DEPARTAMENTO DE CIENCIA ANIMAL

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Treating boar sperm with cholesterol-loaded cyclodextrins or cyclodextrins prior to cryopreservation:

Effects on post-thaw in vitro sperm quality of sperm cryopreserved in different freezing extenders

(Efecto del tratamiento de los espermatozoides de verraco con ciclodextrinas saturadas de colesterol sobre la calidad del semen crioconservado con distintos crioprotectores)

Doctoral Thesis

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Solo si nos detenemos a pensar en las pequeñas cosas

llegaremos a comprender las grandes

José Saramago

Llegará un momento en que creas que todo ha

terminado, ese será el principio

Epicuro

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ABBREVIATION KEY

A	acetamide
AI	artificial insemination
ATP	adenosine triphosphate.
BSA	bovine serum albumin
BTS	Beltsville thawing solution
CASA	computer-assisted sperm analysis system
CITA-IVIA	Centro de Tecnología Animal-Instituto Valenciano de Investigaciones Agrarias
CLC	cholesterol-loaded cyclodextrin
DOGV	Diari oficial de la Generalitat Valenciana
DMA	dimethylacetamide
DMF	dimethylformamide
EG	egg yolk
F	formamide
FE-1	basic freezing extender
FE-2	basic freezing extender with OEP and glycerol

IVF-TCM199	co-incubation medium
L	lactamide
LDL	low density lipoproteins
LEY	lactose egg yolk
LEYGO	lactose egg yolk with OEP and glycerol
LSM	least square means
MA	methylacetamide
MBCD	methyl-β-cyclodextrin
mDPBS	modified Dulbecco´s phosphate-buffered saline
MF	methylformamide
MMP	Mitochondrial membrane potential
mTCM-199	modified M-199 with Earle´s salts and sodium hydrogen carbonate
MTDR	Mitotracker Deep Red 633
MW	molecular weights
OEP	orvus es paste
PBS	phosphate-buffered saline
PI	propidium iodide

RPM	rapid progressively motile sperm
SE	standard error
STR	straightness index
SV	sperm with intact plasma membrane (sperm viability)
TMS	total motile sperm
VAP	average path velocity

ABSTRACT

Cryopreserved boar sperm is not used extensively for artificial insemination due to poor fertility rates of the sperm after freezing and thawing. The sperm membrane is damaged when cooled from body temperature to 5 ºC (cold shock), as well as during the freeze-thaw process. Increasing the cholesterol content of boar sperm membranes could increase their post-thaw survival, similarly to other species that are cold shock sensitive. Cholesterol can be easily added to sperm membranes using cholesterol-loaded cyclodextrins (CLC). Treating sperm from different species susceptible to cold-shock with CLC before cryopreservation improves sperm cryosurvival. Egg yolk and glycerol are common constituents of extenders used for boar sperm cryopreservation. However, conventional freezing extenders could not be the appropriate for CLC-treated sperm.

The aim of this Thesis is to evaluate cryosurvival of CLC or cyclodextrin-treated boar sperm in three different conditions: using conventional freezing extenders, using extenders with alternative concentrations of glycerol and egg yolk and using amides as cryoprotectants.

CLC or methyl- β-cyclodextrin treatment (1 mg/120 x 10^6 sperm) prior to cryopreservation using a conventional freezing extenders provided either slight or no benefit, respectively, to post-thaw sperm plasma membrane integrity (+ 8%; $P < 0.05$) and motility ($P > 0.05$). In addition, sperm from both, good and poor freezers, responded similarly to CLC treatment ($P > 0.05$).

Reduction in egg yolk concentration from 20 to 10% was detrimental for post-thaw sperm viability, even in semen treated with CLC (- 12%; $P < 0.05$). On the other hand, it was observed that traditional concentration of glycerol (3%) was not the appropriate to

freeze CLC-treated sperm (- 13% viable sperm compared to control; $P < 0.05$). Thus, CLC-treated sperm showed a higher tolerance (+ 13 % sperm viability; $P < 0.05$) to high glycerol concentrations (5%) than non-treated sperm.

Regarding the efficacy of amides as cryoprotectants, three of the amides (lactamide, acetamide and formamide) produced deleterious effects in fresh boar sperm ($P < 0.05$). The other amides (methylformamide, dimethylacetamide and dimethylformamide) efficiently improved post-thaw sperm viability (+ 5 to 15 %; $P < 0.05$) but negatively affected the sperm motility (- 11 to 16% total motile sperm; $P < 0.05$) and the sperm fertilizing ability in vitro (dimethylformamide: - 64 % penetration rate; $P < 0.05$), irrespective of the sperm treatment. On the other hand, CLC-treated samples showed better in vitro fertilizing ability than control samples when glycerol was used as cryoprotectant (+ 2 penetrated spermatozoa/oocyte; $P < 0.05$).

The results obtained in this Thesis suggest that conventional freezing protocols should be optimized for CLC-treated boar sperm in order to obtain the benefit of CLC treatment observed in other species sensitive to cold shock.

RESUMEN

Las inseminaciones artificiales en la especie porcina se realizan habitualmente con semen refrigerado, debido a las bajas tasas de fertilidad obtenidas con el semen congelado-descongelado. La membrana del espermatozoide sufre importantes daños cuando es sometida a la fase de enfriamiento desde la temperatura corporal hasta alcanzar los 5 ºC (choque térmico), así como durante el proceso de congelación y descongelación. El aumento del contenido de colesterol en las membranas de los espermatozoides de cerdo podría mejorar su supervivencia tras la descongelación, como sucede en otras especies sensibles al choque térmico. Este incremento en la cantidad de colesterol se puede realizar fácilmente utilizando ciclodextrinas saturadas de colesterol (CLC). El tratamiento con CLC de espermatozoides de varias especies susceptibles al choque térmico antes de la congelación ha conseguido mejorar su supervivencia tras la descongelación. En los protocolos convencionales de congelación de semen porcino se utilizan habitualmente diluyentes de congelación compuestos por yema de huevo y glicerol, sin embargo, puede que estos diluyentes de congelación convencionales no sean los más apropiados para congelar espermatozoides tratados con CLC.

El objetivo de esta Tesis es evaluar la supervivencia a la congelación de los espermatozoides porcinos tratados con CLC o ciclodextrinas utilizando diluyentes de congelación convencionales, utilizando concentraciones alternativas tanto de yema de huevo como de glicerol o utilizando amidas en lugar de glicerol como crioprotectores

Utilizando diluyentes convencionales, el tratamiento con 1mg de CLC o de metil-β-ciclodextrina/120 millones de espermatozoides previamente a la congelación proporcionó una leve mejora de la integridad de la membrana plasmática espermática (+ 8%; $P < 0,05$) y ningún beneficio sobre la movilidad espermática ($P > 0,05$). Además, la respuesta al

tratamiento con CLC fue similar independientemente de si los espermatozoides procedían de verracos buenos o malos congeladores ($P > 0,05$).

