isosmotic environment (the final sperm concentration was approximately 25 x 10^6 sperm/mL). The sperm motility was evaluated as described previously.

Table 1. Protocol for returning the sperm to near isosmotic, after incubating boar sperm with methyl-β-cyclodextrin (MβCD-1 mg, 1 mg/120 x 10^6 sperm/mL) or cholesterol-loaded cyclodextrins (CLC-1 mg, 1 mg/120 x 10^6 sperm/mL) in anisosmotic conditions

Anisosmotic solutions (mOsm/kg)	BTS solution to return to isosmolality (μL)			Ultrapure water (μL)	Semen[a] (μL)	Final osmolality (mOsm/kg)
	370 mOsm/kg	350 mOsm/kg	300 mOsm/kg			
0, 50, 75, 150	300	---	---	---	100	290-315
225	---	300	---	---	100	319
270	---	300	---	40	100	330
300, 350, 370, 425	---	---	300	---	100	300-331
600	---	---	225	75	100	300
800	---	---	133	167	100	300
1200	---	---	---	300	100	300
2400	---	---	---	600	100	300

[a] From samples incubated in their corresponding anisosmotic solution during 5 min at room temperature.

After restoring the sperm to isosmotic conditions, the integrity of the sperm plasma membrane was evaluated using flow cytometry in accordance with a protocol previously described (Purdy and Graham, 2004). For this procedure, 100 µL of each sample was transferred into a tube containing 450 µL BTS, 2.5 µL propidium iodide (PI, a 1 mg/mL stock solution of Component B from the LIVE/DEAD Sperm Viability Kit, L-7011 was prepared in ultra-pure water) and 2.5 µL SYBR-14 [a 10 µM stock solution of Component A from the LIVE/DEAD Sperm Viability Kit was prepared in dimethyl sulfoxide (DMSO)]; the samples were incubated at 37 ºC for 10 min and were filtered through a 40 µm nylon mesh before analysis. The SYBR-14 fluorescence (particles containing DNA, i.e., the live cells) was detected using a 550 long pass (LP) filter combined with a 525 nm band pass (BP) filter (FL1) and the PI fluorescence (non viable cells) was detected using a 645 LP filter combined with a 620 nm BP filter (FL3). The debris was gated out based on the scatter properties and was double gated out based on the SYBR-14 or PI fluorescence. Only the percentage of live sperm was considered in the results (SYBR-14 positive and PI negative).

Experiment 2. The effect of CLC and MβCD treatment before cryopreservation on sperm motility, acrosome integrity and the response to capacitating conditions after freezing-thawing

The single diluted ejaculates from 13 boars (Hypor España) were used for this experiment. The extended (1:1, v:v in BTS) sperm-rich fractions were divided into three aliquots. An aliquot served as a control sample (non-treated), and the other two aliquots were incubated with 1 mg of CLC or MβCD/120 x 10^6 sperm for 15 min at 16 °C, according to the protocol described by Blanch et al. (2009). The samples were centrifuged

and cryopreserved as described previously. After thawing, the samples were incubated in a water bath at 37 °C until the analyses were performed.

Sperm motility was evaluated as described previously. Sperm viability and acrosome integrity were evaluated simultaneously by flow cytometry using a triple fluorescent procedure that was described by Nagy et al. (2003). Briefly, 100 µL of the diluted samples (30×10^6 sperm/mL in PBS) was transferred into culture tubes, and the dyes were added at a final concentration of 100 nM SYBR-14 (100 µM stock solution in DMSO), 1 µg/mL peanut agglutinin-conjugated phycoerythrin solution (1 mg/mL stock solution Phycoprobe R-PE-PNA, P44, Biomeda Co., Foster City, CA) and 12 µM PI (1.5 mM stock solution in PBS). The samples were mixed and incubated at 37 °C in the dark for 10 min. Immediately before analysis, 400 µL PBS was added to each sample, and the samples were remixed before they were run through a flow cytometer. The fluorescence of the SYBR-14, PI and R-PE-PNA were detected using 525, 620 and 575 nm band pass filters, respectively. The analysed sperm were allocated into the following four categories: live sperm with an intact acrosome (SYBR-14+/PI−/R-PE-PNA−), live sperm with a damaged acrosome (SYBR-14+/PI−/R-PE-PNA+), dead sperm with an intact acrosome (SYBR-14−/PI+/R-PE-PNA−) and dead sperm with a damaged acrosome (SYBR-14−/PI+/R-PE-PNA+). Only the viable sperm with an intact acrosome were considered for the results.

The ability of the FT sperm to capacitate in vitro was evaluated in sperm samples that had been incubated for 30 min under non-capacitating (diluted in PBS and incubated in covered tubes) or capacitating (diluted in mTBM and incubated in uncovered tubes) conditions. The samples were incubated in the dark at 39 ºC in a modified atmosphere containing 5% CO_2 and 100% humidity. The pH of the capacitating and non-capacitating

media remained constant at 7.4 for the duration of the incubation period. Sperm capacitation was evaluated using the changes in the plasma membrane fluidity and the intracellular calcium levels.

Plasma membrane fluidity was assessed after staining the sperm with Merocyanine 540 (M540; M24571) and Yo-Pro-1 (Y3603) (Harrison et al., 1996). Each sub-sample was diluted to 3 x 10^6 sperm/mL in PBS or was pre-equilibrated mTBM, stained with 1 µL/mL Yo-Pro-1 (25 nM stock solution in DMSO), and incubated for 30 min in the corresponding medium under the above mentioned conditions. Immediately before analysis, 2.6 µL M540 (1 mM stock solution in DMSO) was added to each sample, and the samples were incubated for 2 min and remixed before flow cytometry analysis. The fluorescence of the M540 and the Yo-Pro-1 was detected using 575- and 525 nm band pass filters, respectively. The analysed sperm were allocated into the following three categories: viable sperm with a stable plasma membrane (Yo-Pro-1−/M540−), viable sperm with an unstable plasma membrane (Yo-Pro-1−/M540+), and dead cells (Yo-Pro-1+). Only the viable sperm (with a stable or an unstable plasma membrane) were considered in the results.

The intracellular calcium levels were evaluated using the fluorescent probes Fluo-3-AcetoMethoxy (Fluo-3-AM; F3021) and PI following the protocol described by Caballero et al. (2009). Briefly, 300 µL thawed-sperm (45 x 10^6 sperm) were incubated with 5 µL/mL Fluo-3-AM (1 mM stock solution in DMSO) at room temperature for 10 min in the dark. Saline medium (2 mL) was added to dilute the suspensions, and the incubation was continued for an additional 20 minutes in the dark at room temperature. This suspension was layered over 3 mL sucrose medium and was centrifuged for 10 min at 300 x g followed by 10 min at 750 x g. The supernatant was aspirated to leave 0.5 mL medium in which the sperm pellet was gently resuspended. The sperm suspension was diluted in PBS or in pre-

equilibrated mTBM to a final concentration of 2 x 10^6 sperm⁄mL, and 5 µL⁄mL PI (1.5 mM stock solution in PBS) was added to stain the dead sperm. Finally, the sperm samples were incubated for 15 min under the above-mentioned conditions before the flow cytometry analysis. In addition, two control sperm samples (positive and negative) were prepared (Harrison et al., 1993). A sample stained with Fluo-3-AM and PI was treated after diluting to 2 x 10^6 sperm/mL using 10 µL/mL calcium ionophore (A23187, 20 µM stock solution in DMSO) to induce an increase in the intracellular calcium, and this sample was used as a positive control (high intracellular calcium levels). The other sample was treated with 10 µL/mL EGTA (200 mM in ultrapure water) to avoid calcium intake and was used as a negative control (low intracellular calcium level). The control settings were adjusted each day during the analyses.

The fluorescence of the Fluo-3-AM and PI was detected using a 525 nm and a 620 nm band pass filter, respectively. The sperm were allocated into four categories, viable sperm with low intracellular calcium (a low Fluo-3 fluorescence signal/PI-), viable sperm with high intracellular calcium (high Fluo-3 fluorescence signal/PI-), dead sperm with low intracellular calcium (low Fluo-3 fluorescence signal/PI+), and dead sperm with high intracellular calcium (high Fluo-3 fluorescence signal/PI+). The results were expressed as the percentage of viable sperm and the percentage of viable sperm with high intracellular calcium.

Experiment 3. The effect of CLC and MβCD treatments on the in vitro fertilizing ability of frozen-thawed boar spermatozoa

The in vitro fertilizing ability of FT boar sperm was evaluated in immature pig oocytes using a homologous in vitro penetration test conducted as described by Martínez et al., (1993), which includes the following steps.

Sperm preparation

The sperm-rich fractions (n = 10) from the CITA-IVIA male boars were treated with CLC, MβCD, or were left untreated (control), and they were cryopreserved and thawed following the protocol described previously.

After thawing, 250 µL of the thawed sperm (37.5 x 10^6 sperm) was washed in 10 mL of mDPBS and centrifuged at 1200 x g for 3 min at 30 ºC. The supernatant was removed, and the sperm pellet was resuspended in mTCM-199.

Oocyte preparation and oocyte sperm co-incubation

The ovaries were obtained at a local slaughterhouse (El Pozo S.A., Alhama de Murcia, Murcia, Spain) from prepuberal gilts weighing approximately 95 kg. The ovaries were transported to the laboratory at 30 ºC in a NaCl solution. In the laboratory, the ovaries were washed three times in a NaCl solution, and the cumulus oocyte complexes (COCs) were collected by slicing the ovaries on a Petri dish containing mDPBS at 37 ºC. The resulting solution containing the COCs was transferred into 15 mL tubes and allowed to settle for 15 min. The supernatant was removed, and the pellet containing the COCs and the follicular cells was resuspended in mDPBS at 37 ºC before transferring to a Petri dish (90 x 14 mm) to identify and select the COCs. The oocytes surrounded by a compact cumulus mass and containing a uniform ooplasm were selected and transferred in groups of 20 into

Petri dishes (35 x 10 mm) containing 2 mL IVF-TCM-199 (equilibrated in an atmosphere of 5% CO_2 in air for at least 2 h prior to use). The sperm (4 x 10^6 sperm at a final concentration of 2 x 10^6 sperm/mL) were added to each of two Petri dishes containing 20 oocytes, and the gametes were co-incubated for 16-18 h at 39 ºC in an atmosphere of 5% CO_2 and 100% humidity. The sperm from a male with a known penetration ability was used as a control on each day of the assay.

At the end of the co-incubation period, the oocytes were transferred to a Petri dish containing mDPBS, and the excess sperm and cumulus cells were removed mechanically by pipetting. The denuded oocytes were placed onto glass slides and were covered with a cover glass that was supported by paraffin wax. The oocytes were fixed in a solution of acetic acid: ethanol (1:3, v:v) for 48-72 h at room temperature.

The fixed oocytes were stained with a solution of 1% (w:v) lacmoid [diluted with 45% (v:v) acetic acid in ultrapure water] and were examined for evidence of sperm penetration under a phase-contrast microscope at a 400X magnification. The immature oocytes with a broken oolema or an abnormal-looking cytoplasm were classified as degenerated and were not evaluated. The healthy immature oocytes at the germinal vesicle stage were considered penetrated when the sperm with swollen or unswollen heads and their corresponding tails were found in the vitellus. The percentage of penetrated oocytes and the number of sperm per oocyte were considered in the results.

2.7. Statistical analyses

Statistical analyses were performed in an SAS system version 9.0 (SAS Institute Inc., Cary, North Caroline, USA). In experiment 1 and experiment 2, the data were analysed using a mixed model analysis of variance (ANOVA). When the ANOVA revealed

a significant effect, the values were compared using the Tukey-Kramer test and were considered to be significant at $P < 0.05$.

In experiment 1, the data were analysed using a mixed model with the male nested within ejaculate as a random effect and treatment (three levels: control, MβCD and CLC), the anisosmotic solution (fourteen levels: 0, 50, 75, 150, 225, 270, 300, 350, 370, 425, 600, 800, 1200 and 2400 mOsm/kg) and their interaction as the fixed effects. The data presented in the figures correspond to the interaction between the fixed effects. In experiment 2, the model included the male as a random effect and the fixed effect of the treatment (for sperm motility and viability assessments) or the treatment and the incubation medium (sperm functionality assessments). The results are presented as the least-squares means (LSM) ± the standard error of the mean (SEM).

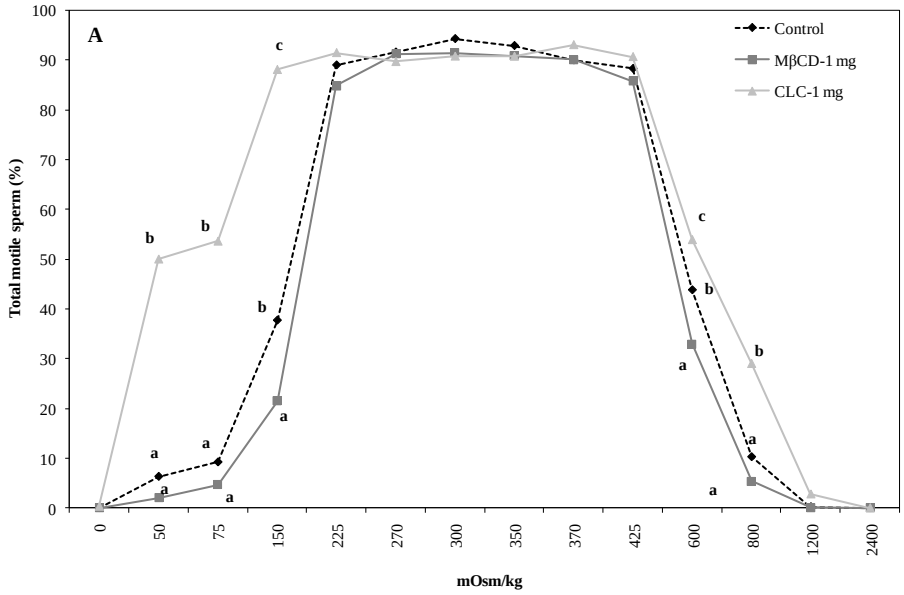
In experiment 3, the penetration rate was analysed using the CATMOD procedure, and the significance was determined using a chi-squared test at $P < 0.05$. The number of sperm per penetrated oocyte was analysed using a mixed model with the male nested within ejaculate as a random effect and the treatment (three levels: control, MβCD and CLC) as a fixed effect. When this analysis showed a significant effect, the means were separated using a Tukey-Kramer test. The means were considered different if $P < 0.05$. The values presented are the LSM ± SEM.

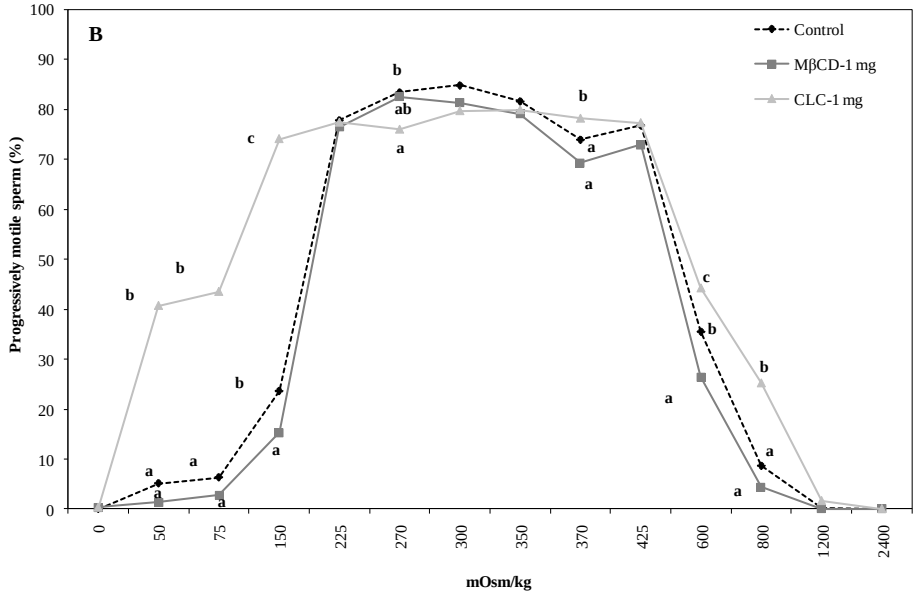
3. Results

Experiment 1. The effect of CLC and MβCD treatments on the osmotic tolerance limits of fresh boar sperm

The fresh sperm treated with CLC exhibited higher percentages of total motile, progressively motile and viable sperm ($P < 0.05$) than the control and the MβCD-treated samples in the hypo-osmotic (50, 75 and 150 mOsm/kg) and the hyper-osmotic (600, 800 mOsm/kg) solutions (Figure 1).

In addition, at least 45% of the sperm treated with CLC maintained the integrity of their plasma membranes when exposed to anisosmotic conditions (50, 75, 150, 600 and 800 mOsm/kg; Figure 1C), whereas the control and the MβCD-treated samples suffered a marked decrease in this parameter when incubated in anisosmotic solutions above 425 mOsm/kg or under 225 mOsm/kg. When the sperm were incubated in a solution of 270 mOsm/kg, the CLC-treated samples exhibited lower percentages of progressively motile sperm ($P < 0.05$) than the control sperm; however, in the other anisosmotic solutions, the CLC-treated sperm presented similar or higher values than the control sperm. On the other hand, the MβCD-treated sperm exhibited similar or lower (150 mOsm/kg; $P < 0.05$) percentages of total, progressively motile and live sperm than the control sperm in all the anisosmotic solutions.