Una reducción de la concentración de yema de huevo de un 20 a un 10% fue perjudicial para la supervivencia de los espermatozoides tras la descongelación, incluidos aquellos que habían sido tratados previamente con CLC (- 12% espermatozoides vivos; $P < 0,05$). Por otro lado, observamos que las concentraciones de glicerol utilizadas habitualmente (3%) no son las más apropiadas para congelar espermatozoides tratados con CLC (- 13 % viabilidad espermática comparando con las muestras control; $P < 0,05$), ya que éstos mostraron una mayor tolerancia (+ 13 % espermatozoides vivos; $P < 0,05$) que las muestras control a las concentraciones de glicerol más altas (5%).

Con respecto a la eficacia de las amidas como crioprotectores para semen porcino, tres de las amidas (lactamida, acetamida y formamida) produjeron efectos perjudiciales durante su incubación con semen fresco ($P < 0,05$). El resto de amidas evaluadas (metilformamida, dimetilacetamida y dimetilformamida) mejoraron eficientemente la viabilidad espermática tras la congelación (+ 5 a 15 %; $P < 0,05$), sin embargo, afectaron negativamente tanto la movilidad espermática (- 11 a 16% móviles totales; $P < 0,05$) como la capacidad de fecundación in vitro (dimetilformamida: - 64 % en la tasa de penetración; $P < 0,05$), independientemente de si el semen fue tratado con CLC o no. Por otro lado, las muestras tratadas con CLC mostraron mejor capacidad de fecundación in vitro que las muestras control cuando se utilizó el glicerol como crioprotector (+ 2 espermatozoides penetrados/ovocito; $P < 0,05$).

Los resultados obtenidos en esta Tesis sugieren que sería necesaria la adecuación de los protocolos de congelación convencionales para congelar semen porcino tratado con CLC con el propósito de alcanzar los claros beneficios obtenidos con dicho tratamiento cuando ha sido evaluado en otras especies sensibles al choque térmico.

RESUM

Les inseminacions artificials en l'espècie porcina es realitzen habitualment amb semen refrigerat, a causa de les baixes taxes de fertilitat obtingudes amb el semen congelat-descongelat. La membrana de l'espermatozoide pateix importants danys quan és sotmesa a la fase de refredament des de la temperatura corporal fins a arribar als 5 ºC (xoc tèrmic), així com durant el procés de congelació i descongelació. L'augment del contingut de colesterol a les membranes dels espermatozoides de porc podria millorar la seva supervivència després de la descongelació, com succeeix en altres espècies sensibles al xoc tèrmic. Aquest increment en la quantitat de colesterol es pot realitzar fàcilment utilitzant ciclodextrines saturades de colesterol (CLC). El tractament amb CLC d'espermatozoides de diverses espècies susceptibles al xoc tèrmic abans de la congelació ha aconseguit millorar la seva supervivència després de la descongelació. En els protocols convencionals de congelació de semen porcí s'utilitzen habitualment diluents de congelació compostos per rovell d'ou i glicerol, però, pot ser que aquests diluents de congelació convencionals no siguin els més apropiats per congelar espermatozoides tractats amb CLC.

L'objectiu d'aquesta Tesi és avaluar la supervivència a la congelació dels espermatozoides porcins tractats amb CLC o ciclodextrines utilitzant diluents de congelació convencionals, utilitzant concentracions alternatives tant de rovell d'ou com de glicerol o utilitzant amides en lloc de glicerol com crioprotectors

Utilitzant diluents convencionals, el tractament amb 1 mg de CLC o de metil-β-ciclodextrina / 120 milions d'espermatozoides prèviament a la congelació va proporcionar una lleu millora de la integritat de la membrana plasmàtica espermàtica (+ 8%; $P < 0,05$) i cap benefici sobre la mobilitat espermàtica ($P > 0,05$). A més, la resposta al tractament amb

CLC va ser similar independentment de si els espermatozoides procedien de verros bons o dolents congeladors (P> 0,05).

Una reducció de la concentració de rovell d'ou d'un 20 a un 10% va ser perjudicial per a la supervivència dels espermatozoides després de la descongelació, inclosos aquells que havien estat tractats prèviament amb CLC (- el 12% espermatozoides vius; P <0,05) . D'altra banda, observem que les concentracions de glicerol utilitzades habitualment (3%) no són les més apropiades per congelar espermatozoides tractats amb CLC (- 13% viabilitat espermàtica comparant amb les mostres control; P <0,05), ja que aquests van mostrar una major tolerància (+ 13% espermatozoides vius; P <0,05) que les mostres control a les concentracions de glicerol més altes (5%).

Pel que fa a l'eficàcia de les amides com crioprotectors per semen porcí, tres de les amides (lactamida, acetamida i formamida) van produir efectes perjudicials durant la seva incubació amb semen fresc (P <0,05). La resta de amides avaluades (metilformamida, dimetilacetamida i dimetilformamida) van millorar eficientment la viabilitat espermàtica després de la congelació (+ 5 a 15%, P <0,05), però, van afectar negativament tant la mobilitat espermàtica (- 11 a 16% mòbils totals; P <0,05) com la capacitat de fecundació in vitro (dimetilformamida: - el 64% en la taxa de penetració; P <0,05), independentment de si el semen va ser tractat amb CLC o no. D'altra banda, les mostres tractades amb CLC van mostrar millor capacitat de fecundació in vitro que les mostres control quan es va utilitzar el glicerol com crioprotector (+ 2 espermatozous penetrats / oòcit; P <0,05).

Els resultats obtinguts en aquesta Tesi suggereixen que seria necessària l'adequació dels protocols de congelació convencionals per congelar semen porcí tractat amb CLC amb el propòsit d'assolir els clars beneficis obtinguts amb el tractament quan ha estat avaluat en altres espècies sensibles al xoc tèrmic.

CHAPTER 1

GENERAL INTRODUCTION

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1. USE OF FROZEN-THAWED SPERM IN PIG PRODUCTION

Pig production is the first Spanish livestock sector with an annual output of more than 6 billion Euros, being Spain the second largest producer in the EU-28 (Ministerio de Agricultura, Alimentación y Medio Ambiente, 2014).

The use of artificial insemination (AI) presents many advantages, including dissemination of genetic material of selected boars and continuous assessment of their reproductive value, improvement of sanitary status of farms, control of reproductive outcomes and reduction in the number of boars (Gadea, 2004). The use of AI is widespread in the pig industry of many European countries, including Spain. Between 80 and 95% of the sows are artificially inseminated in Europe, with Spain reaching a 95% (Riesenbeck, 2011). Indeed, Spain is the second producer of insemination doses worldwide, with a production of 12.5 million insemination doses per year (Echegaray, 2013).

AI can be performed with fresh, refrigerated or frozen-thawed semen. The use of frozen-thawed sperm offers additional benefits. It is an effective method for long-term storage of important genetic material and eliminates the difficulties associated with animal or fresh-semen transport over long distances or extended periods of time. Moreover, the use of frozen-thawed sperm could reduce the risk of disease transmission associated to boar and liquid semen, since the storage of frozen-thawed sperm provides additional time for routine and supplementary tests, and extended observation of boars before shipment (Knox, 2011). However, fresh-refrigerated (15-20 ºC) semen remains the more common AI method in pigs (Johnson et al., 2000) because cryopreservation compromises the fertilizing ability of boar sperm. Indeed, it has been estimated that cryopreservation decreases farrowing rates around 25% and reduces litter size in 2 piglets per litter (Tomás, 2012). However, this

tendency is changing and promising results have been achieved with frozen-thawed sperm, similar to those obtained with fresh semen (Roca et al., 2011). Nevertheless, the cost of AI with frozen-thawed sperm is between 1.3 to 2.4 fold higher than with fresh semen (Tomás, 2012) which represents an important handicap for the commercial use of frozen-thawed doses.

1.1. Stress conditions during the cryopreservation process: cold shock and osmotic stress

Cryopreservation process induces thermal and osmotic stress to the sperm, influencing its viability and fertility. As a result, about 50% of the sperm does not survive the freezing-thawing process and survivors remain with sublethal dysfunctions (Watson, 2000). These dysfunctions reduce sperm longevity and fertilizing ability (Roca et al., 2011).

1.1.1. Cold shock

Cryopreservation protocols include potential harmful steps for sperm cells, such as cooling to 5 ºC, cryoprotectant addition, freezing, thawing and dilution for AI. Changes in sperm volume and temperature alter the properties of the sperm plasmatic membrane.

When sperm are cooled from body temperature to 5 ºC their plasmatic membrane suffers a thermotropic lipid phase transition. The organization of membrane phospholipids, proteins and cholesterol changes and the membrane switches from a liquid-crystalline state to a gel phase conformation (Drobnis et al., 1993). In the liquid-crystalline state, the lipid bilayer is relatively fluid and allows relative freedom for lateral and rotational movement of proteins and lipids within a membrane domain. However, in the gel phase, there is increased order in the phospholipid hydrocarbon chains which decreases membrane fluidity and reduces the mobility of individual membrane components (Parks, 1997). In addition,

the membrane undergoes phase transition over a wide range of temperature, since the different families of phospholipids undergo the phase transition at different temperatures. Hence, when a membrane is cooled to the temperature at which lipids with the highest transition temperature undergo phase transition into the gel state, they will do so and coalesce into micro-domains of "gel" membrane in the otherwise fluid membrane (Hammerstedt et al., 1990). When these lipids are removed from the fluid part of the membrane, changes in lipid-lipid and lipid-protein interactions occur, altering membrane permeability and function (Amann and Pickett, 1987). Further temperature reductions will eventually provoke the entire membrane will undergo the transition into the gel state, but this membrane may possess very different lipid-lipid and lipid-protein interactions compared to the native membrane (Hammerstedt et al., 1990). This process is called cold shock.

Phospholipid and cholesterol composition determine sperm sensitivity to cold shock. Boar sperm membrane has a low ratio of cholesterol: phospholipid compared to other species (Park and Lynch, 1992). Because of this specific composition, the transitions from the liquid to the gel phase during cooling occur at higher temperatures compared to other species (Parks and Lynch, 1992; Drobnis et al., 1993; White, 1993; Brouwers et al., 2005). Thus, boar sperm are very sensitive to cold shock. However, the membrane damage provoked by thermal shock during the cooling phase from 16 ºC to 5 ºC could be diminished by incubating the sperm with seminal plasma prior to cryopreservation (Pursel et al., 1973; Maxwell and Johnson, 1999; Vadnais et al., 2005) and by adding saccharides (such as lactose) and lipids (egg yolk; White, 1993; Buhr et al., 2000) to the freezing extender.

1.1.2. Osmotic stress

The reduction in membrane fluidity associated to low temperatures and the rapid osmotic changes occurring during the freezing and thawing procedures difficult the active transport of molecules across the sperm membrane (ATP-dependent transport). Thus, diffusion and osmosis processes are predominant during freezing and thawing. Diffusion is the process in which molecules tend to reach a homogeneous distribution in the space, and depends on the concentration of solutes and membrane characteristics (thickness and permeability). Osmosis is a special case of diffusion, refering to water movement from low to high concentrated solutions (Ávila-Portillo et al., 2006).

Osmotic stress is one of the main factors causing damage to sperm during cryopreservation. Sperm suffer volume changes associated to addition of cryoprotectants and to freezing and thawing of extracellular water. This osmotic response is lethal when the cell surpasses its osmotic tolerance limits. The first volume adjustment occurs in response to addition of cryoprotectant. Sperm experience an initial fast shrinkage associated with osmotically driven egress of intracellular water, followed by a slower return to the original volume as the penetrating cryoprotectant enter. The second volume adjustment occurs when the extracellular water freezes. Then, an outward movement of water occurs in response to high concentration of extracellular salts resulting from freezing of extracellular water (Hammerstedt et al., 1990). Thawing involves a reversal of these effects, and the consequent inward water flux may cause cell membrane disruption (Holt, 2000). This process is influenced by plasmatic membrane permeability, type and concentration of cryoprotectant and freezing and thawing rates.

1.2. New strategies to improve the quality of frozen-thawed boar sperm

In the past decades a number of studies have aimed to improve the quality and fertilizing ability of frozen-thawed boar sperm. The strategies followed to achieve this objective have been different.

Some authors tried to optimize the freezing-thawing protocols by modifying the freezing extenders (He et al., 2001; Roca et al., 2003; de Mercado et al., 2009), developing new packaging systems (Eriksson and Rodríguez-Martínez, 2000) or optimizing freezing and thawing velocities (Thurston et al., 2003; Hernández et al., 2007).

Some others focused their efforts in optimizing the artificial insemination technique. Consequently, insemination timings (Bolarin et al., 2006; Larsson, 1976; Waberski et al., 1994), and deposition sites (Roca et al., 2002; Bolarin et al., 2006) have been optimized for cryopreserved sperm.

Finally, other studies took into account interindividual differences on sperm resistance to cryopreservation (Holt, 2000), and classified the boars according to the cryopreservation capacity of their sperm, identifying "good" and "bad" boar freezers (Watson, 1995; Thurston et al., 2001; Hernández et al., 2006; Vilagran et al., 2015).

2. ADDING CHOLESTEROL AS AN STRATEGY TO IMPROVE THE QUALITY OF FROZEN-THAWED SPERM

2.1. Effect of cholesterol

The amount of cholesterol in a membrane influences its thermotropic behavior (Johnson et al., 2000). Cholesterol intercalates into the lipophilic core of the membrane, associates with the fatty acyl chains of the phospholipids and inhibits interactions among fatty acids

when the membrane is cooled (Parks, 1997). Therefore, cholesterol prevents the transition of membrane lipids to the gel state at that lipid's phase transition temperature and the membrane remains fluid at temperatures below those that normally would cause a membrane phase transition (de Meyer and Smit, 2009). In that fluid state, the redistribution of membrane components, which causes membrane damage, does not occur. Both the ratio cholesterol: phospholipids and the amount of polyunsaturated fatty acyl chains composing the phospholipids determine the overall fluidity of a membrane (Amann and Pickett, 1987). Cholesterol: phospholipid ratio of the sperm plasma membrane is different between species and determines the resistance of the sperm to the cold shock. Thus, sperm from species with low ratio (boar, stallion, ram, bulls, goat) are susceptible to cold shock while sperm from species with high ratio (rabbits, human) are not (Darin-Bennet and White, 1977; Parks and Lynch, 1992). In model membranes, increasing the cholesterol:phospholipid ratio broadens the lipid phase transition, and reduces membrane leakage and phase separations (reviewed by Drobnis et al. 1993). Therefore, treating sperm with cholesterol before cryopreservation could reduce the sensitivity of sperm membranes to cooling damage by eliminating or minimizing the lateral phase separation of the lipids (Watson, 1981). Cholesterol, a major structural constituent of the membrane, plays an important role as a regulator of membrane function. It reduces membrane permeability, contributes to membrane morphology, enables cell–cell interactions, influences the membrane phase transition, provides suitable microenvironments for membrane-associated proteins and serves as a membrane antioxidant (reviewed by Crockett, 1998). Altogether, cholesterol is one of the principal regulators of membrane fluidity and permeability (Zeng and Terada, 2000).