A
Control
MβCD-1 mg
CLC-1 mg
Total motile sperm (%)
mOsm/kg
0
50
75
150
225
270
300
350
370
425
600
800
1200
2400


B
Control
MβCD-1 mg
CLC-1 mg
Progressively motile sperm (%)
mOsm/kg
0
50
75
150
225
270
300
350
370
425
600
800
1200
2400

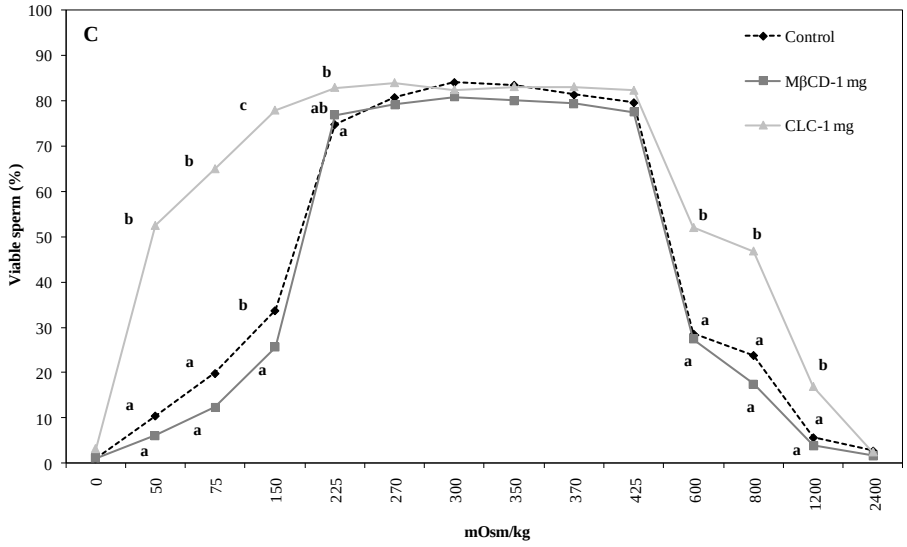


Figure 1. Effect of treatment of boar sperm with methyl-β-cyclodextrin (MβCD-1 mg; 1 mg/120 x 10^6 sperm/mL) or cholesterol-loaded cyclodextrins (CLC-1 mg; 1 mg/120 x 10^6 sperm/mL) on percentages of total motile sperm, SEM = 3.1 (**A**), progressively motile sperm, SEM = 2.9 (**B**) and viable sperm, SEM = 3.2 (**C**). Sperm viability was assessed by SYBR-14/PI fluorescence. Motility parameters were assessed by a CASA system. Different letters (a, b, c) indicate significant differences between treatments in the same osmolality ($P < 0.05$), (n = 10 ejaculates).

Experiment 2. The effect of the CLC and MβCD treatments before cryopreservation on sperm motility, acrosome integrity and the response to capacitating conditions after freezing-thawing

When compared to the control samples, the pre-freezing incubation of boar sperm with MβCD and CLC did not yield any beneficial effects on post-thawed sperm motility or on the percentage of live sperm with an intact acrosome ($P > 0.05$, Figure 2).

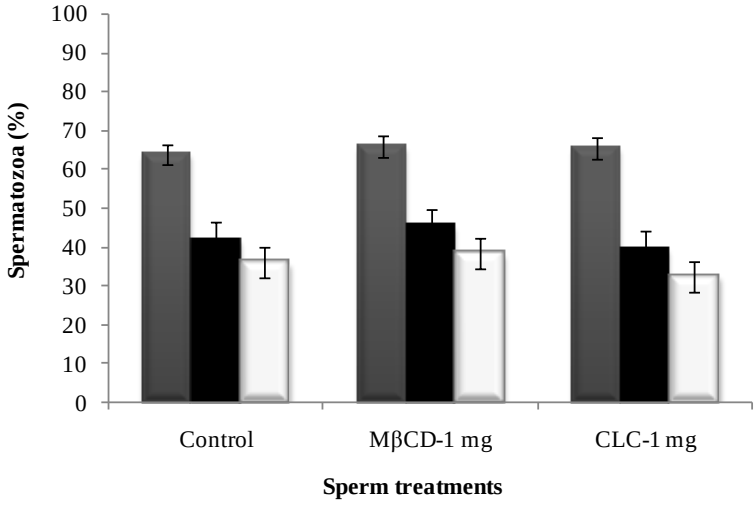


Figure 2. Influence of pre-freezing incubation of boar sperm with methyl-β-cyclodextrin (MβCD-1 mg, 1 mg/120 x 10^6 sperm/mL) or cholesterol-loaded cyclodextrins (CLC-1 mg, 1 mg/120 x 10^6 sperm/mL) on post-thawing percentages of live sperm with an intact acrosome (grey bars), total motile sperm (black bars) and rapid progressively motile sperm (white bars). Live sperm with an intact acrosome were assessed by SYBR-14/PI and R-PE-PNA fluorescence. Motility parameters were assessed by a CASA system. Values are the least square mean ± SEM (n = 13 ejaculates).

To evaluate sperm functionality, the post-thawed sperm samples were incubated under non-capacitating (PBS) or capacitating (mTBM) conditions. The capacitating conditions did not influence the percentage of viable sperm (Figure 4) when compared with the non-capacitating conditions ($P > 0.05$). However, as expected, the conditions induced a significant increase ($P < 0.01$) in the percentages of viable sperm with unstable plasma membranes (Figure 3) or with high intracellular [Ca^{2+}] (Figure 4).

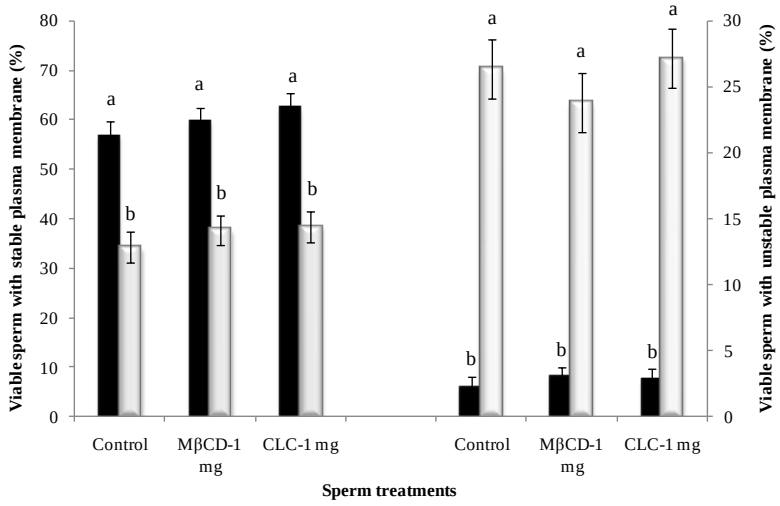


Figure 3. Influence of pre-freezing incubation of boar sperm with methyl-β-cyclodextrin (MβCD-1 mg, 1 mg/120 x 10^6 sperm/mL) or cholesterol-loaded cyclodextrins (CLC-1 mg, 1 mg/120 x 10^6 sperm/mL) on post-thawing percentages of viable sperm with stable plasma membrane (left) and viable sperm with unstable plasma membrane. Thawed sperm were incubated under non-capacitating (PBS, black bars) or capacitating (mTBM, white bars) conditions. Sperm viability and plasma membrane stability were assessed by Yo-pro and Merocyanine-540 fluorescence, respectively. Controls were sperm frozen under standard conditions. Values are the least square mean ± SEM (n = 13 ejaculates). Bars with different letter (a, b) indicate significant differences between post-thaw incubation conditions (non-capacitating vs. capacitating) within the same pre-freezing incubation treatment.

On the other hand, the pre-freezing incubation of sperm with MβCD or CLC did not have an effect on any of these parameters (Figures 3 and 4) when compared with the control (non-treated) samples ($P > 0.05$).

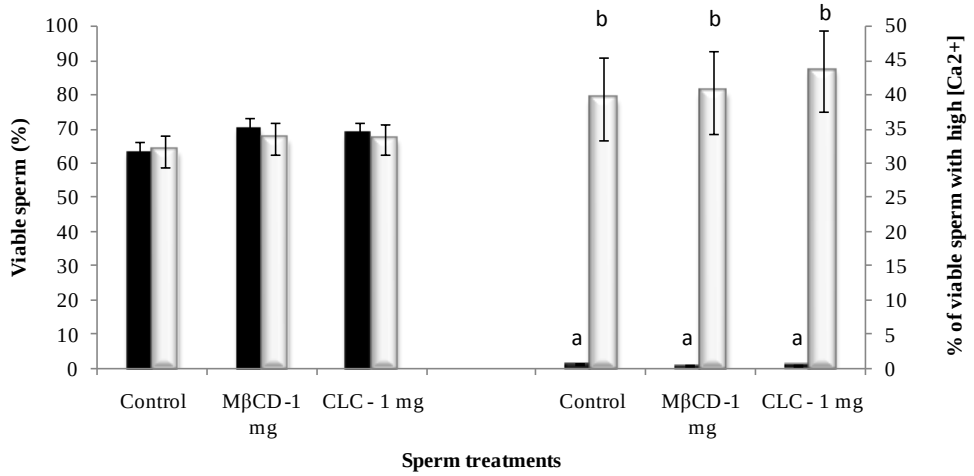


Figure 4. Influence of pre-freezing incubation of boar sperm with methyl-β-cyclodextrin (MβCD-1 mg, 1 mg/120 x 10^6 sperm/mL) or cholesterol-loaded cyclodextrins (CLC-1 mg, 1 mg/120 x 10^6 sperm/mL) on post-thawing percentages of viable sperm (left) and viable sperm with high [Ca^{2+}]. Thawed sperm were incubated under non-capacitating (PBS, black bars) or capacitating (mTBM, white bars) conditions. Sperm viability and calcium concentration were assessed by PI and Fluo-3-AM fluorescence, respectively. Controls were sperm frozen under standard conditions. Values are the least square mean ± SEM (n = 13 ejaculates). Bars with different letter (a, b) indicate significant differences between post-thaw incubation conditions (non-capacitating vs. capacitating) within the same pre-freezing incubation treatment.

Experiment 3. The effect of CLC and MβCD treatments on the in vitro fertilizing ability of frozen-thawed boar spermatozoa

The sperm treated with CLC prior to cryopreservation exhibited a higher penetration ability and greater numbers of sperm per penetrated oocyte than the control or the MβCD-treated sperm ($P < 0.0001$; Table 2).

Table 2. Influence of pre-freezing incubation of boar sperm with methyl-β-cyclodextrin (MβCD-1 mg, 1 mg/120 x 10^6 sperm/mL) or cholesterol-loaded cyclodextrins (CLC-1 mg, 1 mg/120 x 10^6 sperm/mL) on sperm penetration ability of sow's immature oocytes in vitro

Treatment	Penetration rate (%)	Nº sperm/oocyte (LSM ± SEM)
Control	51.5[a] (190/369)	3.3[a] ± 0.5
MβCD-1 mg	52.5[a] (187/356)	3.7[a] ± 0.5
CLC-1 mg	69.4[b] (252/363)	5.0[b] ± 0.5

[a, b] Different superscripts within the same column indicate significant differences between treatments ($P < 0.0001$), (n = 10 ejaculates).

4. Discussion

During both freezing and thawing, the sperm membranes suffer changes caused by the decrease in the temperature (cold shock) and the addition and elimination of cryoprotectants (osmotic stress), which cause a decrease in their biological function. Although the capacity of the sperm to resist these stresses is determined by several factors, the cholesterol: phospholipid ratio of the membrane plays an important role (Watson, 1981; Hoffmann et al., 2009).

The osmotic stress provokes volume excursions in the sperm that are potentially harmful or even lethal when they exceed the sperm osmotic tolerance limits, which are species specific (Meyers, 2005). In this context, cholesterol plays a role in the membrane permeability of the sperm (Raffy and Teissié, 1999). Thus, the amount of cholesterol in the plasma membrane is inversely proportional to the water permeability of the liquid-crystalline bilayer (for a review, see Barenholz, 2002), which permits the adaptation of the membrane to changes in volume (swell and shrink) according to the osmotic conditions. In addition, the cholesterol could act as membrane reinforcement (Dufourc, 2008) by preventing or minimising local membrane ruptures that are produced by local fluctuations in the tensile strength of the membrane when it is subjected to anisosmotic conditions (Muldrew and McGann, 1994). Because a widening of the osmotic tolerance limits was observed when the boar sperm were treated with CLC (1 mg/120 x 10^6 sperm), our results confirm these observations. These results are in agreement with previous reports for boar (Walters et al., 2008), stallion, trout, ram, bull and rabbit sperm (Glazar et al., 2009; Müller et al., 2008; Mocé et al., 2010a; Moraes et al., 2010; Aksoy et al., 2010). Similar to the results reported for trout sperm, treating the boar sperm with MβCD (this treatment removes cholesterol from the membranes; Visconti et al., 1999) did not alter the osmotic

resistance of the boar sperm (Müller et al., 2008). These results, demonstrate that treating the boar sperm with CLC has a beneficial effect on sperm cryosurvival by protecting the sperm from osmotic stress.

Cold shock causes structural changes and brittleness in the plasma membrane when the lipids transition from a fluid to a gel state (Muldrew and McGann, 1990; Drobnis et al., 1993). Cholesterol also plays an important role in sperm resistance to cold shock (Watson, 1981). Indeed, treating the sperm from a cold-shock-sensitive species with cholesterol-loaded cyclodextrins prior to cryopreservation improved sperm cryosurvival (for a review, see Mocé et al., 2010b).

Nevertheless, the results from studies investigating the treatment of boar sperm with CLCs have been contradictory. A number of studies showed that the depletion and the addition of cholesterol (using 2-hydroxypropyl-β-cyclodextrin or methyl-β-cyclodextrin) provoked a pronounced increase in the quality of the boar sperm (acrosome integrity and motility) after freezing-thawing (Zeng and Terada, 2000; 2001) compared to control sperm, although cyclodextrin was more beneficial than CLC treatment. In contrast, later studies did not confirm these results and reported a beneficial effect of cholesterol treatment on the boar sperm quality (viable and non-capacitated sperm) when cold shock was induced (Galantino-Homer et al., 2006) and on the percentage of motile sperm after cryopreservation (Torres et al., 2009).

Our results do not confirm any of the previous reports because neither the addition nor deprivation of cholesterol resulted in an improvement in sperm quality after thawing (sperm motility, sperm viability and acrosome integrity). The reason the boar sperm does not benefit from this treatment like the other cold-sensitive species do is unknown. It is

possible that the differences between the cold-shock sensitive species in their response to the same treatment depend upon or are influenced by the phospholipid composition and distribution of the phospholipids in the plasma membrane (for a review, see Watson 1981).

During the capacitation process, an organised destabilisation of the plasma membrane takes place, where there is a loss of cholesterol from the sperm plasma membrane (Witte and Schäfer-Somi, 2007). For this reason, two different parameters indicative of the capacitation state were measured in the cryopreserved samples: plasma membrane fluidity (M540) and intracellular calcium levels (Fluo-3-AM).

The M540 stain increases its fluorescence when lipids are in a high state of disorder, and it detects changes in plasma membrane fluidity. Therefore, at temperatures above the thermotropic phase transition, a decrease in cholesterol content would increase the lipid disorder in the membrane (increased membrane fluidity), and an increase in cholesterol content would decrease the lipid disorder (decreased membrane fluidity). Nevertheless, the plasma membrane fluidity was similar between the treatments, which corroborates previous reports for bull and trout sperm, although with different stains (Purdy et al., 2005; Müller et al., 2008). The reason why the CLC and cyclodextrin treatments did not modify the membrane fluidity is unknown. A number of studies suggest that for membranes that are already fluid, increasing the cholesterol content of the membrane will not increase the membrane fluidity (Müller et al., 2008). Alternatively, the average membrane fluidity of a sperm population may not change, whereas the average fluidity of individual sperm or even different membrane compartments within each sperm may change (Purdy et al., 2005). It is also possible that because the lipid changes detected by the M540 stain are an early event in the capacitation process (Harrison et al., 1996; Caballero et al., 2009), the differences (if they exist) could not be detected by this stain.

The calcium-sensitive fluorescent probe Fluo-3-AM detects the increase in intracellular calcium levels that takes place when sperm are incubated in vitro under capacitating conditions (Harrison et al., 1993; Caballero et al., 2009). Cholesterol removal during capacitation provokes changes in the fluidity of the sperm plasma membrane that may modulate the Ca^{2+} flux (Visconti et al., 1998). Therefore, the increase in the intracellular calcium level is a marker for a more advanced capacitation state than the lipid changes occurring in the sperm plasma membrane (Harrison et al., 1996). As expected and similar to previous reports (Dubé et al., 2003), when the sperm were incubated under capacitating conditions, the percentage of sperm exhibiting high intracellular calcium levels increased in all of the treatments; however, this response did not depend on the treatment received by the samples prior to cryopreservation (CLC or cyclodextrin treated). These results are similar to those reported for bull and stallion sperm (Purdy and Graham, 2004, and Spizziri et al., 2010, respectively).

Some studies report that the rate of sperm capacitation correlates with the rate of cholesterol efflux from the plasma membrane; sperm with a high cholesterol content are slow to undergo capacitation, whereas sperm with lower cholesterol content are much faster (Gadella et al., 2001). During the freezing process, a decrease in the content of cholesterol in the plasma membrane takes place in CLC-treated and control stallion and ram sperm (Moore et al., 2005, and Mocé et al., 2010a, respectively). Therefore, it is likely that the differences in cholesterol content after cryopreservation between the control and the treated sperm (with CLC or MβCD) are not sufficiently high to detect changes in the capacitation pattern. Nevertheless, it is also likely that under our capacitating conditions, most of the sperm are in an advanced capacitation state where the lipid membrane changes and the intracellular calcium influx have already occurred (Boerke et al., 2008). It should be noted

that we studied the response of the sperm to capacitating conditions at a certain point of incubation (30 min). However, we cannot assume that the results will differ in pattern under different capacitation timing, and intermediate or longer incubation times should be investigated in the future.

In light of these results, it seemed unlikely that these treatments could affect the sperm's ability to penetrate immature oocytes. However, it was surprising that the CLC-treated sperm exhibited a higher penetration ability and that there was a larger number of sperm per penetrated oocyte following this treatment compared with the other treatments. This result is in agreement with previous reports for other species in which CLC-treated sperm bound to the zona pellucida of homologous and/or heterologous oocytes, or they bound to chicken egg perivitelline membranes more efficiently than the control sperm (Moore et al., 2005; Spizziri et al., 2010; Mocé et al., 2010a; Amorim et al., 2009; Moraes et al., 2010). The reason behind the differences between the treatments is unknown. It is possible that the CLC-treated sperm exhibit a higher longevity and penetrate the oocytes more efficiently or that their capacitation pattern is somehow different and that this benefits their fertilizing ability. Nevertheless, this hypothesis will be tested in future studies.

The gamete interaction assays evaluate the effectiveness of the sperm receptors to bind to the oocyte and to the oolemma and to initiate fertilization (Graham and Mocé, 2005). For these reasons, compared with the other in vitro parameters, such as sperm motility or viability, the gamete interaction assays more realistically reflect the in vivo sperm fertilizing ability (Gadea et al., 1998). In pigs, the penetration of immature oocytes provides adequate information on sperm function, and this method may be useful for evaluating the sperm-oocyte interaction during fertilization (Martínez et al., 1996); there is a positive correlation between the in vitro penetration rate and the in vivo boar sperm

fertilizing ability (Martínez et al., 1998). Although these results are encouraging and it is tempting to assume that the CLC-treated sperm will also exhibit a higher fertilizing ability in vivo, in vivo trials must be conducted to definitively determine the usefulness of this treatment for boar sperm cryopreservation.