Cholesterol can be easily added to sperm membranes by using cholesterol-loaded cyclodextrins (CLC). Cyclodextrins are cyclic oligosaccharides obtained by enzymatic degradation of starch. They possess an external hydrophilic face and an internal hydrophobic core that can encapsulate hydrophobic compounds such as cholesterol. These molecules are very efficient stimulating cholesterol removal from membranes of many cell types. In addition, if they are pre-loaded with cholesterol, they can insert cholesterol into cell membranes (Mocé et al., 2010a).

2.2. CLC pre-freezing treatment of sperm

Treating sperm with CLC before cryopreservation improves sperm cryosurvival in cold shock-sensitive domestic species (in stallion, Combes et al., 2000; Moraes et al., 2015; in bull, Mocé and Graham, 2006; Moraes et al., 2010; in donkey, Alvarez et al., 2006; Oliveira et al., 2014; in ram, Mocé et al., 2010b; Naseer et al., 2015; in goat, Konyali et al., 2013). Moreover, CLC treatment improves sperm cryosurvival in wild mammalian species and in fish (carp, Yildiz et al., 2015; dromedary camel, Crichton et al., 2015; bison, Hussain et al., 2013; Asian elephant, Kiso et al., 2012). CLC increases the cholesterol content 2-3 fold in bull, trout, ram and stallion sperm. This additional cholesterol would raise cholesterol: phospholipid ratios to levels similar to those of species not sensitive to cold shock (> 0.8). Generally speaking, treating sperm with CLC prior to cryopreservation improves sperm cryosurvival rates when a single sperm parameter or both motility and viability are assessed. These improvements can be minuscule, as low as two percentage points, or can be as high as 24 percentage points, however most of the studies report increased survival rates of 10-20 percentage points (reviewed by Mocé et al., 2010a).

Additionally, the osmotic tolerance limits of boar, stallion, trout, ram, bull and rabbit sperm are widened when sperm is treated with CLC (Tomás et al., 2011, Glazar et al., 2009; Müller et al., 2008; Moce et al., 2010a; Moraes et al., 2010; Aksoy et al., 2010).

2.3. Treatment of boar sperm with CLC

Boar sperm membranes have low cholesterol content and are very sensitive to cold shock, thus CLC treatment would be expected to benefit boar sperm cryosurvival. Nevertheless, the results of different studies relating the response of boar sperm to CLC treatment are contradictory. Some authors observed that boar sperm cryosurvival was improved by cyclodextrin-alone treatment (treatment that induces cholesterol depletion; Zeng and Terada, 2000) while others reported that treatment with cyclodextrins and cholesterol enhanced cold shock resistance or freezing survival rate (Galantino-Hommer et al, 2006; Bailey et al., 2008; Torres et al., 2009; Lee et al., 2015). Moreover, treating boar sperm with CLC widened the osmotic resistance limits of the fresh sperm and improved the ability of frozen-thawed sperm to adhere to oviductal epithelial cells and penetrate immature oocytes in vitro (Tomás et al., 2011; 2013).

3. CRYOPROTECTANT REQUIREMENTS FOR CLC-TREATED BOAR SPERM

3.1. Cryoprotectants

A cryoprotectant is a molecule that allows a substantial percentage of sperm to survive a freeze-thaw cycle and retain fertilizing capacity (Amann, 1999). Cryoprotectants are

included in cryopreservation extenders to reduce the physical and chemical stresses derived from freezing and thawing sperm cells (Barbas and Mascarenhas, 2009). Cryoprotectants are classified as either penetrating or non- penetrating, according to their ability to cross the plasmatic membrane. Both types can cause a dehydration of the cell by osmotically-induced water egress, but they differ in their capacity to enter the cell and reside in cytoplasm and the membranes (Hammerstedt et al., 1990).

Penetrating cryoprotectants are molecules with low molecular weight that pass through the sperm plasma membrane, and act both intra- and extracellularly. Penetrating cryoprotectants serve as a solute causing osmotic flow of water and as a solvent miscible with water by dissolving salts and sugars present in the cryopreservation extender. The solute role is beneficial through a twofold effect: an extracellular effect consisting of osmotic stimulation of cell dehydration, which decreases the volume of intracellular water available for freezing, and an intracellular effect exerted through its ability to permeate the cell membrane, thus decreasing the intracellular osmotic stress caused by the dehydration (Medeiros et al., 2002). The solvent role is beneficial because penetrating cryoprotectants have a freezing point much lower than water. When extended semen is frozen, crystals of pure water freeze and form “ice blocks” with unfrozen solvent among them containing solutes and sperm. In the presence of permeating cryoprotectants, there is a greater portion of the solvent mixture remaining unfrozen at any given temperature and more space available for the sperm and a lower concentration of solutes in channels of unfrozen solvent. Additionally, penetrating cryoprotectants cause membrane lipid and protein rearrangement, inducing an increase in membrane fluidity (Amann, 1999; Barbas and Mascarenhas, 2009). Permeating cryoprotectants can be toxic and induce membrane damage and decreases in sperm motility depending on their concentration (Medeiros et al.,

2002). The most common penetrating cryoprotectants are glycerol, alcohols (ethylene glycol, propylene glycol), dimethyl sulfoxide and amides.

On the other hand, non-penetrating cryoprotectants are molecules with high molecular weight which do not cross plasma membrane and act only extracellularly (Barbas and Mascarenhas, 2009). They likely modify the plasma membrane by stabilizing membrane phospholipids, rendering a membrane more resistant to temperature-induced damage. Alternatively, they could exert a simple action as a solute, lowering the freezing point of the medium and decreasing extracellular ice formation (Amann, 1999, Fernández-Santos et al., 2007). The most common non-penetrating cryoprotectants are egg yolk, non-fat skimmed milk, sugars, dextrans and methyl cellulose, and are typically present at high concentrations.

3.2. Glycerol

Glycerol is a small polyhydroxylated solute that was discovered to be an effective cryoprotectant for sperm cryopreservation in 1949 (Polge et al., 1949). Glycerol is highly soluble in water because of its ability to form hydrogen bonds with water molecules, and can penetrate across the plasma membrane (Rodríguez-Martínez and Wallgren, 2011). Then, glycerol acts as a penetrating cryoprotectant, decreasing the freezing point and lowering electrolyte concentrations. Glycerol is the most frequent cryoprotectant in boar sperm cryopreservation. Since glycerol disturbs cell metabolism at body temperature, boar sperm are usually exposed to this cryoprotectant at 5 ºC, which, unfortunately, further slows membrane permeation, and glycerol penetrates across the plasma membrane at a low rate (Rodríguez-Martínez and Wallgren, 2011). Then, despite its benefits, glycerol is also cytotoxic causing osmotic stress due to its low permeability through the membrane,

attributed to its high molecular weight (Guthrie et al., 2002). Moreover, glycerol may have a direct effect on the plasma membrane by binding directly to phospholipid head groups, inducing changes in lipid packing structure and hence altering the stability and permeability of the cell membrane (Holt, 2000; Parks and Graham, 1992).

3.3. Alternative cryoprotectants for the cryopreservation of CLC-treated boar sperm

Cholesterol addition to sperm likely alters membrane structure and permeability (Mocé and Graham, 2006). Then, cryopreservation protocols typically used for boar sperm could not be the most appropriate ones for freezing CLC-treated sperm, since treating sperm with CLC modifies membrane permeability to water and cryoprotectants (Li et al., 2006; Glazar et al., 2009). The rate of water movement through the membranes is affected by lipid composition and the cryoprotectant transport can be expected to proceed at a somewhat slower rate than for water, but with similar dependence on membrane structure (Hammerstedt et al., 1990). These changes in membrane composition could alter glycerol requirements for boar sperm treated with CLC.

On the other hand, amides have low molecular weights compared to glycerol and for this reason they readily penetrate the plasma membrane and induce less osmotic damage (Alvarenga et al., 2005). These compounds have been used as alternative cryoprotectants for semen of various species (Alvarenga et al., 2005; Forero-Gonzalez et al., 2012; Futino et al., 2012), including boars. Thus, some studies have investigated the effect of acetamide, dimethylacetamide, methylformamide and dimethylformamide on boar semen cryopreservation (Wilmut and Polge, 1977; Bianchi et al., 2008; Kim et al., 2011; Buranaamnuay et al., 2011; Malo et al., 2012) but the findings have been contradictory.

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CHAPTER 2

OBJECTIVES

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The general aim of this Thesis was to determine if treating boar spermatozoa with cholesterol-loaded cyclodextrin prior to cryopreservation in different freezing extenders improved post-thaw sperm cryosurvival. The aim was itemized into the following specific objectives:

1. To evaluate the response of boar sperm to the treatment with cholesterol-loaded cyclodextrins or cyclodextrins alone using a conventional freezing extender.

2. To evaluate the effect of glycerol and egg yolk concentrations on the cryosurvival of boar sperm treated with cholesterol-loaded cyclodextrins or cyclodextrins alone.

3. To evaluate the effect of amides on the cryosurvival and in vitro fertilizing ability of boar sperm treated with cholesterol-loaded cyclodextrins or cyclodextrins alone.

CHAPTER 3

STUDY 1

Response of boar sperm to the treatment with cholesterol-loaded cyclodextrins added prior to cryopreservation

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ABSTRACT

Cryopreserved boar sperm is not used extensively for artificial insemination, due to the poor fertility rates of the sperm after freezing and thawing. The sperm membrane is damaged as the cells are cooled from body temperature to 5 ºC (cold shock), as well as during the freeze-thaw process. Increasing the cholesterol content of boar sperm membranes could help them survive cryopreservation, similar to sperm from other species that are cold shock sensitive. The aim of this study was to determine the optimal cholesterol-loaded cyclodextrin (CLC) concentration to use for boar sperm cryopreservation, and the influence of CLCs on the cryosurvival of sperm from boars classified as good or poor freezers. Treating boar sperm with 1 mg of CLC/120 x 10^6 sperm slightly improved ($P < 0.05$) the percentage of viable sperm after freezing-thawing. On the other hand, sperm, from both good and poor freezers, responded similarly to CLC treatment. Nevertheless, additional studies will be needed to study the effect of this treatment on other parameters of sperm quality.

1. INTRODUCTION

Although great genetic gain can be achieved using frozen semen from only the best sires, frozen-thawed boar semen is not routinely used by the industry. The reason for this is that our current cryopreservation technologies for boar sperm do not yield sufficient or reliable fertility rates to be economical for commercial swine production (Bailey et al. 2008). Although cryopreservation can cause damage to different sperm attributes, much of that damage is a result of plasma membrane destabilization that occurs during cooling and freezing (Hammerstedt et al. 1990). When sperm are cooled from body temperature to 5ºC

the organization of membrane phospholipids, proteins and cholesterol is modified when the membrane undergoes the thermotropic lipid phase transition from a liquid-crystalline to a gel phase (Drobnis et al. 1993). In the liquid-crystalline state, the lipid bilayer is relatively fluid which allows proteins and lipids relative freedom for lateral and rotational movement within a membrane domain. In the gel phase, however, there is increased order in the phospholipid hydrocarbon chains which decreases membrane fluidity and reduces the mobility of individual membrane components (Parks 1997). In addition, the membrane undergoes the phase transition over a wide temperature range, since the different phospholipid families undergo the phase transition at different temperatures. What this means, is that when a membrane is cooled to the temperature at which the lipids with the highest transition temperature undergo a phase transition into the gel state, they will do so and coalesce into microdomains of 'gel' membrane in the otherwise fluid membrane (Hammerstedt et al. 1990). As these lipids are removed from the fluid membrane, changes in lipid-lipid and lipid-protein interactions occur which alter membrane permeability and function (Amann and Pickett 1987). As temperature is reduced further, eventually the entire membrane will undergo the transition into the gel state, but this membrane may possess very different lipid-lipid and lipid-protein interactions than the native membrane (Hammerstedt et al. 1990).

The amount of cholesterol in a membrane influences its thermotropic behavior (Johnson et al. 2000). Cholesterol intercalates into the lipophilic core of the membrane, associating with the fatty acyl chains of the phospholipids and inhibits fatty acid interactions with other fatty acids when the membrane is cooled (Parks 1997). Cholesterol, therefore, prevents lipids from undergoing the phase transition to gel state at that lipid's phase transition temperature and the membrane remains fluid at temperatures below what

would normally cause a membrane phase transition (de Meyer and Smit 2009). In that fluid state, the redistribution of membrane components, which cause membrane damage, does not occur. Therefore, sperm from species that contain higher membrane cholesterol:phospholipid ratios possess fluid membranes at low temperatures and as such these membranes do not exhibit damage at such temperatures, making these sperm resistant to 'cold shock' (Darin-Bennett and White 1977). Boar sperm plasma membranes are more sensitive to cold shock than sperm from many other species, since they have a very low cholesterol: phospholipid ratio, that results in their membranes undergoing the phase transition at relatively higher temperatures during cooling, making them very susceptible to damage during cooling (Parks and Lynch 1992).