In conclusion, treating the boar sperm with cholesterol-loaded cyclodextrin (1 mg/120 x 10^6 sperm) widens the osmotic tolerance limits of the sperm. This treatment does not improve the sperm quality after cryopreservation and does not modify the response of the sperm to capacitating conditions. However, the CLC-treated sperm penetrate the sow's immature oocytes in vitro more efficiently than the control or MβCD-treated sperm. Although these results are encouraging, more research on this topic needs to be undertaken to derive definitive conclusions about the usefulness of this treatment for boar sperm cryopreservation.

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Chapter 4: Study 2

Effect of a pre-freezing treatment with cholesterol-loaded cyclodextrins on boar sperm longevity, capacitation dynamics, ability to adhere to porcine oviductal epithelial cells in vitro and DNA fragmentation dynamics

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Abstract

The aim of this work was to examine how a pre-freezing treatment with cholesterol-loaded cyclodextrins (CLC) affects boar sperm longevity, capacitation dynamics, ability to bind to a porcine telomerase-immortalised oviductal epithelial cell line (TERT-OPEC) in vitro and DNA integrity dynamics after freeze-thawing. Although the samples treated with CLC exhibited lower sperm quality than the control samples ($P < 0.05$) immediately after thawing, these differences disappeared ($P > 0.05$) after long-term incubation (26 h at 37 or 16 ºC). Additionally, the CLC-treated sperm underwent similar capacitation and DNA fragmentation dynamics as the control sperm ($P > 0.05$). However, CLC-treated sperm were better able to bind to TERT-OPEC in vitro ($P < 0.0001$). In conclusion, the pre-freezing treatment of boar sperm with CLC enhanced the ability of the sperm to bind to TERT-OPEC in vitro, which could have an effect on the establishment of the sperm reservoir in the ampullary-isthmic junction in vivo. Additionally, frozen-thawed sperm can be stored at 16 ºC for at least 6 h without a significant observable decline in sperm quality, which could be beneficial for the transport of thawed diluted doses of spermatozoa from the laboratory to the farm.

1. Introduction

One of the main advantages of frozen-thawed semen is that they will maintain their fertilizing ability for an indefinite period if handled properly (Medeiros et al., 2002). However, the cryopreservation process also has a cost in terms of sperm quality and sperm fertilizing ability. The damages induced by the cryopreservation process are numerous, and they affect several sperm structures (Hammerstedt et al., 1990; Medeiros et al., 2002).

These damages not only lead to cell death, but they also alter the functionality of the surviving population (Watson, 2000).

Competent sperm are necessary for the establishment of a functional sperm reservoir (SR) in the ampullary-isthmic junction (Rodriguez-Martinez, 2007) until ovulation occurs. However, the size of the SR after insemination with frozen-thawed boar sperm is markedly reduced, which could in turn compromise the fertility (Abad et al., 2007). The sperm plasma membrane plays an important role in capacitation and fertilization events (Stein et al., 2004; Rodriguez-Martinez, 2007), and for this reason, it is important that the sperm possess an intact and competent plasma membrane to fertilize an oocyte. Paradoxically, this structure is also very sensitive to the freeze-thawing processes, and it suffers from destabilisation and/or disruption when the temperature is decreased and the membrane undergoes a phase transition from the fluid to the gel phase (Graham and Mocé, 2005). Although several factors influence the thermotropic behaviour of membranes, the cholesterol:phospholipid ratio plays an important role in the resistance of sperm to cold shock (Darin-Bennett and White, 1977; Watson, 1981). Among domestic species, boar sperm are considered very sensitive to cryodamage because of the high ratio of unsaturated:saturated fatty acids and low cholesterol:phospholipid ratio in their membranes (Parks and Lynch, 1992).

Cholesterol not only plays an important role in the thermotropic behaviour of membranes (Ohvo-Rekilä et al., 2002), but it also participates in the signalling mechanisms that control sperm capacitation. Capacitation is an essential step for the successful interaction of the sperm with the female genital tract and the completion of the fertilization-related fusion processes (Cross, 1998; Visconti et al., 1999a; Visconti et al., 1999b; Travis and Kopf, 2002). The sperm capacitation process is a sequence of complex molecular

mechanisms that occurs in vivo while the sperm reside in the female reproductive tract. A modification in the architecture of the sperm plasma membrane occurs during capacitation, and the loss of cholesterol from the plasma membrane plays an important role in triggering the process (Witte and Schöfer-Somi, 2007). Thus, cholesterol efflux increases the permeability and fluidity of the bilayer, which in turn is important for calcium influx (Visconti and Kopf, 1998) and for events taking place downstream of capacitation.

Cryopreservation induces capacitation-like changes in frozen-thawed sperm that are collectively known as cryo-capacitation (Bailey et al., 2000). The frozen-thawed sperm exhibit an altered membrane state that is functionally similar to capacitated and/or acrosome-reacted sperm (Maxwell and Johnson, 1997; Green and Watson, 2001). Thus, some of the changes observed in frozen-thawed sperm are due to a loss of cholesterol from the plasma membrane (Cerolini et al., 2001) and an increase in the intracellular calcium content (Green and Watson, 2001; Kadirvel et al., 2009). These events take place during the capacitation process as well. Cryo-capacitation is an undesirable event and is associated with the reduced viability (between 0-4 h; Waberski et al., 1994) and poor survival of cryopreserved sperm in the female reproductive tract (Bailey et al., 2000).

Several studies have used cholesterol-loaded cyclodextrins in cold-shock sensitive species to increase the number of spermatozoa that survive the cryopreservation process and to enhance membrane stability in the surviving spermatozoa (for a review, see Mocé et al., 2010b).

Cholesterol-loaded cyclodextrins have also been used for boar sperm cryopreservation, although previously reported results vary between studies (Zeng and Terada, 2000; 2001; Tomás et al., 2011). Nevertheless, this treatment increases the osmotic

tolerance limits of boar sperm (Tomás et al., 2011), minimises cold shock damage (Galantino-Hommer et al., 2006) and improves the ability of frozen-thawed sperm to penetrate immature oocytes in vitro (Tomás et al., 2011). CLC-treated sperm exhibited a better penetration ability in vitro than control sperm; this held true even when the sperm were of equal quality (in terms of motility and viability) immediately after thawing (Tomás et al., 2011). It is possible that the advantage of CLC-treated sperm over the control samples could be due to differences in longevity or capacitation dynamics.

The aim of the present investigation was to determine the effect of CLC treatment on the longevity and capacitation dynamics of boar spermatozoa. Additionally, because the establishment of the sperm reservoir in the ampullary-isthmic junction is important for sow fertility, we studied the effect of CLC on the ability of the sperm to bind to a porcine telomerase-immortalised oviductal epithelial cell line (TERT-OPEC; Hombach-Klonisch et al., 2006) in vitro. Finally, as DNA integrity is of major importance in embryo production, we studied the DNA integrity dynamics of frozen-thawed boar sperm.

2. Materials and methods

2.1. Reagents and media

All the chemicals were of analytical grade. Unless otherwise stated, all the media components and the acridine orange fluorochrome were purchased from Sigma-Aldrich Quimica, S.A. (Madrid, Spain) or Sigma-Aldrich Company Ltd. (Gillingham, UK) and the media were prepared under sterile conditions with purified water (18.2 megohm/cm, Milli-Q water purification system; Millipore Co, Billerica, MA). The other fluorochromes were purchased from Invitrogen S.A. (Barcelona, Spain).

The basic medium used for sperm extension was Beltsville Thawing Solution (BTS; Minitub Ibérica, S.L., Tarragona, Spain). The basic medium used for sperm freezing was lactose-egg yolk extender (LEY), which was composed of 80% (v:v) β-lactose solution (310 mM in water) and 20% (v:v) egg yolk (pH = 6.2 and 330 ± 5 mOsm/kg; Westendorf et al., 1975).

To evaluate the capacitation status, sperm samples were incubated in capacitating medium (Tyrode's albumin lactate pyruvate medium (mTALP) containing 114.06 mM NaCl, 3.2 mM KCl, 8 mM calcium lactate·$6H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.35 mM $NaH_2PO_4 \cdot H_2O$, 25.07 mM $NaHCO_3$, 1.85 mL/L of 60% sodium lactate, 1.1 mM sodium pyruvate, 5 mM glucose, 2 mM caffeine, 0.3% (w:v) BSA, 0.1% (w:v) polyvinyl alcohol, 0.2% (v:v) phenol red solution (0.5%; w:v) and 0.25% (v:v) antibiotic solution (stock solution was composed of 14 mM streptomycin and 84 mM penicillin G); pH = 7 and 275-300 mOsm/kg; Rath et al., (1999) with slight modifications (Silvestre et al., 2007)). The capacitating medium was equilibrated in the dark at 39 ºC under a modified atmosphere containing 5% CO_2 and 100% humidity for 15 min before use.

To wash the sperm, a saline medium and a sucrose medium were used (Harrison et al., 1993). The compositions of these media are detailed in Tomás et al. (2011).

For the in vitro culture of TERT-OPEC, a commercial medium (Dulbecco´s Modified Eagle's Medium/Ham's F-12 Nutrient Mixture; DMEM/F-12) was supplemented with 1% (v:v) L-glutamine, 1% (v:v) antibiotic-antimycotic solution, 0.004% (v:v) insulin, 10% heat inactivated foetal bovine serum (FBS) and 0.1% (w:v) 17-β-oestradiol (sDMEM/F-12). For the TERT-OPEC binding assay, a modified Tyrode's albumin lactate pyruvate medium supplemented with serum (TALPs; Fazeli et al. 1999) was used. This

medium was composed of 2 mM $CaCl_2$, 3.1 mM KCl, 0.4 mM $MgCl_2 \cdot 6H_2O$, 100 mM NaCl, 25 mM $NaHCO_3$, 0.3 mM $NaH_2PO_4 \cdot 2H_2O$, 10 mM HEPES, 21.6 mM sodium lactate, 1 mM sodium pyruvate, 1.2% (w:v) BSA and 1% (v:v) antibiotic-antimycotic solution supplemented with 10% (v:v) FBS.

To evaluate the DNA integrity, sperm samples were diluted and stored in TNE buffer (0.01 M Tris HCl, 0.15 M NaCl, 1 mM EDTA disodium salt; pH = 7.4; Evenson et al., 1994). To analyse the samples, the sperm were treated with an acid solution (0.08 N HCl, 0.15 M NaCl, 0.1% (v:v) Triton X-100; pH = 1.2; Evenson et al., 1994) and stained with acridine orange staining solution (AO-solution; 0.15 M NaCl, 0.126 M Na_2HPO_4, 0.011 M EDTA disodium salt, 0.037 M citric acid, 6 µg/mL AO; pH = 6; Evenson et al., 1994).

2.2. Preparation of cholesterol-loaded cyclodextrins

Cholesterol-loaded cyclodextrins (CLC) were prepared according to the protocol developed by Purdy and Graham (2004a) as described elsewhere (Tomás et al. 2011).

A working solution of CLC crystals (50 mg/mL in BTS) was freshly prepared on the day of the experiment.

2.3. Sourcing, collecting and handling of the ejaculates

Healthy mature Pietrain or crossbred (Landrace x Pietrain) boars (n = 15) of proven fertility housed in a commercial insemination station (CIAR, Centro de Inseminación, Peñarroya de Tastavins, Teruel, Spain) and fertile Pietrain boars (n = 3) housed at the experimental farm at the Centro de Tecnología Animal-Instituto Valenciano de Investigaciones Agrarias (CITA-IVIA, Segorbe, Spain) were used as ejaculate donors.

The boars were housed in individual climate-controlled pens (15 to 25 °C), fed with a commercial diet and provided with water *ad libitum*. The protocols that were used fulfilled the European regulations for the care and use of animals for scientific purposes (EC Directive 2010/63/EU).

The sperm-rich ejaculate fractions were collected using the "gloved-hand" method, extended (1:1, v:v) in BTS and evaluated for conventional semen characteristics.

The extended semen samples from the CIAR were transferred to 100 mL plastic tubes, cooled to 16 °C, packaged in insulated containers and sent by courier service to the sperm cryopreservation laboratory at the CITA-IVIA. The extended semen samples arrived at the CITA-IVIA between 18 and 20 h after ejaculate collection. When the ejaculates came from the CITA-IVIA, the extended semen was transported to the laboratory at 22 ºC less than 30 min after collection, and it was maintained at 22 ºC for a minimum of 2 h and at 16 ºC for a maximum of 20 h before cryopreservation.

2.4. Sperm cryopreservation

Immediately after reception, the concentration (Pursel and Johnson, 1974), motility and membrane integrity of the samples were evaluated. Only ejaculates with greater than 75-80% motile sperm and sperm with intact plasma membranes were selected for cryopreservation.

The ejaculates from three different boars were mixed (using equal numbers of sperm) to constitute a pool. Each pool was split into two aliquots; one aliquot served as a control (non-treated) and the other was treated with 1 mg of CLC per 120 x 10^6 sperm

(Blanch et al., 2012). The aliquots were incubated with CLC for 15 min at 16 ºC (Blanch et al., 2009).

The sperm samples were then cryopreserved according to the protocol described by Westendorf et al. (1975). Briefly, each aliquot (control and CLC-treated) was centrifuged at 800 g for 10 min at 16 °C, and the supernatant was removed by aspiration. The sperm pellet was suspended in LEY extender to yield a concentration of 1.5 x 10^9 sperm/mL. After cooling to 5 °C within 120 min, the extended sperm were diluted (2:1, v:v) in LEY-Glycerol-Orvus ES Paste (OEP) extender (89.5% (v:v) LEY, 1.5% (v:v) OEP (Equex STM; Nova Chemical Sales Inc., Scituate, MA, USA) and 9% (v:v) glycerol) to yield final concentrations of 10^9 sperm/mL, 3% glycerol and 0.5% OEP. The cooled sperm was then equilibrated with glycerol for 15 min before packaging into 0.5 mL straws that were sealed with polyvinyl alcohol. The straws were frozen using a programmable freezer (Mini-Digitcool, IMV, Humeco, Huesca, Spain) in accordance with the protocol described by Carvajal et al. (2004).

The straws were thawed in a water bath at 37 ºC for 30 s and then diluted in BTS (1:1, v:v; 37 ºC) prior to the analyses.

2.5. Sperm assessment

Sperm motility

Sperm motility was evaluated objectively using a computer-assisted sperm analysis system (ISAS®, Proiser R+D, Paterna, Spain) following the protocol described by Tomás et al. (2011). The percentages of total motile sperm (VAP > 10 µm/s) and sperm with rapid progressive motility (VAP > 50 µm/s and STR > 75%) were recorded.

Flow cytometry analyses

Flow cytometry analyses were performed at room temperature under a dimmed light using an EPICS XL flow cytometer (Coulter Corporation Inc, Miami, FL, USA) equipped with standard optics a 15 mW 488 nm argon ion laser (Cyonics, Coherent, Santa Clara, CA, USA), and EXPO 2000 software (Coulter Corporation). Although initially cell distributions were selected on the basis of forward and side scatter (L-shaped profile which mostly would be related to spermatozoa), this gating procedure is associated with artifacts as has been shown previously (Petrunkina and Harrison, 2010; Petrunkina et al., 2010; Petrunkina and Harrison, 2011). Therefore, sequentially we also analysed the impact of non-spermatozoon events and were able to demonstrate that in our case it was negligible because it was below the threshold reported in the above-quoted papers.

The green fluorescence of SYBR-14, Fluo-3-AcetoMethoxy (Fluo-3-AM, F-14218) and AO were detected using a 550 nm long pass (LP) filter combined with a 525 nm (bandwidth 505-545) band pass (BP) filter (FL1). The red fluorescence of propidium iodide (PI) and AO were detected using a 645 nm LP filter combined with a 620 nm (bandwidth 605-635) BP filter (FL3). Each sample was filtered through a 40 µm nylon mesh before analysis to remove large clumps of cells and debris.

The integrity of the sperm plasma membrane was evaluated by double vital staining with PI (1 mg/mL in ultrapure water) and SYBR-14 (10 µM in dimethyl sulfoxide, DMSO) (LIVE/DEAD Sperm Viability Kit, L-7011), and flow cytometry was performed in accordance with a previously described protocol (Purdy and Graham, 2004b; Tomás et al., 2011). The photomultiplier (PMT) value of the detector in FL1 was set at 650 V and in FL3 at 691 V, FL1-FL3 compensation was 41.4%, and FL3-FL1 compensation was 5.5%. A minimum of 10,000 events/sample were analyzed. The percentage of non DNA-containing

events (PI and SYBR-14 negative) was below 3% (Figure 1), and was not considered for the calculations to avoid an overestimation in the proportion of live (PI negative) cells (Petrunkina and Harrison, 2010; Petrunkina et al., 2010). Only the percentage of live sperm was considered in the results (SYBR-14 positive and PI negative).

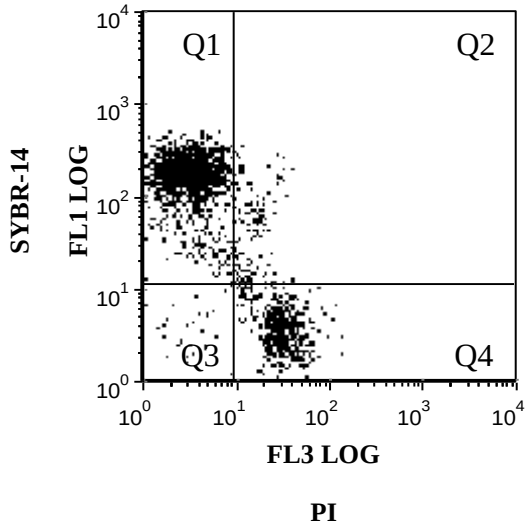


Figure 1. Histogram and quadrants of the stain SYBR-14/PI. Q1 represents the number of events SYBR-14 positive and PI negative, Q2 the number of events SYBR-14 and PI positive, Q3 the number of events PI and SYBR-14 negative (unstained events), and Q4 the number of events PI positive and SYBR-14 negative. The percentage of unstained events (Q3) was below 3% for the analyzed samples.

The intracellular calcium levels were evaluated using the fluorescent probes Fluo-3-AM and PI following the protocol described by Harrison et al. (1993) and modified by Caballero et al. (2009) with slight modifications. Briefly, 150 x 10^6 sperm (300 µL) were diluted in 700 µL BTS and incubated with 3 µL/mL Fluo-3-AM (1 mM in DMSO) in the dark at room temperature for 10 min. A saline medium (4 mL) was added to dilute the

suspensions, and the incubation was continued for an additional 20 minutes in the dark at room temperature. This suspension was then layered over 6 mL of sucrose medium and centrifuged for 10 min at 300 x g followed by 10 min at 750 x g at room temperature (22 ºC). The supernatant was aspirated, and the sperm pellet was gently resuspended in 0.2 mL of PBS. The sperm suspension was diluted in pre-equilibrated mTALP to a final concentration of 2 x 10^6 sperm/mL, and 5 µL/mL PI (1 mg/mL stock solution in ultrapure water) was added to stain the dead sperm. Finally, the sperm samples were incubated in the dark for 24 h at 39 ºC in a modified atmosphere containing 5% CO_2 and 100% humidity. Additionally, a positive control sample (high intracellular calcium) was prepared (Harrison et al., 1993) as follows: a sample stained with Fluo-3-AM and PI was diluted to 2 x 10^6 sperm/mL and treated with 5 µL/mL calcium ionophore (A23187, 40 µM stock solution in DMSO) to induce an increase in the intracellular calcium. The control settings were adjusted each day during the analyses. The PMT value of the detector in FL1 was set at 862 V and in FL3 at 714 V, and FL3-FL1 compensation was 4.3%. A minimum of 10,000 events/sample were analyzed. A washing step is recommended in order to eliminate the alien material when it is not possible to use DNA stains in the protocols (Petrunkina and Harrison, 2010). In our case, the protocol included a washing step (the samples were washed through a sucrose medium after the incubation with Fluo-3-AM) therefore, the impact of alien particles was negligible (< 3%) also for this stain. The results were expressed as the percentage of dead sperm (PI +) from the total sperm population and the percentage of viable sperm (PI -) with high intracellular calcium (with respect to the total viable sperm population).

To evaluate the integrity of the sperm DNA, a sperm chromatin structure assay (SCSA) was performed in accordance with a protocol described previously (Evenson et al.,

1994). Aliquots (1 mL) were diluted in TNE buffer to a concentration of 2 x 10^6 sperm/mL, frozen in liquid nitrogen vapour and transferred to a -80 ºC freezer where they were stored until further processing and flow cytometric analysis (Johannisson et al., 2009). The TNE-diluted samples were thawed in a 37 ºC water bath, placed on crushed ice and subjected to partial DNA denaturation in situ by mixing 200 µL of the sample with 400 µL of an acid solution for 30 s followed by subsequent staining with 1.2 mL of AO solution. The AO-stained samples were analysed within 3 min. Sperm without detectable levels of DNA fragmentation emit a green fluorescence, and those with moderate to high DNA fragmentation emit various levels of red fluorescence (Didion et al., 2009). The PMT value of the detector in FL1 was set at 675 V and in FL3 at 966 V. A minimum of 10,000 events/sample were analyzed. Data were stored as list mode files, and further analyses of the parameters were performed using the FCS Express software version 3 (De Novo Software, Los Angeles, CA) (Hernández et al., 2006). The extent of DNA denaturation, expressed as the DNA fragmentation index (DFI), was calculated based on the ratio of red:total (red+green) fluorescence for each sperm in the sample (Evenson et al., 2002). For each sample, four parameters were evaluated (Evenson et al., 2002): mean DFI and standard deviation (SD-DFI) expressed in channels of fluorescence; the percentage of sperm with detectable DFI (% DFI), and the percentage of sperm with high DNA stainability (% HDS).

Sperm-oviductal porcine epithelial cell (OPEC) binding assay

To evaluate the binding ability of the sperm to OPEC in vitro, a sperm-OPEC binding assay was performed according to the method described by Green et al. (2001) with slight modifications. For the experiment, a porcine telomerase-immortalised oviductal epithelial cell line (TERT-OPEC; Hombach-Klonisch et al., 2006) was used. The in vitro

culture of TERT-OPEC was initiated from frozen cryovials (gift of Prof. Hombach-Klonisch to AF). The frozen cryovials of TERT-OPEC were thawed in a water bath at 37 ºC and the cells were then transferred to a tissue culture flask (75 cm^2) containing sDMEM/F-12. The cultures were incubated at 37 ºC in an atmosphere of 100% humidity and 5% CO_2 in air. The culture medium was refreshed every 48-72 h until confluency, and TERT-OPEC reached confluence after 7-14 days. Cells from one flask that had reached confluence were rinsed three times with PBS in the absence of Ca^{2+} and Mg^{2+} (Life Technologies LTD, Paisley, UK) and detached by incubating with 3 mL of a commercial trypsin-EDTA solution (0.5 mg/mL porcine trypsin and 0.2 mg/mL EDTA) for 5 min at 37 ºC. Trypsinised cells were diluted with 9 mL sDME/F-12 and washed once by centrifugation at 300 x g for 5 min at 4 ºC. The supernatant was discarded, and the cells were resuspended in 1 mL of TALPs. The concentration of TERT-OPEC was measured using a counting chamber, and the viability was evaluated by mixing a sample of the cells (0.2 mL) with an equal volume of a commercial preparation of 0.4% Trypan blue solution. After 3 min of incubation at room temperature, a 30 µL aliquot was transferred to a Neubauer chamber, and cells were counted under a light microscope using the 10X objective. The samples were diluted in TALPs to a final concentration of 2 x 10^6 viable cells/mL.

For the TERT-OPEC-sperm co-incubation, 200 µL of a sperm sample (200 x 10^6 sperm/mL) was added to an eppendorf tube containing an equal volume of the TERT-OPEC suspension. The incubation, recovery and evaluation of the sperm-TERT-OPEC complexes were performed according to the protocol described by Green et al. (2001). Briefly, the TERT-OPEC-sperm suspension was gently mixed and incubated for 30 min with rotation at 39 ºC. After co-incubation, sperm-TERT-OPEC complexes were washed

over a two-step iso-osmotic Percoll density gradient and recovered at the interface between the 35% and 70% Percoll layers (Green et al., 2001).

Finally, the sperm-TERT-OPEC complexes were fixed 1:1 (v:v) with a solution of 2% PBS-formaldehyde (w:v), and a 10 μL aliquot of the fixed sample was placed on a microscope slide with a coverslip to count the number of sperm bound per TERT-OPEC under a light microscope using the 40X objective. A total of 250 sperm-TERT-OPEC complexes per pool and treatment were evaluated.

2.6. Experimental design

A total of 12 pools of semen were used for each one of the experiments with the exception of experiment 3, in which 10 pools of semen were used. The pools were treated with CLC, cryopreserved and thawed as described previously.

Experiment 1. The effect of a pre-cryopreservation CLC treatment on sperm resistance to incubation at 37 or 16 ºC for up to 26 h after freeze-thawing

In this experiment, the evolution of sperm motility and the integrity of the sperm plasma membrane under long-term incubation were studied. After the first evaluation (time 0 after thawing), each sperm sample (diluted 1:1, v:v; in BTS) was split into two subsamples, both of which were incubated for up to 26 h. One of the subsamples was incubated in a cold storage chamber set at 16 ºC, and the other sample was incubated in a water bath at 37 ºC. One aliquot from each of the subsamples was removed at 0.5, 1.5, 3, 4.5, 6 and 26 h, and sperm motility and the integrity of the sperm plasma membrane were evaluated according to the protocol described previously.

Experiment 2. The effect of CLC treatment on sperm capacitation dynamics

In this experiment, capacitation dynamics were evaluated by measuring the intracellular calcium concentration in samples incubated under capacitating conditions for 24 h. Samples were prepared and stained with Fluo-3-AM and PI according to the protocol described previously. Aliquots were removed and analysed at 15 min and 0.5, 1, 2, 3, 4, 5 and 24 h of incubation.

Experiment 3. The effect of CLC treatment on the ability of freeze-thawed boar sperm to bind a porcine telomerase-immortalised oviductal epithelial cell line (TERT-OPEC) in vitro

For this experiment, three straws per treatment (from the same pool) were thawed, and the contents were mixed. The samples were diluted to 25 x 10^6 sperm/mL in BTS and were washed over a two-step iso-osmotic Percoll density gradient (2 mL of 70% Percoll overlaid with 2 mL of 30% Percoll). The sperm suspension (4 mL) was layered over the Percoll density gradient and centrifuged at 400 x g for 20 min followed by centrifugation at 250 x g for 10 min (Pertoft, 2000). The supernatant was aspirated, the sperm pellet was gently resuspended, the sperm concentration was evaluated as described previously and the samples were diluted in TALPs to a final concentration of 200 x 10^6 sperm/mL. The samples were maintained at 37 ºC prior to incubation with TERT-OPEC. The sperm-TERT-OPEC binding assay was performed according to the protocol described previously.

Experiment 4. The effect of CLC treatment on sperm DNA integrity after thawing

For this experiment, an aliquot (150 µL; 75 x 10^6 sperm) of frozen-thawed sperm was diluted in 850 µL of BTS. Aliquots from this solution were diluted in pre-equilibrated

mTALP to a final concentration of 22.5 x 10^6 sperm/mL and incubated for up to 24 h at 39 ºC in a modified atmosphere containing 5% CO_2 and 100% humidity. Aliquots were removed at 15 min and 0.5, 1, 2, 3, 4, 5 and 24 h of incubation, diluted in TNE buffer and stored until analysis. The analyses were performed following the protocol described previously.

2.7. Statistical analyses

Statistical analyses were performed with the SAS system version 9.0 (SAS Institute Inc., Cary, North Carolina, USA). The data were analysed using a mixed model analysis of variance (ANOVA) including the pool of semen as a random effect. When the ANOVA revealed a significant effect, the values were compared using the Tukey-Kramer test and were considered to be significant at $P < 0.05$. The results are presented as least squares means (LSM) ± the standard error of the mean (SEM).

In experiment 1, to evaluate the differences between times within a treatment and a storage temperature and the differences between storage temperatures within a time and a treatment, the fixed factors of time and storage temperature and their interaction were included in the model. To evaluate the differences between treatments within a time and a storage temperature, the treatment, temperature and their interaction were included as fixed effects. In experiments 2 and 4, the model included the incubation time (to evaluate the differences within a treatment) or the treatment (to evaluate the differences within an incubation time) as a fixed effect. In experiment 3, the model included the sperm treatment as a fixed effect.

3. Results

Experiment 1. The effect of pre-cryopreservation CLC treatment on sperm resistance to incubation at 37 or 16 ºC for up to 26 h after freeze-thawing

The effect of post-thaw incubation time (0, 0.5, 1.5, 3, 4.5, 6 and 26 h) and post-thaw storage temperature (37 ºC *vs.* 16 ºC) on the percentages of live, total motile and progressively motile sperm are shown in Tables 1, 2 and 3, respectively. When evaluated immediately after thawing, the samples treated with CLC prior to cryopreservation were of lower quality than the control samples ($P < 0.05$). Nevertheless, these differences disappeared soon after the incubation started because both treatments presented similar sperm quality after 30 min or 1.5 h.

When the samples were incubated at 37 ºC up to 26 h after thawing, the percentages of live, of total motile and of progressively motile sperm in control samples declined significantly ($P < 0.05$) after 4.5 h of incubation (with respect to the value at time 0). However, CLC-treated samples exhibited similar percentages of live sperm from 0 h to 4.5 h of incubation ($P > 0.05$), and these samples only exhibited significantly lower percentages of total motile and progressively motile sperm after 26 h of incubation at 37 ºC ($P < 0.05$).

Table 1. Effect of post-thaw incubation time and post-thaw storage temperature on the percentage of viable sperm in samples treated with cholesterol-loaded cyclodextrins (CLC) (1 mg CLC/120 x 10^6 sperm) or not treated (Control) prior to cryopreservation

Time	37 ºC		16 ºC		
(hours)	Control	CLC	Control	CLC	SEM[1]
0	63.1 [a]	58.5 [a*]	63.1	58.5[*]	2.2
0.5	62.6 [a]	57.7 [a]	57.7	54.9	2.3
1.5	61.4 [a]	57.3 [a]	59.5	56.0	2.3
3	58.5[ab]	55.2 [a]	60.6	57.0	2.6
4.5	54.4[bc]	53.5[ab]	61.4[†]	57.5	2.8
6	48.7 [c]	48.5 [b]	60.3[†]	54.9[*†]	3.4
26	3.0 [d]	4.5 [c]	58.6[†]	55.1[†]	2.0
SEM[2]	2.7	2.4	2.7	2.4	

[a, b, c, d] Indicate significant differences within the same column ($P < 0.05$). [*]Indicates significant differences ($P < 0.05$) between treatments within an incubation time and a storage temperature (37 or 16 ºC). [†]Indicates significant differences between storage temperatures (37 or 16 ºC) within an incubation time and a treatment ($P < 0.05$). [1]Data correspond to the double interaction (treatment x storage temperature). [2]Data correspond to the double interaction (time x storage temperature), (n = 12 pools of semen).

Table 2. Effect of post-thaw incubation time and post-thaw storage temperature on the percentage of total motile sperm in samples treated with cholesterol-loaded cyclodextrins (CLC) (1 mg CLC/120 x 10^6 sperm) or not treated (Control) prior to cryopreservation

Time	37 ºC		16 ºC		
(hours)	Control	CLC	Control	CLC	SEM[1]
0	48.8 ^{a}	40.2^{abc*}	48.8 ^{a}	40.2 ^{a*}	2.0
0.5	50.8 ^{a}	44.0 ^{a}	48.4 ^{a}	38.7 ^{a*}	2.8
1.5	48.1 ^{a}	47.8 ^{a}	47.3 ^{a}	43.9 ^{a}	2.3
3	44.3^{ab}	42.3^{ab}	47.8 ^{a}	40.8 ^{a}	2.6
4.5	36.8^{bc}	33.5^{bc}	44.9 ^{a}	40.9 ^{a}	3.3
6	29.6 ^{c}	31.7 ^{c}	42.9^{a†}	36.6^{ab}	3.3
26	0.0 ^{d}	0.0 ^{d}	30.3^{b†}	28.0^{b†}	1.4
SEM[2]	2.7	2.5	2.7	2.5	

$^{a, b, c, d}$ Indicate significant differences within the same column ($P < 0.05$). *Indicates significant differences ($P < 0.05$) between treatments within an incubation time and a storage temperature (37 or 16 ºC). †Indicates significant differences between storage temperatures (37 or 16 ºC) within an incubation time and a treatment ($P < 0.05$). [1]Data correspond to the double interaction (treatment x storage temperature). [2]Data correspond to the double interaction (time x storage temperature), (n = 12 pools of semen).

As expected, the loss of sperm quality was less pronounced when the samples were stored at 16 ºC than when the samples were incubated at 37 ºC. Indeed, when the samples were stored at 16 ºC, they contained similar percentages of live sperm over time ($P > 0.05$) irrespective of the treatment. Additionally, a decline ($P < 0.05$) in the percentage of total motile and progressively motile sperm was only observed after 26 h of storage in CLC-treated samples and after 26 h (total motile sperm) and 6 h of storage (progressively motile sperm), in control samples (with respect to the values at time 0).

The control samples stored at 16 ºC contained higher percentages ($P < 0.05$) of live sperm (Table 1) after 4.5, 6 and 26 h of incubation, higher percentages ($P < 0.05$) of total motile sperm (Table 2) after 6 and 26 h of incubation, and higher percentages ($P < 0.05$) of progressively motile sperm (Table 3) after 26 h of incubation than the samples stored at 37 ºC. However, the CLC-treated samples exhibited temperature-dependent differences ($P < 0.05$) in the percentage of live sperm at 6 and 26 h of incubation; such differences were observed in the percentages of total motile and progressively motile sperm only after 26 h of incubation.

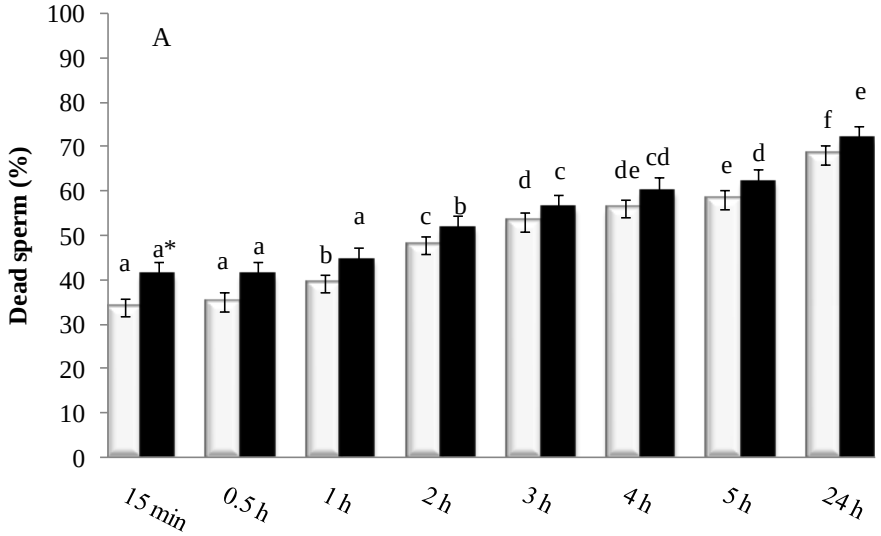
Table 3. Effect of post-thaw incubation time and post-thaw storage temperature on the percentage of progressively motile sperm in samples treated with cholesterol-loaded cyclodextrins (CLC) (1 mg CLC/120 x 10^6 sperm) or not treated (Control) prior to cryopreservation

Time	37 ºC		16 ºC		
(hours)	Control	CLC	Control	CLC	SEM[1]
0	40.7 ^{a}	34.3^{abc*}	40.7 ^{a}	34.3^{a*}	1.9
0.5	43.5 ^{a}	38.3 ^{a}	38.1^{ab}	31.2^{ab*}	2.6
1.5	42.7 ^{a}	41.1 ^{a}	37.1^{ab}	36.1 ^{a}	2.3
3	38.4^{ab}	35.0^{ab}	36.8^{ab}	34.3 ^{a}	2.7
4.5	30.3^{bc}	27.4^{bc}	34.9^{ab}	30.9^{ab}	3.1
6	24.4 ^{c}	25.6 ^{c}	30.5^{bc}	28.5^{ab}	3.1
26	0.0 ^{d}	0.0 ^{d}	21.3^{c†}	22.1^{b†}	1.2
SEM[2]	2.5	2.4	2.5	2.4	

$^{a, b, c, d}$ Indicate significant differences within the same column ($P < 0.05$). *Indicates significant differences ($P < 0.05$) between treatments within an incubation time and a storage temperature (37 or 16 ºC). †Indicate significant differences between storage temperatures (37 or 16 ºC) within an incubation time and a treatment ($P < 0.05$). [1]Data correspond to the double interaction (treatment x storage temperature). [2]Data correspond to the double interaction (time x storage temperature), (n = 12 pools of semen).

Experiment 2. The effect of CLC treatment on sperm capacitation dynamics

To evaluate sperm capacitation dynamics, the post-thawed sperm samples were incubated under capacitating conditions for 24 h. As expected, the percentage of sperm with a high intracellular calcium concentration (with respect to the live population) and the percentage of dead sperm gradually increased over time ($P < 0.05$), exhibiting the highest values after 24 h of incubation under capacitating conditions (Figure 2). However, the samples treated with CLC prior to freezing contained a lower percentage of sperm with high intracellular calcium and a higher percentage of dead sperm after 15 min of incubation ($P < 0.05$), although thereafter the values were similar ($P > 0.05$) for both treatments (Figure 2).