Cyclodextrins can encapsulate hydrophobic compounds, such as cholesterol, and transfer the cholesterol into membranes down a concentration gradient (Zidovetzki and Levitan 2007). Treating sperm, from several cold-shock susceptible species, with cholesterol-loaded cyclodextrins (CLC) prior to cryopreservation improves sperm cryosurvival rates (reviewed by Mocé et al. 2010a). Since boar sperm membranes have low cholesterol content and are very sensitive to cold shock, it seems probable that increasing the cholesterol content of boar sperm, using CLC technology, should benefit boar sperm cryosurvival.

The aim of this study is to determine if treating boar sperm with CLC prior to cryopreservation would improve post-thaw sperm survival; and to study the influence of CLCs on the cryosurvival of sperm from boars classified as good or poor freezers.

2. MATERIALS AND METHODS

2.1. Reagents and media

All chemicals were reagent grade and purchased from Sigma-Aldrich (Madrid, Spain), except for SYBR-14 and Propidium Iodide (PI), which were purchased from Invitrogen (Barcelona, Spain) and Orvus Es Paste (OEP; Equex STM) which was purchased from Nova Chemical Sales Inc. (Scituate, MA, USA).

Beltsville Thawing Solution (BTS; Minitub ibérica, S.L., Tarragona, Spain) was used to initially dilute the semen. BTS supplemented with bovine serum albumin (BSA: 0.6%; w/v) was used to dilute and evaluate the samples after thawing.

Lactose-egg yolk (LEY; Westendorf et al. 1975) was used as the freezing extender, composed of 80 mL of lactose (11% ; w/v) and 20 mL of egg yolk (356 mOsm, pH=6.2). The extender was centrifuged (12,000 x g, 20 min, 5 ºC), the supernatant recovered and filtered sequentially through 5 μm, 3 μm and finally 1.2 μm pore filters (Millipore ibérica, Madrid, Spain). The glycerol containing extender consisted of LEY (89.5%; v/v) supplemented with 1.5% (v/v) OEP and 9% (v/v) glycerol (LEYGO: 1,812 mOsm, pH=6.2; Westendorf et al. 1975).

2.2. Preparation of cholesterol-loaded cyclodextrins (CLC)

Methyl-β-cyclodextrin (MBCD) was preloaded with cholesterol (CLC) as described by Purdy and Graham (2004). Working solutions of CLC and MBCD diluted in BTS (5%; w/v) were made according to the protocol described by Purdy and Graham (2004).

2.3. Semen collection and dilution

Mature Pietrain boars (n = 11) housed either at a commercial farm (Los Golliznos, Caminreal, Teruel, Spain) or at the experimental farm of Centro de Tecnología Animal (CITA-IVIA, Segorbe, Castellón, Spain) were used for these experiments. Boars ranged from 12 to 24 months of age and underwent regular weekly semen collection. Boars were fed with commercial diet (once/day) and provided water ad libitum. All boars were maintained under the guidelines approved by the Ethics Committee from the Instituto Valenciano de Investigaciones Agrarias and fulfilled the European regulations for the Care and Use of Animals for Scientific Purposes (EC Directive 86/609/ECC).

Semen was collected by the gloved-hand method. The sperm-rich fractions were collected in prewarmed thermo flasks and the gel fraction was withheld on a gauze tissue covering the flask. The initial percentage of motile sperm was determined for each ejaculate (as indicated below) and only ejaculates containing greater than 75 % motile sperm were processed for freezing. Semen was extended (1:1, v/v) in BTS at 37 ºC and cooled to 22 ºC over 2 to 3 hours.

2.4. Cryopreservation and thawing

Semen samples were processed to be frozen in straws as described by Westendorf et al. (1975) with minor modifications, as indicated below. Semen, diluted in BTS, was slowly cooled from 22ºC to 16ºC over 2 hours. After cooling, the samples were centrifuged (800 x g, 10 min, 16ºC) and the sperm pellets resuspended with cooled LEY extender (16ºC) to a concentration of 225 x 10^6 spermatozoa/mL. After further slow cooling to 5ºC over 120 min, the spermatozoa were diluted (2:1, v:v) with cooled LEYGO extender (5ºC) resulting in a final sperm concentration of 150 x 10^6 cells/mL and 3% of glycerol. Samples were

equilibrated for 15 min before being packed into 0.5 mL straws (IMV® Technologies, L'Aigle, France) and frozen in liquid nitrogen vapor 4 cm above static liquid nitrogen for 20 min. The straws were then plunged into liquid nitrogen for storage.

Straws were thawed in a water bath at 39ºC for 30 sec. After thawing, sperm motility and viability were determined 10 min after thawing and again after 1 h of incubation at 39 ºC, as described below.

2.5. Sperm evaluation

The sperm concentration in each ejaculate was determined using a Neubauer counting chamber (Paul Marienfield, GmbH & Co., Lauda-Koenigshofen, Germany).

The percentages of motile sperm (total and progressively motile) in each sample were determined using a computer-assisted sperm analysis system (CASA; ISAS, version 1.0.17, Proiser, Valencia, Spain) operating at 25 video frames per second (25 Hz), with settings of particle area = from 10 to 80 µm, and search radius = 11 µm. Sperm were defined as non-motile if their average path velocity (VAP) was lower than 10 µm/sec; and sperm were considered progressively motile if they exhibited a VAP > 45 µm/sec and a straightness index (STR) ≥ 45%. Sperm motility was assessed from a 5 µL sub-samples, which were placed on a Makler chamber (Counting Chamber Makler, Sefi-Medical Instruments) pre-warmed to 39ºC on a thermal plate and a minimum of 200 sperm from three different fields were captured to be analyzed using a x 10 negative phase contrast objective and a Nikon Eclipse 90i microscope (Nikon Corporation Instruments Company; IZASA, Barcelona, Spain) connected to the computer through a monochrome video camera Basler A312f (Basler Vision Technologies, Proiser, Paterna, Valencia, Spain). Individual sperm tracks were visually assessed to eliminate possible debris and misdiagnosed tracks.

For each sample, the sperm concentration was adjusted with BTS-BSA to 25 x 10^6 sperm/mL, prior to assessment.

The percentage of viable (plasma membrane intact) sperm (SV) in each frozen-thawed sample was determined using flow cytometry, as described by Purdy and Graham (2004). Briefly, straws were thawed and each sample was diluted with BTS-BSA to 25 x 10^6 sperm/ mL. The samples were prepared for flow cytometric analysis by transferring 0.1 mL of sperm to a tube containing 0.45 mL BTS-BSA diluent, 2.5 µL SYBR-14 (10 µM solution in DMSO) and 2.5 µL PI (1.5 mM solution in Milli-Q water). The samples were incubated for 10 min at 22ºC, filtered through a 40 µm nylon mesh and analyzed using an Epics XL-MCL flow cytometer (Coulter Corporation Inc., Miami, FL, USA) equipped with an argon laser (Cyonics, Coherent, Santa Clara, CA, USA) tuned to 488 nm at 15 mW power. Data were analyzed using EXPO 32 ADC software (Beckman Coulter Inc., Miami, FL, USA). Fluorescence from 10,000 cells was measured using a 550 long pass filter combined with a 525 nm band pass filter to detect SYBR-14, and a 645 nm long pass filter with a 620 nm band pass filter to detect PI. Using this protocol, all live cells stain with SYBR-14, permitting cells to be distinguished from egg yolk particles, but only non-viable cells stain with PI. Events showing only green fluorescence were considered as viable sperm (stained with SYBR-14) and events exhibiting green and red fluorescence (stained with SYBR-14 and PI) or only red fluorescence (stained with PI) were considered as nonviable sperm.