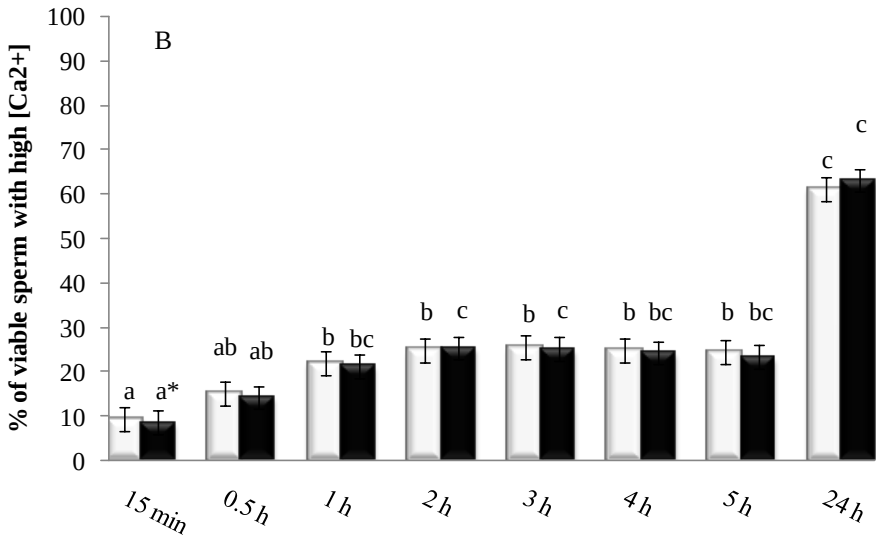


Figure 2. Effect of incubation under capacitating conditions (A) on the post-thaw percentages of dead sperm and (B) on the percentage of viable sperm with high [Ca^{2+}] with respect to the viable sperm population in samples treated with cholesterol-loaded cyclodextrins (■ CLC) (1 mg of CLC per 120 x 10^6 sperm) or not treated (□ Control) prior to cryopreservation. Values are the least squares means ± SEM (n = 12 pools of semen). Bars of the same colour with different letters (a, b, c, d, e, f) indicate significant differences between incubation times, and an asterisk indicates significant differences between treatments within an incubation time (SEM = 2.3 and 0.5 for the percentage of dead sperm and for the percentage of viable sperm with a high calcium concentration, respectively; $P < 0.05$).

Experiment 3. The effect of CLC treatment on the ability of frozen-thawed boar sperm to bind TERT-OPEC in vitro

The sperm treated with CLC prior to cryopreservation adhered to TERT-OPEC in greater numbers than the control sperm ($P < 0.0001$; Table 4).

Table 4. Effect of pre-freezing incubation of boar sperm with cholesterol-loaded cyclodextrins (1 mg CLC/120 x 10^6 sperm) on the ability of frozen-thawed sperm to adhere to a porcine telomerase-immortalised oviductal epithelial cell line (TERT-OPEC) in vitro

Treatment	Number of sperm attached/TERT-OPEC
Control	17.3 ± 1.5^{a}
CLC	21.8 ± 1.5^{b}

Values are the least squares means ± SEM (n = 250 TERT-OPEC for each pool of semen and treatment. Different letters indicate a significant difference between treatments ($P < 0.05$), (n = 10 pools of semen).

Experiment 4. The effect of CLC treatment on sperm DNA integrity after thawing

The incubation of sperm with CLC prior to freezing did not have any effect ($P > 0.05$) on the parameters measured with the sperm chromatin structure assay (Table 5). Additionally, these parameters remained very low throughout the incubation.

Table 5. Effect of incubation time on the post-thaw sperm chromatin structure assay (SCSA) in samples treated with cholesterol-loaded cyclodextrins (CLC) (1 mg CLC/120 x 10^6 sperm) or not treated (Control) prior to cryopreservation

Treatment	Parameter	Incubation time					
		15 min	0.5 h	1 h	2 h	5 h	24 h
Control	% HDS	1.6 ± 0.2	1.4 ± 0.2	1.7 ± 0.2	1.6 ± 0.2	1.7 ± 0.2	1.7 ± 0.2
	% DFI	0.7 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.2
	Mean DFI	387.6 ± 2.6	389.9 ± 2.6	388.1 ± 2.6	388.2 ± 2.6	388.7 ± 2.6	386.9 ± 2.6
	SD-DFI	19.4 ± 0.7	18.9 ± 0.7	19.6 ± 0.7	18.8 ± 0.7	19.2 ± 0.7	18.6 ± 0.7
CLC	% HDS	1.6 ± 0.2	1.5 ± 0.2	1.6 ± 0.2	1.6 ± 0.2	1.8 ± 0.2	1.7 ± 0.2
	% DFI	0.6 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	0.7 ± 0.2	0.7 ± 0.2
	Mean DFI	387.6 ± 2.6	390.1 ± 2.6	388.5 ± 2.6	388.9 ± 2.6	388.0 ± 2.6	389.1 ± 2.6
	SD-DFI	19.0 ± 0.7	18.9 ± 0.7	18.8 ± 0.7	18.7 ± 0.7	19.4 ± 0.7	18.8 ± 0.7

SCSA parameters: percentage of sperm with high DNA stainability, % HDS; percentage of sperm with detectable DFI, % DFI; mean DNA fragmentation index, mean DFI; standard deviation of DFI, SD-DFI. Values are least squares means ± SEM, (n = 12 pools of semen).

4. Discussion

Despite the numerous advantages that sperm cryopreservation presents, the freeze-thawing process is harmful and results in a product with suboptimal quality and fertilizing ability. Thus, sperm cryoinjury, including the capacitation-like changes induced in the sperm during the freeze-thawing processes, lead to shortened sperm longevity and impaired sperm transport in the female genital tract (Bailey et al., 2000). Although several factors influence the resistance of sperm to cold shock damage and participate in the process of sperm capacitation, cholesterol plays important roles in both events (Cross, 1998; Watson, 1981).

Due to the importance of cholesterol, cyclodextrins pre-loaded with cholesterol have been used to modify the cholesterol content of sperm plasma membranes with the objective of increasing sperm cryosurvival in different species (for a review, see Mocé et al., 2010b). In a previous study, boar sperm treated with these compounds contained similar percentages of motile sperm and sperm with intact acrosomes and plasma membranes compared to control samples immediately after thawing, although CLC-treated sperm penetrated immature oocytes in vitro more efficiently than control sperm (Tomás et al., 2011). Nevertheless, sperm longevity during incubation seems to be a better indicator of the fertilization rate (Dorado et al., 2007) because the ability of sperm to maintain motility for long periods of time indicates a greater likelihood that they will survive in the female genital tract, undergo capacitation and fertilize an oocyte (Fiser et al., 1991). For this reason, we studied the effect of CLC treatment on the longevity of sperm in vitro.

Despite the differences observed between treatments at time 0, control and CLC-treated samples behaved similarly throughout the 26 h of incubation at 37 ºC. These results

are in agreement with previous reports on ram sperm incubated at 38.5 ºC for 3 h (Mocé et al., 2010a). However, even when significant differences between control and CLC-treated ram sperm were not observed at any incubation time, the CLC-treated samples maintained greater percentages of motile sperm for 3 h and did not lose as much motile sperm throughout the incubation time (-3%) as the control samples (-9%; Mocé et al, 2010a). A similar phenomenon was observed in the boar sperm, where CLC-treated samples maintained their quality parameters for longer even when their initial quality (time 0) was lower than the quality of the control samples. Thus, after 4.5 h of incubation at 37 ºC, the CLC-treated samples had lost 2 and 7 percentage points of viable and total motile sperm, respectively, while the control samples had lost 9 and 14 percentage points for these parameters.

As expected, sperm quality decreased in a time-dependent manner when the frozen-thawed samples were incubated at 37 ºC, which is similar to what was observed in previous reports (Peláez et al., 2006). However, our data indicate that the frozen-thawed samples maintain their motility for at least 6 h and their viability for 24 h when they are stored at 16 ºC. Thus, after 26 h of storage, the samples contained approximately 55% viable sperm and 28% total motile sperm. Similar results were observed for frozen-thawed bull sperm (Underwood et al., 2009); however, to our knowledge, previous studies have not been performed with frozen-thawed boar sperm. Because the dilution of frozen-thawed boar semen is a pre-requisite for artificial insemination, the fact that the samples maintain their quality for at least 6 h could be interesting for the transport of thawed-diluted (or partially thawed-diluted) doses from the laboratory to the farm because the conditions for thawing and handling the straws are easier to control in the laboratory than on the farm.

Nevertheless, further studies are required to determine whether this storage period will affect other functionality parameters and/or the fertilizing ability of the sperm.

Cryopreservation induces capacitation-like changes in sperm (Bailey et al., 2000), several of which are likely due to a loss of membrane cholesterol. Additionally, one of the initial steps in sperm capacitation is a loss of cholesterol from the plasma membrane, after which a cascade of events takes place that allows the sperm to acrosome react and fertilize the oocyte (Cross, 1998). Some authors suggested that the capacitation interval is inversely proportional to the cholesterol content of the plasma membrane (for a review, see Davis, 1981). For this reason, we studied how CLC treatment affected capacitation dynamics. Because the sperm will have to reside in the female genital tract for several hours after insemination and before fertilization, control and CLC-treated samples were incubated in capacitating conditions for 24 h after thawing to simulate the in vivo conditions and to study the capacitation dynamics. The intracellular calcium level was chosen as a parameter that is indicative of the capacitation state at each time point, and it was measured using a fluorescence technique (Fluo-3-AM and PI) and flow cytometry (Harrison et al., 1993).

As expected, the percentage of viable cells with high intracellular calcium concentrations increased over time, which was similar to what was observed in previous studies (Hossain et al., 2011). At the same time, the percentage of dead sperm increased throughout the incubation. With respect to the CLC treatment, the control samples exhibited higher percentages of viable sperm with high intracellular calcium after 15 min of incubation. However, this difference may be too small to be physiologically relevant, especially considering that both types of sperm behaved similarly for the remaining incubation period. These results are in agreement with those reported for bull sperm (Purdy and Graham, 2004b), although the parameter studied was different (ability to acrosome

react after 3 h of incubation under capacitating conditions). Thus, it appears that this treatment does not counteract the damaging cryo-capacitation effect of the cryopreservation process in boars as well, and it is likely that additional damage is induced in the membrane during freeze-thawing that also contributes to the cryo-capacitated state (Purdy and Graham, 2004b).

Although the in vitro analyses attempt to mimic the conditions that the sperm will encounter in vivo, fertilization is a complex process in which the sperm are sequentially exposed to various environments along the female reproductive tract before they encounter and fertilize the oocytes (Rodriguez-Martinez, 2007). Once the sperm are deposited in the female genital tract, a functional sperm reservoir (SR) is established in the ampullary-isthmic junction (Rodriguez-Martinez, 2007), and this reservoir is markedly reduced when frozen-thawed sperm are used (Abad et al., 2007). The main function of the SR is to maintain a selected population of viable sperm for an extensive period of time (up to 36 h in pigs; Smith, 1998), which will better coordinate sperm capacitation at the time of ovulation (for a review, see Petrunkina et al., 2007). Thus, sperm capacitation in vivo is a gradual multi-step process that takes place during sperm transport along the female genital tract (Rodriguez-Martinez et al., 2001). The sperm that adhere to the oviductal epithelial cells of the SR are characterised by their intact acrosomes (Fazeli et al., 1999), low internal free calcium, reduced tyrosine phosphorylation of membrane proteins (Petrunkina et al., 2001) and normal chromatin structure (Ellington et al., 1999). The mechanisms involved in sperm release from oviductal epithelial cells are still unknown, although they appear to be related to an endocrine signal (Hunter, 1995) and coincide with the capacitation process and sperm hyperactivation (Hunter et al., 1998; Fazeli et al., 1999). Therefore, the ability of sperm to bind oviductal epithelial cells has great importance in vivo for establishing the

reservoir of sperm that will participate in the fertilization process; for this reason, we used an in vitro model based on the ability of frozen-thawed sperm to bind TERT-OPEC in vitro. Several authors demonstrated that these cells could be used to study spermatozoa-oviduct epithelial cell interactions because this cell line is not dedifferentiated and has not lost the expression of functional receptors (Hombach-Klonisch et al., 2006). Additionally, when these cells are incubated with sperm, the alteration in the expression of some genes ([1]*ADM*, *HSPA8* and *PGES*) is similar to what was described for primary oviductal epithelial cells (Aldarmahi et al., 2012). Sperm-oviductal epithelium binding assays evaluate the plasma membrane for multiple functions prior to the fertilization process (Waberski et al., 2005), and this in vitro binding test has proven very useful for the assessment of sperm quality given its high sensitivity for detecting differences (Petrunkina et al., 2007; De Pauw et al., 2002). In our study, treatment with CLC enhanced the ability of sperm to bind TERT-OPEC. Although the difference between CLC-treated and control samples (4.5 bound sperm) may not be physiologically relevant, this observation should not be downplayed based on the sensitivity of the test.

The results obtained in this experiment seem to contradict those obtained with the Fluo3-AM-PI technique, in which both types of sperm underwent capacitation in the same time frame, and they differ with respect to other studies that have shown a higher number of uncapacitated sperm bound to oviductal epithelial cells. This contradiction may have occurred in our study because the oviductal epithelial cells may possess a mechanism that prevents a premature increase in the intracellular calcium concentration, as demonstrated in a previous study (Dobrinski et al., 1996). In that study, sperm incubated for up to 6 h in the absence of oviductal epithelial cells exhibited higher intracellular calcium concentrations

[1]*ADM, Adrenomedullin; HSPA8, Heat-shock 70k-Da protein 8; PGES, Prostaglandin E synthase*

than the sperm attached to oviductal epithelial cells (Dobrinski et al., 1996), and the maintenance of low intracellular calcium during the co-incubation of sperm with these cells was attributable to direct membrane contact between the oviductal epithelial cells and the sperm (Dobrinski et al., 1997).

The difference between control and CLC-treated sperm with regard to their binding ability to TERT-OPEC in vitro could be due to the potential participation of cholesterol in the mechanism by which the oviductal epithelial cells prevent the premature capacitation of sperm stored in the SR. Cholesterol participates in the stabilisation of the sperm plasma membrane, and it could prevent the passive influx of calcium ions that takes place when cholesterol is removed from the membrane due to an increase in membrane fluidity and permeability (Cross, 1998). The maintenance of a low intracellular calcium concentration in sperm after binding to an oviductal epithelial cell and the delay in capacitation may explain the higher number of CLC-treated sperm bound to TERT-OPEC. Whether the difference we observed between treatments in vitro will have implications for fertility in vivo should be examined in future studies. Because cryopreservation markedly reduces the ability of sperm to establish a sperm reservoir in the oviduct (Abad et al., 2007), treatments that improve the association between sperm and oviductal epithelial cells could have a positive effect on the fertilizing ability of the sperm.

The freeze-thawing process induces extensive lipid peroxidation in sperm, which will ultimately lead to the functional impairment of proteins and DNA (Brouwers et al., 2011). The alterations induced in DNA in turn affect the early embryonic development (for a review, see Barbas and Mascarenhas, 2009). Because sperm nuclear fragmentation/degeneration negatively affects male fertility (Evenson et al., 2002), we also studied how treatment with CLC affected DNA fragmentation dynamics by incubating the

samples for 24 h in capacitating conditions in an attempt to partially mimic the conditions that the sperm will be subjected to once insemination occurs.

We observed a low proportion of sperm with fragmented DNA (< 1% DFI) after cryopreservation, which is in agreement with previous reports (Hernández et al., 2006). Indeed, a cut-off point of 2-7 % was established to differentiate between fertile and infertile samples of boar semen (Andrabi, 2007). The fragmentation index remained very low during all the incubations, which agrees with a recent study in which frozen-thawed boar sperm were incubated at 37 ºC for a shorter time period (4 h; Gosálvez et al., 2011). The small amount of fragmentation in response to incubation seems to be due to the absence of protamine 2 and the presence of 10 cysteine residues in protamine 1, which stabilise the chromatin structure in the spermatozoa (Gosálvez et al., 2011). However, the SD-DFI, which measures the extent of sperm DNA fragmentation, was also very low (approximately 20); this is also in agreement with previous reports for frozen-thawed boar sperm (Hernández et al., 2006). Additionally, treating the sperm with CLC prior to cryopreservation did not result in a higher DNA fragmentation index, which agrees with previous reports on equine epididymal sperm (Pamornsakda et al., 2011). Although the presence and formation of cholesterol oxidation products (oxysterols) has been recently demonstrated in bovine sperm, the freeze-thaw procedure did not cause an elevation of oxysterol levels (Brouwers et al., 2011). Therefore, it seems reasonable to conclude that both types of sperm presented similar DNA fragmentation dynamics, considering that oxysterol generation is disrupted following cryopreservation (for a review, see Aitken, 2011).

In conclusion, treating boar sperm with cholesterol-loaded cyclodextrin prior to cryopreservation does not modify sperm longevity when incubated at 37 or 16 ºC for up to

26 h, although CLC-treated sperm were able to maintain their quality parameters for longer when incubated at 37 ºC. Frozen-thawed sperm stored at 16 ºC maintain their quality parameters (motile sperm and sperm with intact plasma membranes) at acceptable values for at least 6 h, which could be beneficial for the transport of thawed-diluted doses from the laboratory to the farm. Additionally, this treatment did not alter the capacitation or DNA fragmentation dynamics in sperm incubated under capacitating conditions in vitro. However, this treatment enhanced the ability of the sperm to bind to TERT-OPEC in vitro, which could have an effect on the establishment of the sperm reservoir in the ampullary-isthmic junction in vivo. These results are encouraging and warrant further investigation; specifically, CLC-treated sperm should be used in fertility trials to assess the in vivo fertilizing potential of these spermatozoa.