2.6. Statistical analyses

Data were analyzed similarly in all the experiments with minor modifications described in each experiment. Data from each observation time (10 min and 1 h) were

analyzed separately. Treatment differences in the percentages of viable or motile sperm were determined by analysis of variance (Mixed procedure; SAS, version 9.0, 2002, SAS Institute Inc., Cary, NC, USA). When this analysis showed a significant effect, the means were separated using Tukey test. Means were considered different if $P < 0.05$. The values presented are the least square means ± the standard error.

2.7. Experimental design

2.7.1. Experiment 1: Effect of CLC concentration on boar sperm cryosurvival

Ten ejaculates were collected, as described above, and each ejaculate was split into 9 aliquots. Three of the aliquots were treated with three different concentrations of MBCD (2 mg, 3 mg or 6 mg/120 x 10^6 cells), five aliquots were treated with five different concentrations of CLC (1 mg, 2 mg, 3 mg, 6 mg or 12 mg/120 x 10^6 cells) and the ninth aliquot was used as the control sample (non-treated). The treated samples were incubated with MBCD or CLC for 15 min at 22ºC (before cooling to 16 ºC) and were then cryopreserved, as described above.

Data (sperm motility and sperm viability) were analyzed using a mixed model with ejaculates as a random effect, and treatment as a fixed effect, with 9 levels (Control, MBCD 2, 3 or 6 and CLC 1, 2, 3, 6 or 12).

2.7.2. Experiment 2: Effect of CLC treatment on sperm cryosurvival from males classified as good or poor freezers

Semen from eleven boars (one ejaculate/boar) was collected, as described above, and each ejaculate was split into two fractions: one fraction was used untreated (control)

and the other was treated with 1 mg of CLC/120 x 10^6 cells, as described above. Samples were then processed and frozen, as described in Experiment 1.

To determine the effect of CLC treatment on the cryosurvival of the sperm from males classified as good or poor freezers, boars were divided into two groups according to the post-thaw percentages of viable and total motile sperm, with boars having semen that exhibited >50% viable sperm and >30% total motile sperm after thawing classified as "good freezers" (n=4) and the other boars (n=7) classified as "poor freezers". These threshold values were chosen according to the raw means presented by the control samples for these parameters at time 10 after thawing. Data were analyzed using a GLM model including cholesterol treatment (two levels: control and CLC), boar group (two levels: good and poor freezer) and their interaction as fixed effects.

3. RESULTS

3.1. Experiment 1: Effect of CLC concentration on boar sperm cryosurvival

Treating boar sperm with 1 mg CLC prior to cryopreservation resulted in higher percentages of viable (10 min and 1 hr) and total motile sperm after 1 hr ($P<0.05$), but not total motile sperm ($P>0.05$) after thawing (Table 1). However, treating boar sperm with high concentrations of CLC or MBCD (above >3 mg/120 x 10^6 sperm) resulted in lower percentages of viable and motile sperm than control samples ($P<0.05$; Table 1).

Table 1. Percentages of viable (SV), total motile (TMS) and progressively motile sperm (RPM; least square means ± standard error) 10 min after thawing and after 1 h of incubation at 39ºC when boar spermatozoa were treated with different concentrations (mg/120 x 10^6 sperm) of methyl-β-cyclodextrin (MBCD), or methyl-β-cyclodextrin pre-loaded with cholesterol (CLC) and cryopreserved in Lactose-egg yolk diluent. n = 10.

Semen treatment	**Conc. (mg)**	**SV (%)** 10 min	**SV (%)** 1 hr	**TMS (%)** 10 min	**TMS (%)** 1 hr	**RPM (%)** 10 min	**RPM (%)** 1 hr
Control	0	53 ± 3^{b}	32 ± 3^{b}	29 ± 4^{a}	19 ± 3^{b}	26 ± 4^{ab}	16 ± 3^{abc}
MBCD	2	54 ± 3^{ab}	33 ± 3^{b}	30 ± 4^{a}	18 ± 3^{bc}	28 ± 4^{a}	15 ± 3^{bcd}
MBCD	3	51 ± 3^{b}	32 ± 3^{b}	25 ± 4^{ab}	16 ± 3^{bcd}	22 ± 4^{abc}	12 ± 3^{cde}
MBCD	6	38 ± 3^{c}	22 ± 3^{c}	20 ± 4^{bc}	11 ± 3^{de}	18 ± 4^{bcd}	8 ± 3^{ef}
CLC	1	61 ± 3^{a}	40 ± 3^{a}	32 ± 4^{a}	25 ± 3^{a}	30 ± 4^{a}	22 ± 3^{a}
CLC	2	58 ± 3^{ab}	40 ± 3^{a}	25 ± 4^{ab}	22 ± 3^{ab}	23 ± 4^{abc}	19 ± 3^{ab}
CLC	3	53 ± 3^{b}	36 ± 3^{ab}	19 ± 4^{bc}	17 ± 3^{bcd}	17 ± 4^{cd}	15 ± 3^{bcd}
CLC	6	39 ± 3^{c}	24 ± 3^{c}	13 ± 4^{cd}	11 ± 3^{cde}	11 ± 4^{de}	10 ± 3^{def}
CLC	12	21 ± 3^{d}	12 ± 3^{d}	9 ± 4^{d}	6 ± 3^{e}	8 ± 4^{e}	5 ± 3^{f}

The results are presented as least square mean values ± standard error (LSM ± SE); $^{a-f}$ Column means for each observation time with uncommon superscripts are different at $P<0.05$.

3.2. Experiment 2: Effect of CLC treatment on sperm cryosurvival from males classified as good or poor freezers

Samples from boars classified as "good freezers" (n = 4) exhibited higher percentages of viable and motile sperm than samples from "poor freezers" (n = 7) ($P < 0.05$; Table 2). CLC treatment showed slight, although not significant, improvement in the percentages of viable sperm after thawing (+4%), total motile (+8%) progressively motile sperm (+9%) and viable sperm (+6%) after 1h of incubation at 39 ºC in sperm from "good freezers", but the improvements were lower for sperm from "poor freezers" (+7% viable sperm after thawing and +6% total motile sperm after 1 hr of incubation).

Table 2. Percentages of viable (VS), total motile (TMS), progressively motile sperm (RPM; least square means ± standard error) 10 min and 1 h after thawing, when spermatozoa from boars whose sperm normally cryopreserve well (good freezers) or poorly (poor freezers) were cryopreserved using normal methods (control) or were treated with 1 mg/120 x 10^6 sperm of methyl-β-cyclodextrin pre-loaded with cholesterol (CLC) prior to cryopreservation. n = 4 categorized as good freezers and 7 categorized as poor freezers.