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Chapter 5: Study 3

In vivo fertilizing ability of frozen-thawed boar sperm treated with cholesterol-loaded cyclodextrins prior to cryopreservation

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Abstract

The use of frozen-thawed (FT) sperm in artificial insemination in pigs is scarce. Treating the sperm from boars with cholesterol-loaded cyclodextrins (CLC) prior to cryopreservation enhanced the penetration ability of immature oocytes in vitro, and this parameter has been positively related to the in vivo fertilizing ability in this species. The aim of this study was to compare the in vivo fertilizing ability of boar sperm treated prior to freezing with 0 (control) or 1 mg of CLC/120 x 10^6 sperm (CLC). The fertilizing ability of FT sperm was compared in hormonally treated (eCG/hCG) weaned sows inseminated once (cervical insemination) at fixed-times after hCG injection: 37 h (experiment 1) or 30 h (experiment 2). In experiment 1, both treatments exhibited similar fertility (67.7 and 55.9% for control and CLC; $P > 0.05$) but CLC showed lower ($P < 0.05$) litter size (11.3 ± 0.9) than control sperm (13.6 ± 0.8). In experiment 2, pregnancy and farrowing rates (65.2 and 66.7% for control and CLC) and litter size (12.9 ± 1 and 11.3 ± 1 for control and CLC) were similar ($P > 0.05$) for both treatments. Moreover, the fertilizing ability of FT boar sperm was similar at both asynchronies (30 and 37 h). These results indicate that FT control and CLC-treated sperm present similar in vivo fertilizing ability at the asynchronies studied, and that the fertilizing ability of FT boar sperm do not decrease when they are inseminated at an asynchrony recommended for fresh sperm (30 h after hCG injection).

1. Introduction

Commercial use of frozen-thawed (FT) boar sperm is not a reality as in the case of dairy cattle. Unfortunately for the pig industry, the lowered fertility and the additional costs associated to the use of FT compared to diluted fresh semen, limit the use of FT sperm

basically to research. Nevertheless, the potential advantages associated to the use of FT semen are numerous, i.e. for reducing risk of disease, for gene transfer, for facilitating international sperm exchange and for preservation and regeneration of swine genetics (for a review, see Knox, 2011).

Unfortunately, the freezing-thawing process provokes also damages in the sperm that have been extensively studied and evidenced (for a review, see Bailey et al., 2000; Watson, 2000). Extensive research has been conducted over the years with the objective of enhancing the quality of FT boar sperm and of increasing the number of functional sperm available for fertilization. To achieve these objectives, several fronts have been tackled to try to include most of the factors that may have an impact on the fertilizing ability of FT sperm (male, female and artificial insemination techniques). The strategies have focused on improving the artificial insemination (AI) technique (Roca et al., 2003), establishing optimal timing between insemination and ovulation (Bolarín et al., 2006), developing new packaging systems for the sperm (Eriksson et al., 2001), optimizing the freezing-thawing protocols (Carvajal et al., 2004; Hernández et al., 2007) or modifying the composition of the freezing diluents to reduce the damage during the cryopreservation process (de Mercado et al., 2009; Garcia et al., 2010; Groβfeld et al., 2008; Yamaguchi et al., 2009). Although the advances towards increasing the fertilizing ability of FT sperm are remarkable, the use of this type of sperm commercially is still utopian. Unfortunately, the shortened lifespan FT sperm exhibits (for a review, see Holt, 2000) forces to use this type of sperm in the peri-ovulatory period in order to achieve high fertility levels (Bolarín et al., 2006; 2009). This is a handicap for the use of FT sperm in farms, since it requires of changes in the management schemes used by commercial farms and, as a consequence, cryopreserved sperm is not an attractive product.

The sperm plasma membrane appears to be one of the structures suffering extensive damage during the freezing-thawing process. Therefore, the alterations in the sperm plasma membrane structure and properties may be partly responsible for the decrease in the fertilizing ability of FT sperm (for a review, see Watson, 2000). Cholesterol plays important roles in both sperm cold-shock resistance as well as in sperm functionality and capacitation and for this reason it has been used in cryopreservation protocols for the sperm from several cold-shock sensitive species (reviewed by Mocé et al., 2010). In boar sperm, the addition of cholesterol-loaded cyclodextrins (CLC) prior to cryopreservation enhanced the penetration ability of immature homologous oocytes in vitro (Tomás et al., 2011) and increased the binding ability of the sperm to a porcine telomerase-immortalised oviductal epithelial cell line (TERT-OPEC) in vitro (Tomás et al., 2012). Nevertheless, the effect this treatment may have on the sperm fertilizing ability in vivo is still unknown.

The aim of this work was to study the effect of pre-freezing treatment of boar sperm with cholesterol-loaded cyclodextrins on their in vivo fertilizing ability when sows were inseminated at two different asynchronies after ovulation induction (30 or 37 h).

2. Materials and Methods

2.1. Reagents and media

All the chemicals were of analytical grade. Unless otherwise stated, all the media components were purchased from Sigma-Aldrich Quimica, S.A. (Madrid, Spain), and the media were prepared under sterile conditions (HH48, Holten LaminAir, Allerod, Denmark) using purified water (18.2 megohm-cm, Milli-Q water purification system, Millipore Co,

Billerica, MA). The fluorochromes were purchased from Invitrogen S.A. (Barcelona, Spain).

The basic medium used for the sperm extension was Beltsville Thawing Solution (BTS; Minitub Ibérica, S.L., Tarragona, Spain). The basic medium used for the sperm freezing was lactose-egg yolk extender (LEY), which was composed of 80% (v:v) β-lactose solution (310 mM in water) and 20% (v:v) egg yolk (pH = 6.2 and 330 ± 5 mOsm/kg; Westendorf et al., 1975).

2.2. Preparation of cholesterol-loaded cyclodextrins

Cholesterol-loaded cyclodextrins (CLC) were prepared according to the protocol developed by Purdy and Graham (2004), as described elsewhere (Tomás et al., 2011).

The working solution of the CLC crystals (50 mg/mL BTS) was freshly prepared each day that sperm were cryopreserved.

2.3. Animals

A total of 44 multiparous (2 to 8 parity) crossbred sows of proven fertility housed at the experimental farm at the "Centro de Tecnología Animal-Instituto Valenciano de Investigaciones Agrarias" (CITA-IVIA, Segorbe, Spain) were used in the experiments.

A total of 24 healthy mature Pietrain or crossbred (Landrace x Pietrain) boars of proven fertility housed at a commercial insemination station (CIAR, Centro de Inseminación, Peñarroya de Tastavins, Teruel, Spain), and a total of 3 fertile Pietrain boars housed at CITA-IVIA (Segorbe, Spain) were used as ejaculate donors.

The animals were housed in climate-controlled pens (15 to 25 °C), were fed with a commercial diet in accordance with the guidelines for the nutritional requirements for adult boars or sows and received water *ad libitum*. The protocols that were used fulfilled the European regulations for the care and use of animals for scientific purposes (EC Directive 2010/63/EU).

2.4. Collecting and handling of the ejaculates

The sperm-rich ejaculate fractions were collected using the gloved-hand method, and the fractions were extended (1:1, v:v) in BTS and evaluated for conventional semen characteristics.

The extracted semen from CIAR was transferred to 100 mL plastic tubes, cooled at 16 °C, packaged in insulated containers and sent by courier service to the sperm cryopreservation laboratory at the CITA-IVIA research centre. The extended semen arrived at the laboratory between 18 and 20 h after the ejaculate collection. When the ejaculates came from the CITA-IVIA farm, the extended semen was transported to the laboratory at 22 ºC in less than 30 min after collection, and it was maintained at 22 ºC for a minimum of 2 h and stored at 16 ºC for a maximum of 20 h before cryopreservation.

2.5. Sperm Cryopreservation

Immediately after reception, the concentration, motility and membrane integrity of the samples were evaluated. Only ejaculates containing greater than 75 to 80% motile sperm and sperm with an intact plasma membrane were selected for cryopreservation. The ejaculates from three different boars were mixed in equal sperm numbers to constitute a pool. Then, each pool of semen was divided into two aliquots. An aliquot served as a

control (non-treated) and the other one was incubated with 1 mg of CLC/120 x 10^6 sperm (Blanch et al., 2012) for 15 min at 16 °C.

The sperm samples were cryopreserved according to the protocol described by Westendorf et al. (1975). Briefly, the extended sperm rich fractions were centrifuged at 800 x g for 10 min at 16 °C and the supernatant, containing mostly seminal plasma, was removed by aspiration. The sperm pellet was suspended in the LEY extender to yield a concentration of 1.5 x 10^9 sperm/mL. After cooling to 5 °C within 120 min, the extended sperm were diluted (2:1, v:v) in the LEY-Glycerol-Orvus Es Paste extender [89.5% LEY, 9% glycerol (v:v) and 1.5% OEP (Equex STM; Nova Chemical Sales Inc., Scituate, MA, USA)] to a final concentration of 10^9 sperm/mL, 3% glycerol and 0.5% OEP. The cooled sperm was equilibrated for 15 min and packaged into 0.5 mL straws. The straws were frozen using a programmable freezer (Mini-Digitcool, IMV, Humeco, Huesca) in accordance to the protocol described by Carvajal et al. (2004) and they were stored in liquid nitrogen until used.

The straws were thawed in a water bath at 37 ºC for 30 s and then diluted in BTS (1:1, v:v; 37 ºC) before the analyses were performed.

2.6. Sperm assessments

The sperm concentration in each ejaculate was evaluated using an improved Neubauer chamber (Marienfield, Germany) in aliquots that were fixed with a solution of 0.25% glutaraldehyde in Dulbecco's phosphate-buffered saline (Pursel and Johnson, 1974).

The thawed sperm were incubated in a water bath at 37 ºC for 1.5 h. The analyses were performed at 0 h and at 1.5 h.

Motility was evaluated in samples diluted with BTS-BSA (6 mg/mL BSA) to a final concentration of 30 x 10^6 sperm/mL. The sperm motility was evaluated objectively using a computer-assisted sperm analysis system (ISAS®, Proiser R+D, Paterna, Spain) following a procedure previously described (Tomás et al., 2011). The percentages of total motile sperm (TMS; VAP > 10 µm/s) and rapid progressively motile sperm (RPMS; VAP > 50 µm/s and STR > 75%) were recorded.

The analysis of the integrity of the sperm plasma membrane was performed at room temperature under a dimmed light in an EPICS XL flow cytometer (Coulter Corporation Inc, Miami, FL, USA) equipped with standard optics and an argon ion laser (Cyonics, Coherent, Santa Clara, CA, USA) with 15 mW laser power at 488 nm and EXPO 2000 software (Coulter Corporation). Forward and side scatter were recorded in a linear mode for a total of 10,000 events per sample, and only sperm-specific events, which appeared in a typically L-shape scatter profile, were positively gated for the analysis. This parameter was evaluated by a double vital staining with propidium iodide (1 mg PI/mL in ultrapure water) and SYBR-14 (10 µM in DMSO) (LIVE/DEAD Sperm Viability Kit), in accordance with a protocol previously described (Tomás et al., 2011). Only the percentage of live sperm was considered in the results (SYBR-14 positive and PI negative).

2.7. Preparation of the insemination doses

To prepare each insemination dose of FT sperm, 12 straws per pool and treatment were thawed in a water bath at 37 ºC for 30 s in constant agitation. Then, the content of the straws was diluted slowly with pre-warmed (37 ºC) BTS to a final volume of 80 mL. Each insemination dose contained 6 x 10^9 sperm and the sperm were inseminated within 20 min of thawing.

2.8. Estrus synchronization, ovulation induction, insemination and pregnancy diagnosis

The estrus was induced in sows by IM injection of 1000 IU eCG (Folligon® 5000, Intervet International B.V., Madrid) 24 h after weaning followed 72 h later with im injection of 750 IU hCG (Veterin Corion® 3000, Divasa-Farmavic, Barcelona) to stimulate follicular development and induce ovulation (Roca et al., 2003). Sows were inseminated (cervical insemination) once at 30 or 37 h after hCG application, depending on the experiment. For the inseminations, standard foam tipped AI catheters (Minitub Ibérica, S.L., Tarragona, Spain) were used.

Pregnancy was determined by real-time ultrasonography (Agroscan A16, Humeco, Huesca, Spain) 28 days after insemination. All the pregnant sows were allowed to carry litters to term and farrowing rates and litter sizes were recorded.

2.9. Experimental design

The insemination doses were prepared and the artificial inseminations were performed in accordance to the protocols previously described. In each insemination session the sows were distributed in two groups: one group was inseminated with FT control sperm and the second group was inseminated with FT CLC-treated sperm. In order to minimize the effect of the sow as much as possible, they were rotated to be inseminated with each of the treatments whenever possible.

Experiment 1: Fertilizing ability of frozen-thawed sperm inseminated in weaned sows 37 h after hCG injection

A total of 37 sows were used in this experiment and 68 inseminations were performed over 16 sequential insemination sessions. In this experiment, sows were inseminated once 37 h after hCG application (Bolarín et al., 2009).

Experiment 2: Effect of CLC treatment on the fertilizing ability of frozen-thawed sperm inseminated in weaned sows 30 h after hCG injection

A total of 28 sows were used in this experiment and 46 inseminations were performed over nine sequential insemination sessions. In this experiment, sows were inseminated once 30 h after hCG application.

2.10. Statistical analyses

Statistical analyses were performed in SAS system version 9.0 (SAS Institute Inc., Cary, North Caroline, USA). The average values of semen quality for FT sperm were analyzed using PROC UNIVARIATE analyses. The data are showed as the mean and the standard deviation.

Data for litter size (live and total born) were analyzed using a mixed model analysis of variance (ANOVA), including the sow as random and the sperm treatment as fixed effects. When the ANOVA revealed a significant effect, the values were compared using the Tukey-Kramer test and were considered to be significant when $P < 0.05$. Results are presented as least-squares means (LSM) ± the standard error of the mean (SEM).

The pregnancy and the farrowing rates were analysed using the FREQ procedure, and the significance was determined using a chi-squared test at $P < 0.05$.

3. Results

The average sperm quality of FT insemination doses used in both experiments is summarized in Table 1.

Table 1. Quality of frozen-thawed (FT) sperm (control or treated with 1 mg of cholesterol-loaded cyclodextrins-CLC/120 x 10^6 sperm, prior to cryopreservation) used for the inseminations

Time	0 h				1.5 h*			
Treatment	FT control		FT treated-CLC		FT control		FT treated-CLC	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
% TMS	51.2	9.5	44.5	9.7	48.4	8.9	46.9	11.1
% RPMS	42.6	8.6	37.6	8.2	42.3	7.8	40.9	10.9
% Viable sperm	62.0	6.6	59.0	9.0	59.8	9.5	55.6	8.1

*Samples were incubated in a water bath at 37 ºC for 1.5 h. % TMS: percentage of total motile sperm; % RPMS: percentage of rapid progressively motile sperm; % Viable sperm: percentage of sperm presenting an intact plasma membrane (propidium iodide negative). The viable sperm were assessed with the double staining SYBR-14/propidium iodide and flow cytometry. Motility parameters were assessed by a CASA system. Values showed are the means and standard deviation (SD), (n = 17 pools of semen).

Experiment 1: Fertilizing ability of frozen-thawed sperm inseminated in weaned sows 37 h after hCG injection

The pregnancy and farrowing rates were similar between treatments (Table 2; $P > 0.05$) when sows were inseminated 37 h after hCG application. However, when the sows were inseminated with CLC-treated sperm the number of total piglets born dropped (-2 total piglets born; $P < 0.05$) compared to the litter size obtained with control semen. Nevertheless, the number of piglets born alive was similar ($P > 0.05$) between treatments.

Table 2. Fertility, farrowing rates and litter size (total and live piglets born) of weaned hormonally treated sows inseminated once with frozen-thawed sperm (control or treated with cholesterol-loaded cyclodextrins prior to cryopreservation) and conventional insemination (cervical) 37 h after hCG injection

Parameter	Frozen-thawed sperm	
	Control	CLC-treated
No. Inseminations	34	34
Pregnancy rate (%)	67.7 (23/34)	55.9 (19/34)
Abortion rate (%)	4.4 (1/23)	5.3 (1/19)
Farrowing rate (%)	64.7 (22/34)	52.9 (18/34)
Live born litter size (LSM ± SEM)	12.4 ± 0.8	10.6 ± 0.9
Total born litter size (LSM ± SEM)	13.6 ± 0.8[a]	11.3 ± 0.9[b]

CLC-treated: samples were treated with cholesterol-loaded cyclodextrins (1 mg/120 x 10^6 sperm) prior to cryopreservation. Values in the same row with different superscript (a, b) are significantly different ($P < 0.05$).

Experiment 2: Effect of CLC treatment on the fertilizing ability of frozen-thawed sperm inseminated in weaned sows 30 h after hCG injection

The pregnancy and farrowing rates as well as the litter size were similar between treatments when sows were inseminated 30 h after hCG injection (Table 3; $P > 0.05$). Surprisingly neither the fertility and farrowing rates nor the litter size decreased at this longer asynchrony for any of the sperm treatments, and even the fertility and farrowing rates increased for the CLC-treated sperm.

Table 3. Fertility, farrowing rates and litter size (total and live piglets born) of weaned hormonally treated sows inseminated once with frozen-thawed sperm (control or treated with cholesterol-loaded cyclodextrins prior to cryopreservation) and conventional insemination (cervical) 30 h after hCG injection

Parameter	Frozen-thawed sperm	
	Control	CLC-treated
No. Inseminations	23	23
Pregnancy rate (%)	65.2 (15/23)	65.2 (15/23)
Abortion rate (%)	0 (0/15)	0 (0/15)
Farrowing rate (%)	65.2 (15/23)	65.2 (15/23)
Live born litter size (LSM ± SEM)	11.3 ± 0.9	10.1 ± 0.9
Total born litter size (LSM ± SEM)	12.9 ± 1.0	11.3 ± 1.0

CLC-treated: samples were treated with cholesterol loaded cyclodextrins (1 mg/120 x 10^6 sperm) prior to cryopreservation.

4. Discussion

The optimal time of insemination depends on the lifespan of a sufficient number of fertile sperm to ensure fertilization, and on the speeds of sperm transport and of the sperm capacitation (for a review, see Johnson et al., 2000).

Precisely, some of the problems cryopreserved sperm present are their reduced lifespan and the capacitation-like state of their plasma membrane (Bailey et al., 2000; Green and Watson, 2001; Maxwell and Johnson, 1997), as a consequence of the structural and functional changes suffered by the membrane during the cryopreservation process.

Many studies have been conducted over the years with the objective of increasing the number of sperm surviving the cryopreservation process as well as the functionality of the FT sperm. The use of cholesterol-loaded cyclodextrins enhanced the survival of the sperm from different cold-shock sensitive species to the cryopreservation process (for a review, see Mocé et al., 2010). Although the response of boar sperm is variable between studies (in terms of motility parameters and of plasma membrane integrity), CLC-treated sperm penetrated more efficiently immature sows' oocytes in vitro than control sperm (Tomás et al., 2011). Since a positive correlation between the in vitro penetration rate and the in vivo boar sperm fertilizing ability has been observed (Martinez et al., 1998), it was likely that the CLC treatment could affect the in vivo fertilizing ability of the sperm as well.

Due to the alterations in the sperm functions, the FT-doses contain larger numbers of sperm than the liquid-stored doses and, in addition, cryopreserved sperm needs to be delivered within a short period of time before the ovulation time expected (Roca et al., 2011). These practices force to introduce modifications in the management schemes usually

followed and involve an increase in the cost of the insemination doses, resulting in a reduced use of FT sperm in the pig industry. Several strategies have been suggested with the objective of offering a product economically attractive, such as performing single inseminations in sows hormonally treated to synchronize the ovulation (for a review, see Roca et al., 2011).

The optimal insemination time for FT sperm when the weaned sows are treated with eCG-hCG and inseminated once has been established in 37 h after hCG injection (Bolarín et al., 2009). The results obtained in our study after one single cervical insemination with 6 x 10^9 FT sperm are similar to those reported in other studies (62 to 65% farrowing rate and 9.4 to 9.8 total born/litter) using weaned hormonally treated sows inseminated twice (cervical or post-cervical) after hCG injection with 5 or 7.5 x 10^9 FT sperm/dose (Casas et al., 2010; Garcia et al., 2010). Therefore, acceptable results were achieved in our study with FT sperm with single artificial inseminations, which involves savings in money and time.

Despite the higher in vitro fertilizing ability of CLC treated sperm (Tomás et al., 2011), this treatment did not improve the in vivo fertilizing ability of the FT-sperm when inseminated 37 h after hCG injection. These results follow the trend observed in other species for CLC-treated sperm where the fertility of this type of sperm was similar or lower to that exhibited by FT-control sperm (for a review, see Mocé et al., 2010). The reason why the sperm treated with CLC do not exhibit higher fertilizing ability than control sperm in vivo is not known. It would be tempting to assume that the lower percentage of motile sperm exhibited by CLC-treated samples immediately after thawing could explain this result. However, based on the fact that both types of sperm presented similar quality 1.5 h after incubation at 37 ºC, it seems unlikely that this lower motility at time 0 could justify

the behavior of FT CLC-treated sperm. Moreover, recent studies show that the pregnancy rate and the embryonic survival do not change when the sows are inseminated with FT-doses containing between 1 and 4 x 10^9 motile sperm (Spencer et al., 2010), numbers reached in our study with both types of sperm (between 2.67 and 3 x 10^9 motile sperm for CLC-treated and control sperm, respectively).

To our knowledge, all the fertility trials conducted up to date with CLC-treated sperm used insemination protocols developed for control sperm. However, some authors suggested that the CLC-treated sperm might have altered capacitation requirements before they can acrosome react in vivo (for a review, see Mocé et al., 2010).

Thus, when using protocols developed for FT-control sperm, the timing of insemination with respect to the expected time of ovulation could not be optimal for the CLC-treated sperm. In addition, apart from the low fertility rate of FT sperm compared with liquid-stored semen and the higher numbers of FT sperm required in the insemination doses (Roca et al., 2011), the modification in the timing of insemination is a handicap for the extensive use of cryopreserved sperm for many farms (Mocé et al., 2010). Thus, the insemination timings for fresh sperm and FT sperm should be as similar as possible when attempting the use of FT sperm in commercial farms. For this reason, in the second experiment we studied the fertilizing ability of FT sperm inseminated at the asynchrony recommended for fresh sperm (30 h after hCG injection).

At this asynchrony, the pregnancy and farrowing rates increased (9 and 12 percentage points, respectively) for the CLC-treated samples, compared to the values they exhibited with the asynchrony of 37 h. Therefore, FT CLC-treated sperm fertilize more efficiently when longer asynchronies are used. This would corroborate, at least partially,

that the use of insemination timings developed for FT-control sperm might be not adequate when CLC-treated sperm is used. However, it is still to be determined if intermediate or longer asynchronies will be more adequate for this type of sperm.

Unexpectedly, control samples were equally efficient in fertilizing at both asynchronies (30 and 37 h). Taking into account that with this treatment most ovulations occur (at least in winter) within a window of 40 to 42 h (Bolarín et al., 2009), and that FT sperm are functional for about 6 to 8 h (Waberski et al., 1994), with the 37 h asynchrony the presence of functional FT sperm is assured around the periovulatory period, when the fertility is higher (Bolarín et al., 2006; 2009). Nevertheless, based on the results we observed for the 30 h asynchrony with FT-control sperm, it seems that this type of sperm would be able to remain functional for longer in vivo (at least 10 or 12 h), at least in our work conditions (the doses were inseminated immediately after dilution with BTS).

In conclusion, our results show that it is possible to perform a unique fixed-time insemination with FT control sperm in weaned hormonally treated sows 30 h after hCG injection, without negatively affecting the overall fertility results. Moreover, the FT-treated CLC sperm benefits from the long asynchrony (30 h instead of 37 h), which could indicate that the use of insemination timings developed for FT-control sperm might be not adequate when CLC-treated sperm is used. Nevertheless, further studies should be conducted to determine if longer or intermediate asynchronies are more suitable for this type of sperm. Overall, the results are encouraging since the use of longer asynchronies for FT sperm will mean a better organization and management of insemination when these types of doses are used and would allow using timings similar to the liquid-stored semen, which nowadays is one of the handicaps hindering the use of cryopreserved sperm in the pig industry.

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Chapter 6

General Discussion

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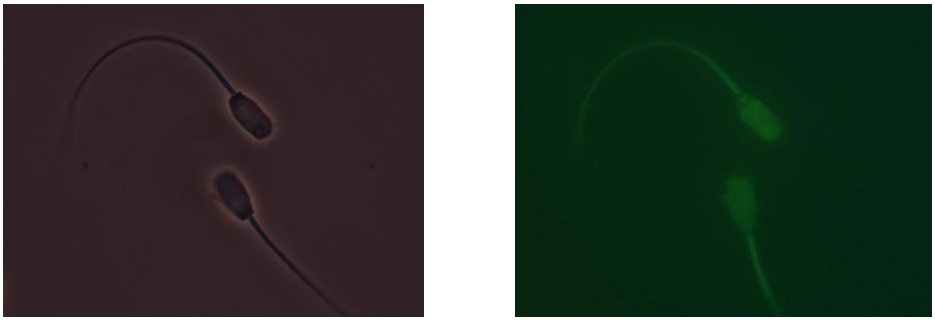
As it has been previously indicated, through the cryopreservation process several changes in the sperm occur that in the end will cause a decrease in the sperm functionality. The reduced fertility generally associated to the use of frozen-thawed (FT) boar sperm along with the cost of the doses make the use of this type of sperm unattractive for the farmers. Continued interest in boar FT semen is based on its success in the dairy industry (Knox, 2011), and for this reason extensive research has been conducted over the years with the objectives of enhancing the quality of FT boar sperm and of increasing the number of functional sperm available for fertilization.

During the process of cryopreservation the sperm membrane is the structure suffering the most intensive damage as a result of the different stresses they are subjected to (thermal and osmotic), and the greater or less survival will partially depend on the capacity of the membrane to adapt to these stresses. This adaptability has been linked to the composition of the membrane. Thus, those species presenting a high content of cholesterol in their sperm membranes, exhibit higher resistance to thermal and osmotic stresses.

This Thesis is based on the determination of the effect of the treatment of boar sperm with cholesterol-loaded cyclodextrins (CLC) prior to cryopreservation on the number of sperm surviving the cryopreservation process and the functionality and the fertilizing ability of the survivors.

1. Treating boar sperm with cholesterol-loaded cyclodextrins (CLC) increases the cholesterol content of the sperm plasma membrane

The incorporation of cholesterol to the sperm plasma membrane was proven by incubating the sperm with 1 mg CLC/120 x 10^6 sperm, in which 10% of the cholesterol loaded into the cyclodextrin was labeled with the fluorescent molecule 22- (N- (7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)- 23, 24- bisnor- 5- cholen- 3β- ol (NBD; Invitrogen S.A., Barcelona, Spain). When boar sperm were visualized under a fluorescent microscope, cholesterol could be visualized throughout the entire sperm although some membrane compartments appeared to be more heavily labeled (Figure 1). These results are similar to those previously observed in bull and stallion sperm (Purdy and Graham, 2004a; Moore et al., 2005).



A) B)

Figure 1. Boar sperm incubated with 1 mg of cholesterol-loaded cyclodextrins (CLC) per 120 x 10^6 sperm with 10% of the cholesterol loaded into the cyclodextrin labeled with NBD. A) negative phase contrast image (1000X) B) fluorescent image (1000X).

It was previously demonstrated that treating the sperm of different species (bull, stallion, ram and trout) with CLC increased 2 to 3 fold the content of cholesterol in their membranes, without detrimental effects on the sperm quality (Purdy and Graham, 2004a; Moore et al., 2005; Müller et al., 2008; Mocé et al., 2010). This effect was also observed in boar sperm, where CLC-treated sperm (1 mg CLC/120 x 10^6 sperm) presented higher cholesterol content in their membranes (up to 1.5 fold) than control sperm immediately after the treatment (unpublished results), after storage of the sperm at 16 °C for 24 h and after cryopreservation (Tomás et al., 2012). The opposite effect was observed when the boar sperm were treated with 1 and 6 mg methyl-β-cyclodextrin (MβCD) per 120 x 10^6 sperm, since this treatment reduced the content of cholesterol in membranes 0.9 and 0.7 fold, respectively (unpublished results).

Therefore, by increasing the cholesterol content of the boar sperm plasma membrane in this proportion, the cholesterol: phospholipid ratio should increase from 0.26 (Parks and Lynch, 1992) to 0.4, a value similar to that observed in other species with an intermediate ratio (0.45 in bull sperm; Darin-Bennett and White, 1977) and with higher resistance to cold-shock than boar sperm (Watson, 1981). Taking into account that the cholesterol: phospholipid ratio of the species showing the highest resistance to the cold shock is close to 1 (0.88-0.99; Darin-Bennett and White, 1977; Watson, 1981), it is reasonable to believe that boar sperm would achieve a greater tolerance to cold-shock by increasing the cholesterol to these values. In order to achieve these ratios, the cholesterol content in the boar plasma membrane should increase 3 to 4 fold. Nevertheless, treating boar sperm with concentrations above 1 mg CLC/120 x 10^6 resulted in a gradual decrease of the sperm quality after cryopreservation (Blanch et al., 2012). In addition, other authors observed that when the cholesterol increased 4 to 5 fold in bull sperm, a detrimental effect on the sperm

survival to the cryopreservation process was evident (Purdy and Graham, 2004a). It is therefore likely that the increase in the content of cholesterol above a certain value will affect the structural organization of the membrane, which will in turn modify its functionality.

2. Effect of the treatment with cholesterol-loaded cyclodextrins (CLC) on the resistance of the boar sperm membrane to osmotic stress

Cholesterol plays an important role in stabilizing membranes by their interaction with the fatty acyl chains of the phospholipids. An increase in the content of cholesterol in the membrane tends to make the membrane less fluid above the transition temperature for phospholipids (in boar at 24 ºC; Parks and Lynch, 1992), and below the transition temperature the cholesterol keeps the membrane in a fluid state by preventing the hydrocarbon fatty acyl chains of the membrane from binding to one another, thereby offsetting the drastic reduction in fluidity that would otherwise occur at low temperatures (Lodish et al., 2000).

In our study, when fresh boar sperm were treated with CLC (1 mg/120 x 10^6 sperm) they showed higher resistance to hypo- and hyper-osmotic conditions than sperm treated with MβCD or than control sperm (Chapter 3, Study 1). At least 45% of the CLC-treated sperm maintained the integrity of their plasma membrane when exposed to anisosmotic conditions (50, 75, 150, 600 and 800 mOsm/kg). In addition, a minimum of 40% progressively motile and a minimum of 50% total motile sperm were observed when CLC-treated sperm were exposed to 50, 75, 150 and 600 mOsm/kg. On the other hand, CLC-treated sperm exhibited higher resistance to hypo-osmotic conditions (50, 75, and 150 mOsm/kg) than to hyper-osmotic conditions (600, 800, 1200 mOsm/kg).

These results are similar to those observed in CLC-treated sperm of different species, such as stallion, trouts, ram, bull and rabbit (Glazar et al., 2009; Müller et al., 2008; Mocé et al., 2010; Moraes et al., 2010; Aksoy et al., 2010), including boar (Walters et al., 2008). Indeed, a positive correlation between the cholesterol content of the plasma membrane and the resistance of fresh sperm to hypo-osmotic stress was reported in trouts (Müller et al., 2008). Therefore, treating boar sperm with CLC improves the ability of the membrane to adapt to volume changes probably by decreasing the membrane permeability to water, as has been previously observed during cooling in bull sperm (Li et al., 2006).

Since one of the stresses the sperm are subjected to during the cryopreservation process is osmotic, this increment in the resistance of the membrane to anisosmotic conditions could increase the cryotolerance of boar sperm.

3. Effect of treatment with cholesterol-loaded cyclodextrins (CLC) on the motility and plasma membrane integrity of frozen-thawed boar sperm

Treating boar sperm prior to cryopreservation with CLC did not affect the quality of the sperm after freezing-thawing (Chapter 3, Study 1). Thus, CLC-treated sperm presented similar percentages of live sperm with an intact acrosome (65%), total motile (40%) and rapid progressively motile sperm (30%) than control samples or MβCD-treated sperm.

Ejaculates are composed by a mixture of sperm in different degree of capacitation to ensure a greater potential for fertilizing an oocyte (for a review, see Curry, 2000). Nevertheless, the summary statistics provided by the CASA system do not show the variability of the sperm comprising each sample, but just the average parameters. Thus, the evaluation of the subpopulations (they are a group of sperm within the ejaculate exhibiting

a similar motility pattern) in the samples give more information about their quality by studying the modifications in the frequencies of these subpopulations (for a review, see Martínez-Pastor et al., 2011). Therefore, two samples presenting similar percentages of motile sperm could have very different subpopulation's frequencies. In a preliminary study (Tomás et al., 2010), we observed the existence of three subpopulations in the FT samples based on three parameters provided by the CASA system (VAP, mean velocity (µm/s); LIN, linearity coefficient (%); WOB, wobble coefficient (%)). Although the characteristics of these subpopulations were similar for the control and the CLC-treated sperm, the frequency of these subpopulations was different between these treatments. Thus, the CLC-treated samples exhibited a lower proportion (-10%) of sperm in the subpopulation with characteristics indicative of the hyperactivation pattern, subpopulation that has been related to the capacitation state of sperm (Suarez, 1996) and is characterized by high VAP values (Abaigar et al., 1999).

On the other hand, under natural circumstances the sperm will have to reside in the female genital tract for a while before being able to fertilize the oocytes. After natural mating or conventional AI in pigs sperm are deposited in the cervix and once they traverse the uterus and reach the oviduct, part of these sperm become trapped within the distal portion of the isthmus (sperm reservoir, SR), in which they reside under protective conditions until ovulation (for a review, see Petrunkina et al., 2007).

For the fertilization success, it is necessary the presence of functional sperm in the oviduct at the exact time when the ovulation occurs. This synchronization between sperm and oocytes will depend on the time of insemination respect to the time of the ovulation, on the longevity of the sperm in the female genital tract, on the release of the sperm from the

SR and on the capacitation of the sperm in the optimal time. Since the longevity of the sperm is one of the factors influencing the fertilization success, the in vitro longevity of the sperm was evaluated over time (incubated at 37 ºC up to 24 h) in the Chapter 4 (Study 2).

Although initially the sperm treated with CLC showed lower percentages of live and motile sperm than control samples, these differences disappeared soon after the incubation started (30 min). In addition, the CLC-treated samples maintained their quality for longer (up to 6 h) than the control sperm (4.5 h of incubation). These results are similar to those observed in ram sperm (Mocé et al., 2010). Considering that the longevity of the samples in vitro reflects a greater likelihood of the sperm to survive in the female genital tract, undergo capacitation and fertilize an oocyte (Fiser et al., 1991), these results seem promising.

Likewise, the in vitro longevity of the FT sperm incubated at 16 ºC was evaluated, as a strategy for the storage of the doses after thawing. We observed that the quality of the samples remained in acceptable values up to 6 h at this temperature, irrespective of the treatment (55-60% of live sperm; 37-42% of total motile sperm; 29-31% of progressively motile sperm). This conservation would facilitate the handling of FT doses in the farm.

4. Effect of treatment with cholesterol-loaded cyclodextrins (CLC) on the structure and functionality in vitro of frozen-thawed boar sperm

Treating boar sperm with CLC prior to cryopreservation did not affect the percentages of total and progressively motile sperm or the integrity of the sperm plasma membrane immediately after thawing. Nevertheless, the incorporation of cholesterol produces a change in plasma membrane properties (Müller et al., 2008), and these changes could have some consequences on the sperm functionality.

To assess the structure and functionality sperm in vitro, several essential aspects of the sperm involved in the functionality have been evaluated in this Thesis.

4.1. Effect of cholesterol-loaded cyclodextrins (CLC) on the capacitation status and capacitation dynamics of frozen-thawed boar sperm

The molecular and physiological changes suffered by the sperm during their residence in the female genital tract and that ultimately confer them the ability to fertilize the oocytes are known as capacitation. Changes in the sperm motility patterns (Suarez, 1996), an increase in intracellular Ca^{2+} (involved in sperm signal transduction and the acrosome reaction; Visconti et al., 1998), and a disorganization of the lipids of the membrane (Flesch et al., 2001) have been described to occur during the capacitation process. These changes are initiated by the removal of part of the cholesterol from the sperm plasma membrane (Cross, 1998), which in turn produces changes in the membrane fluidity that will favor the changes suffered by the membrane during the capacitation process. As it has been previously indicated, the plasma membrane of the sperm after freezing-thawing has been altered in such a way that the sperm are in an advanced capacitation-like state (Collin et al., 2000; Green and Watson, 2001; Cormier and Bailey, 2003).

Therefore, CLC-treated sperm could present a lower number of capacitated sperm after freezing-thawing due to the increase in the cholesterol content of their membrane, and hopefully the number of sperm able to keep their characteristics for longer would increase in these samples. Nevertheless, this increase in the cholesterol content could also have some consequences in the capacitation dynamics of the sperm, taking into account the differences observed in the capacitation interval between species presenting different

cholesterol: phospholipid ratio in their sperm membranes (Davis, 1981). If this was the case and the CLC-treated samples presented a retarded capacitation, then the AI timing could not be adequate for these samples and it could be advisable its modification.

For the evaluation of the capacitation status after cryopreservation, we chose two different parameters indicative of the capacitation state: plasma membrane fluidity (M540) and intracellular calcium concentration (Fluo-3-AM).

Neither CLC nor MβCD treatments modified the percentages of viable sperm with unstable plasma membrane or the percentage of viable sperm with high intracellular calcium concentration, compared to the control samples (Chapter 3, Study 1). These results are similar to those reported by other authors in different species (Purdy and Graham, 2004b; Spizziri et al., 2010).

The reason membrane fluidity was not modified by CLC treatment (as a priori is expected) is not known. It may be that the sperm cells exhibit a plateau of their membrane fluidity (Müller et al., 2008) or that the average membrane fluidity of a sperm population may not change while those of individual sperm or even different compartments within each sperm do (Purdy et al., 2005).

However, after insemination the sperm have to reside in the female genital tract for several hours before fertilization and they have to maintain their function and to capacitate at the right time to be synchronized with the oocytes. Thus, we studied also the capacitation dynamics by means of Fluo-3-AM (Chapter 4, Study 2) in sperm incubated up to 24 h under capacitating conditions after freezing-thawing in an attempt to partially simulate the in vivo conditions.

The sperm pre-treated with CLC exhibited a lower percentage of sperm with high intracellular calcium concentration and a higher percentage of dead sperm after 15 min of incubation than control sperm, although for the rest of the incubation the values were similar between treatments. Therefore, although the pre-treatment of boar sperm with CLC showed a lower percentage of viable sperm with high intracellular calcium concentration at 15 min after thawing, this treatment did not modify the capacitation dynamics over time.

Similarly, the percentage of sperm with high intracellular calcium concentrations was not modified by the CLC-treatment in bulls and stallions either (Purdy and Graham, 2004b; Spizziri et al., 2010). It is possible that there is additional damage to sperm membranes, as a result of cryopreservation, inducing the cryo-capacitated state that added cholesterol does not counteract (Purdy and Graham, 2004b).

4.2. Effect of cholesterol-loaded cyclodextrins (CLC) on the ability of frozen-thawed boar sperm to adhere to oviductal epithelial cells in vitro

Of the several millions of sperm normally ejaculated or inseminated, only a small proportion becomes trapped within in the isthmus, in a storage site denominated ‘sperm reservoir’ (Hunter, 1981). This SR selects the fertilizing-competent sperm population, modulates sperm capacitation and regulates sperm transport to minimize polyspermic fertilization. On the other hand, cryopreservation alters the ability of the sperm to interact normally with the structures of the female genital tract, as fewer cryopreserved sperm attach to the oviductal epithelial cells (OPEC) than fresh sperm (for a review, see Bailey et al., 2000). Therefore, the ability of the sperm to bind to the OPEC in vitro could be related to the ability of the sperm to form the oviductal SR. This type of in vitro binding test has a

high sensitivity for detecting differences (Petrunkina et al., 2007; De Pauw et al., 2002), and for this reason we decided to use this test as one of the functional assays (Chapter 4, Study 2).

The sperm pre-treated with CLC showed a higher number of sperm attached per oviductal epithelial cell (+5) than the control sperm, which means that 26% more CLC-treated sperm attached per OPEC, compared to the control sperm. This difference may not seem physiologically important, although treatments helping to improve the association between the sperm and the OPEC could exert a positive influence in the fertilizing ability of the sperm through the establishment of an appropriate SR.

In addition, the sperm attached to oviductal epithelial cells are characterized by their uncapacitated state (Fazeli et al., 1999), and binding occurs preferentially to viable cells with low intracellular calcium (Petrunkina et al., 2001), so this test in vitro may indirectly evaluate the capacitation state of the sperm. According to this argument, the membrane of sperm pre-treated with CLC would be less capacitated than the control sperm. This difference was found at 15 min after thawing, where the CLC-treated sperm showed a lower proportion of viable sperm with high intracellular calcium concentration (-0.8%), which means 9% less CLC-treated sperm in this population compared to the control sperm. This slight difference in the capacitation status could explain the slight difference observed between treatments in the number of sperm attached per oviductal epithelial cell.

In addition, it has been observed that the direct contact of the sperm membrane with the oviductal epithelial cells is responsible for the prevention of a premature increase in intracellular calcium concentration (Dobrinski et al., 1996), and therefore of the capacitation process.

4.3. Effect of cholesterol-loaded cyclodextrins (CLC) on the DNA integrity dynamics of frozen-thawed boar sperm

Normal sperm genetic material is required for successful fertilization and for normal embryo and fetal development. On the other hand, the sperm forming the SR are those showing normal chromatin structure (Ellington et al., 1999).

The freeze-thaw process induces also extensive lipid peroxidation and the products released during the oxidative stress damage the sperm chromatin (Brouwers et al., 2011). Cholesterol can be oxidized and some of the oxysterol species are toxic (Brouwers et al., 2011). For these reasons, we studied the effect of CLC treatment on the DNA integrity dynamics of boar sperm after cryopreservation (Chapter 4, Study 3).

Our results show a low DNA fragmentation index in this species, similarly to the results reported by different authors (Hernández et al., 2006). In addition, the integrity of the DNA did not worsen when the sperm were incubated in capacitating conditions at 37 ºC over time (up to 24 h), similarly to previous studies (Gosalvez et al., 2011). On the other hand, the sperm treatment had no effect on this parameter probably due to the lack of oxysterols formation after freezing/thawing as has been recently reported (Brouwers et al., 2011).

5. Effect of treatment with cholesterol-loaded cyclodextrins (CLC) on the fertilizing ability of frozen-thawed boar sperm

All the changes the sperm suffer during their residence in the female genital tract have the aim of preparing the sperm to fertilize the oocytes. Therefore, after sperm capacitation the sperm will be enabled to penetrate and fertilize the oocyte.

The fertilizing ability of the sperm may be studied in vitro or in vivo.

5.1. Effect of cholesterol-loaded cyclodextrins (CLC) on the ability of frozen-thawed boar sperm to penetrate immature oocytes in vitro

There are different methods to assess the fertilizing ability of the sperm in vitro, which are more affordable and economical than in vivo AI trials. In this work, we have used a homologous in vitro penetration test with immature pig oocytes (Martinez et al., 1993) to evaluate the in vitro fertilizing ability of FT sperm (control, MβCD- and CLC-treated sperm; Chapter 3, Study 1).

The CLC-treated sperm showed higher penetration rate (+17%) and number of sperm per penetrated oocyte (+2), than control or MβCD-treated sperm. The results obtained with this in vitro assay have been correlated with the fertility that the samples present in vivo, and for this reason this assay is a good tool to assess the sperm fertilizing ability of the doses (Martinez et al., 1998).

5.2. Effect of cholesterol-loaded cyclodextrins (CLC) on the fertilizing ability of frozen-thawed boar sperm in vivo

From the results observed in all the in vitro assays we conducted we did not expect that the capacitation timing of CLC-treated sperm would be very different from that of control sperm, therefore we could use a similar AI timing for both types of sperm. With respect to the fertilizing ability, we expected that the CLC-treated sperm would present an enhanced fertilizing ability compared to the control sperm, taking into account the results obtained in the in vitro penetration assay.

Thus, we conducted a trial in vivo using sows of proven fertility (Chapter 5, Study 3). The weaned sows were hormonally treated with eCG-hCG to synchronize and induce the ovulation. In a first trial, the sows were inseminated once 37 h after hCG injection, which is the timing of insemination recommended for FT doses (Bolarín et al., 2009). The results we obtained with FT sperm with this asynchrony are within the values reported by other authors in similar conditions but performing two AIs (Casas et al., 2010; Garcia et al., 2010). However, CLC-treated sperm presented lower farrowing rate (-12%; $P > 0.05$) and total piglets born (-2; $P < 0.05$) than the control sperm, results that were unexpected. The reason why this happened is unknown, but some authors suggested that cholesterol treatment might modify certain sperm domains, but not those important for sperm fertilization (Müller et al., 2008). Nevertheless, others suggested that the CLC-treated sperm might have altered capacitation requirements before they can acrosome react in vivo (Spizziri et al., 2010). Although the in vitro assays try to mimic the in vivo conditions, they study only a few of the steps required for the sperm to be fertile but none of them evaluates

the whole process (the interaction of the sperm with all the structures of the female genital tract until fertilization).

The optimal timing of insemination will ensure that functional sperm are present in the moment of ovulation and therefore it is determinant in the fertilization success. Up to date, all the studies conducted with CLC-treated sperm in different species used the asynchronies recommended for control sperm, and it is likely that CLC-treated sperm will have different requirements to undergo the capacitation process or to acquire the ability to fertilize an oocyte (Spizziri et al., 2010). On the other hand, the changes in the management of the females associated to the use of FT sperm are one of the handicaps inhibiting the extensive use of this type of semen. Therefore, one of the goals for FT semen is the use of AI timings as close as possible to those used for fresh semen. For this reason, the timing of insemination was reduced 7 h (30 h after hCG injection) in a second trial, which is an insemination timing recommended for fresh semen (Roca, personal communication).

At this insemination timing the farrowing rate of CLC-treated sperm improved respect to the first trial (from 53% to 65%), matching the fertility results obtained with control sperm which surprisingly maintained the values obtained in the first trial. These results suggest that the CLC-treated sperm might need different AI timings, and not those developed for the control sperm.

Considering that the ovulation occurs in 40 to 42 h after hCG injection (Bolarín et al., 2009) and that the FT sperm can be functional between 6 to 8 h once inseminated (Waberski et al., 1994; Wongtawan et al., 2006), then when the inseminations are carried out 37 h after hCG injection, the sperm should remain functional for 3 to 5 h after insemination. However, when the insemination timing is 30 h after hCG injection, the

sperm will have to maintain their functionality for 10 to 12 h in order to fertilize the oocytes. Since the FT control sperm was equally efficient in fertilizing in both trials and the CLC-treated sperm was more efficient at 30 than at 37 h of asynchrony, it means that FT sperm are able to maintain their functionality for at least 10 to 12 h after insemination, at least in our working conditions.

Although some of the differences observed in the in vitro assays between control and CLC-treated sperm did not seem very relevant at first, the use of this treatment results in sperm with different functionality in vivo. These small differences observed in vitro (lower proportion of sperm with a motility pattern indicative of hyperactivation and a possible lesser degree of capacitation state determined by a higher adherence to OPEC) could finally be important in the sperm functionality in vivo and could explain the improvement in the fertility results observed for CLC-treated sperm when they were inseminated 7 h earlier. Nevertheless these arguments should be carefully considered because the control sperm maintained their fertilizing ability when the insemination timing was reduced in 7 h, and different insemination timings should be tested in the future to determine if CLC-treated sperm will fertilize better at other timings.

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Chapter 7

Conclusions

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The conclusions obtained in this Thesis are detailed next:

1. The treatment of boar sperm with cholesterol-loaded cyclodextrins (CLC; 1 mg/120 x 10^6 sperm) widens the sperm osmotic tolerance limits. At least 45% of the fresh sperm treated with CLC maintained the integrity of their plasma membrane and the motility when exposed to anisosmotic conditions (50, 75, 150, 600 mOsm/kg).

2. The pre-freezing treatment of boar sperm with CLC did not have any effect on the percentages of the sperm surviving the process of cryopreservation neither after thawing nor in long term incubation. Although initially the CLC-treated sperm presented lower percentages of live and of motile sperm, these parameters equaled to those presented by the control samples over time. In addition, CLC-treated sperm maintained their quality for longer (+1.5 h) than control sperm during the incubation at 37 ºC. On the other hand, the frozen-thawed sperm doses can be stored at 16 ºC for at least 6 h without affecting these quality parameters and this could be useful for the transport of the doses from the laboratories or insemination centers to the farms.

3. The pre-freezing treatment of boar sperm with CLC did not modify the capacitation status and did not alter the capacitation dynamics. This treatment did not have any negative effect on the chromatin integrity either. However, this treatment improved the ability of frozen-thawed sperm to adhere to oviductal epithelial cells in vitro (+5 numbers of attached sperm/cell).

4. The pre-freezing treatment of boar sperm with CLC did enhance the ability of frozen-thawed sperm to penetrate immature oocytes in vitro (+17 penetration rate and +2 numbers of sperm per penetrated oocyte).

5. The pre-freezing treatment of boar sperm with CLC did not improve the fertilizing ability (farrowing rate and number of total piglets born) of the frozen-thawed sperm in vivo for the insemination timings used. Nevertheless, by decreasing the hCG injection-insemination interval (from 37 to 30 h) the farrowing rate of the CLC-treated sperm improved (+12% farrowing rate), although the difference in the total number of piglets born (2 piglets) remained between treatments. In addition, the control samples were equally efficient to fertilize the oocytes at both insemination timings. These results might show that CLC-treated sperm may require more capacitation time in the female reproductive tract and that weaned sows hormonally synchronized (eCG-hCG) can be inseminated once with frozen-thawed sperm 30 h after hCG injection without detrimental effects on the fertilizing ability.

Appendix: media

Beltsville thawing solution (BTS; Johnson et al., 1988)

- 205 mM glucose
- 20.39 mM NaCl
- 5.4 mM KCl
- 15.01 mM $NaHCO_3$
- 3.35 mM EDTA
- 50 µg/mL kanamycin sulphate
- pH = 7.2, 290 ± 5 mOsm/kg

Lactose-egg yolk extender (LEY; Westendorf et al., 1975)

- 80% (v:v) β-lactose solution (310 mM in water)
- 20% (v:v) egg yolk
- pH = 6.2, 330 ± 5 mOsm/kg

LEY-Glycerol-Orvus ES Paste

- 89.5% (v:v) lactose-egg yolk extender
- 1.5% (v:v) Orvus ES Paste
- 9% (v:v) glycerol

Phosphate-buffered saline (PBS)

- 137 mM NaCl
- 2.7 mM KCl
- 0.86 mM Na2HPO4

- 6.4 mM Na2HPO4·7H2O
- pH = 6.8, 292 ± 2.2 mOsm/kg

Modified Tris-buffered medium (mTBM; Caballero et al., 2009)

- 131.1 mM NaCl
- 3 mM KCL
- 7.5 mM $CaCl_2 \cdot 2H_2O$
- 20 mM Tris
- 11 mM glucose
- 5 mM sodium pyruvate
- 1 mM caffeine
- 0.2% BSA (fraction V)
- pH = 7.4, 299 ± 1.6 mOsm/kg
- Equilibrate the medium in the dark at 39 ºC under a modified atmosphere containing 5% CO_2 and 100% humidity for 15 min before use.

Saline medium (Harrison et al., 1993)

- 137 mM NaCl
- 10 mM glucose
- 2.5 mM KOH
- 20 mM Hepes
- 1 mg/mL polyvinyl alcohol (PVA)
- 1 mg/mL polyvinylpirrolidone (PVP)
- Adjust pH to 7.5 with NaOH

Sucrose medium (Harrison et al., 1993)

- 220 mM sucrose
- 10 mM NaCl
- 10 mM glucose
- 2.5 mM KOH
- 20 mM Hepes
- 0.5 mg/mL polyvinyl alcohol (PVA)
- 0.5 mg/mL polyvinylpirrolidone (PVP)
- Adjust pH to 7.5 with NaOH

NaCl solution

- 0.9% NaCl (w:v)
- 70 µg/mL kanamycin sulphate
- pH = 7.2-7.4, 280-300 mOsm/kg

Modified Dulbecco's phosphate-buffered saline (mDPBS)

- Dulbecco's phosphate-buffered
- 4 mg/mL BSA (fraction V)
- 0.34 mM sodium pyruvate
- 5.5 mM D-glucose
- 70 µg/mL kanamycin sulphate
- pH = 7.2-7.4, 280-300 mOsm/kg

Modified M-199 (mTCM-199)

- M-199 with Earle's salts and 26.19 mM sodium hydrogen carbonate
- 12% heated foetal calf serum (inactivated at 56 ºC for 30 min)
- 0.91 mM sodium pyruvate
- 3.05 mM D-glucose
- 2.92 mM calcium lactate
- 50 UI/mL penicillin-G
- 30 µg/mL streptomycin sulphate
- pH = 7.8, 280-300 mOsm/kg

In vitro fertilization medium (IVF-TCM199)

- mTCM-199
- 2 mM caffeine
- 5.84 mM calcium lactate
- pH = 7.4, 280-300 mOsm/kg

Oocyte fixative solution

- Acetic acid: ethanol (1:3, v:v)

Staining solution for fixed oocytes

- 1% (w:v) lacmoid diluted with 45% (v:v) acetic acid in ultrapure water

Tyrode's albumin lactate pyruvate medium (mTALP; Rath et al., 1999 with slight modifications by Silvestre et al., 2007)

- 114.06 mM NaCl
- 3.2 mM KCl
- 8 mM calcium lactate·$6H_2O$

- 0.5 mM $MgCl_2 \cdot 6H_2O$
- 0.5 mM $NaH_2PO_4 \cdot H_2O$
- 25.07 mM $NaHCO_3$
- 1.85 mL/L sodium lactate (60%)
- 1.1 mM sodium pyruvate
- 5 mM glucose
- 2 mM caffeine
- 0.3 % (w:v) BSA
- 0.1% (w:v) polyvinyl alcohol
- 0.2% (v:v) phenol red solution (0.5 %, w:v)
- 0.25% (v:v) antibiotic solution (14 mM streptomycin and 84 mM penicillin-G)
- pH = 7, 275-300 mOsm/kg
- Equilibrate the medium in the dark at 39 ºC under a modified atmosphere containing 5% CO_2 and 100% humidity for 15 min before use.

Dulbecco's modified Eagle's Medium/Ham's F-12 Nutrient Mixture supplemented (sDMEM/F-12)

- Dulbecco's modified Eagle's Medium/Ham's F-12 Nutrient Mixture
- 1% (v:v) L-glutamine
- 1% (v:v) antibiotic-antimycotic solution
- 0.004% (v:v) insulin
- 10% heat inactivated foetal bovine serum
- 0.1% (w:v) 17-β-oestradiol

Tyrode's albumin lactate pyruvate supplemented (TALPs; Fazeli et al., 1999)

- 2 mM $CaCl_2$
- 3.1 mM KCl
- 0.4 mM $MgCl_2 \cdot 6H_2O$
- 100 mM NaCl
- 25 mM $NaHCO_3$
- 0.3 mM $NaH_2PO_4 \cdot 2H_2O$
- 10 mM Hepes
- 21.6 mM sodium lactate
- 1 mM sodium pyruvate
- 1.2% (w:v) bovine serum albumin
- 1% (v:v) antibiotic-antimycotic
- 10% (v:v) foetal bovine serum

TNE buffer (Evenson et al., 1994)

- 0.01 M Tris HCl
- 0.15 M NaCl
- 1 mM EDTA disodium salt
- pH = 7.4

Acid solution (Evenson et al., 1994)

- 0.08 N HCl
- 0.15 M NaCl
- 0.1% (v:v) Triton X-100
- pH = 1.2

Acridine orange staining solution (AO-solution; Evenson et al., 1994)

- 0.15 M NaCl
- 0.126 M Na_2HPO_4
- 0.011 M EDTA disodium salt
- 0.037 M citric acid
- 6 µg/mL acridine orange
- pH = 6