Semen treatment	**Freezing group**	**VS (%)** 10 min	**VS (%)** 1 h	**TMS (%)** 10 min	**TMS (%)** 1 h	**RPM (%)** 10 min	**RPM (%)** 1 h
Control		52 ± 3	34 ± 3	32 ± 3	20 ± 4	29 ± 3	16 ± 4
CLC		57 ± 3	37±3	31 ± 3	27 ± 4	27 ± 3	20 ± 4
	Good freezers	61 ± 3*	44 ± 3*	40 ± 4*	33 ± 5*	36 ± 4*	28 ± 5*
	Poor freezers	47 ± 3	26 ± 2	23 ± 3	14 ± 4	20 ± 3	8 ± 3
Control	Good freezers	59 ± 5	42 ± 4	41 ± 5	29 ± 7	38 ± 5	24 ± 6
CLC	Good freezers	63 ± 5	47 ± 4	39 ± 5	37 ± 7	35 ± 5	32 ± 6
Control	Poor freezers	44 ± 4	26 ± 3	23 ± 4	11± 5	20 ± 4	8 ± 5
CLC	Poor freezers	51 ± 4	26 ± 3	23 ± 4	17 ± 5	20 ± 4	8 ± 5

The results are presented as least square mean values ± standard error (LSM ± SE);

*Sperm attribute between 'good freezers' and 'poor freezers' is different for each observation time at $P<0.05$.

4. DISCUSSION

Cryopreservation can induce many types of cellular damage, although damage to the structural integrity of the plasma membrane is probably the most critical. Cholesterol plays an important role on cold-shock resistance (Watson 1981). Thus, increasing the cholesterol content of sperm has been previously used in cryopreservation protocols for sperm from several species that exhibit cold-shock sensitivity and cholesterol-treated sperm maintained greater numbers of cells surviving cryopreservation (reviewed by Mocé et al. 2010a). Therefore, attempting to increase the amount of cholesterol in boar sperm, to improve cryopreservation, seems reasonable, as boar sperm plasma membrane composition has a low cholesterol:phospholipid ratio and these sperm are sensitive to cooling damage (Darin Bennett and White 1977; Parks and Lynch 1992). In order to develop a protocol for adding CLCs to boar sperm, it is necessary to determine the optimal CLC concentration to use. The CLC concentration that optimized the percentage of plasma membrane intact sperm both immediately after thawing and 1 h after incubation and the percentage of total motile sperm 1 h after thawing is 1 mg/120 x 10^6 sperm. This is similar to the optimal CLC concentration reported by others for boar sperm (1.5 mg; Torres et al. 2009) and for sperm from other species (usually between 1 and 3 mg CLC/ 120 x 10^6 sperm) including bull (Purdy and Graham 2004), ram (Mocé et al. 2010b), goat (Barrera-Compean et al. 2005), stallion (Moore et al. 2005), and donkey (Álvarez et al. 2006) sperm. On the other hand, CLC-treatment increased boar sperm motility and viability only 6 to 8 percentage points after thawing, which is a much smaller benefit than was observed for other species, as most studies reported that CLC-treatment increased sperm cryosurvival rates by 10 to 20 percentage points (reviewed by Mocé et al. 2010a). Perhaps the differences between boar sperm and sperm from other cold-shock sensitive species, in their response to CLC

treatment, may be due to differences in the specific phospholipids that compose the sperm plasma membranes from these different species (Parks and Lynch 1992). The membrane composition of boar sperm is different from that of bull and ram sperm (both species which positively respond to CLC treatment) and is more similar to sperm from some of the more cold-shock resistant species such as human and rabbit (Watson 1981). It is therefore likely that results obtained after CLC treatment will be different for sperm from different species, regardless of merely whether the sperm are sensitive to cold-shock or not, depending on the phospholipid composition of the sperm membrane, and this should be addressed in future experiments. Unlike ram sperm (Mocé et al. 2010b), treating boar sperm with cyclodextrins alone (a treatment that decreases the cholesterol content of the sperm membrane; Companyó et al. 2007) did not decrease sperm quality after thawing. Indeed, this treatment was neither beneficial nor detrimental to boar sperm in our study, which is contrary to previous reports in which the addition of cyclodextrin alone to extenders containing egg yolk improved (+ 27 to 32 percentage points in total motile sperm; Zeng and Terada 2000; 2001) or decreased (- 10 percentage points in total motile sperm; Bailey et al. 2008) the sperm quality after cryopreservation. However, when an egg yolk-free extender was used, this treatment resulted in decreased sperm viability (-19 percentage points) after cold shocking the sperm (Galantino-Homer et al. 2006). The differences seen between the results of different investigators may be due to differences between the breeds or males used, or to differences in the protocols used to treat the sperm with the cyclodextrins, since some added CLCs to the sperm in the presence of egg yolk during the incubation, 15 min or 3 h, while others in the absence of egg yolk, or to the amount of egg yolk (5 or 20%) that was included in the freezing extender.

Differences, in the ability of sperm from individual sires, to survive cryopreservation have been well documented (Holt 2000; Roca et al. 2006). In our study, sperm from 4 boars exhibited higher sperm survival rates, and the boars producing that sperm were classified as "good freezers". Casas et al. (2010) similarly observed boar differences in sperm progressive motility and viability after cryopreservation. Although there can be many reasons why sperm from different sires cryopreserve differently, one reason may be due to differences in plasma membrane composition, and differing amounts of cholesterol in sperm membranes of different sires, could play a role in the ability of sperm, from different sires, to survive cryopreservation. Therefore, it seems reasonable to expect that CLC treatment to be more effective for freezing sperm from individual stallions (Moore et al. 2005) or lines of mice (Loomis and Graham 2008) which normally do not survive cryopreservation well, than for sperm that do. For this reason, it was expected that sperm from boars classified as "poor freezers", would benefit more from CLC treatment than sperm from boars which cryopreserved well. This, however, was not case, as although CLC treatment slightly improved the percentage of viable, total and progressively motile sperm, these differences were not significant. Moreover, the response to CLC treatment was similar for sperm from both the good and poor freezing groups. Perhaps it would be important to study the effect CLCs using more males to make a definitive conclusion, since only four boars were classified as 'good freezers' in this study and the effect of an individual male may be more important than the effect of an artificial classification (good or poor) the sample was assigned. In addition, it would be interesting to determine if differences in the composition of the sperm plasma membranes exist between males classified as good or poor freezers, to determine the role of cholesterol in sperm cryopreservation for boar sperm.

In conclusion, treating sperm with cyclodextrins alone or with cholesterol-loaded cyclodextrins provided either no or only slight benefit, respectively, to sperm plasma membrane integrity and motility, after thawing. This response is not dependant on the original ability of the sperm to survive cryopreservation. However, future studies are needed to determine if CLC treatment might affect other sperm quality parameters as well as the impact it may have on sperm functionality.

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CHAPTER 4

STUDY 2

Egg yolk and glycerol requirements for freezing boar spermatozoa treated with methyl-β-cyclodextrins or cholesterol-loaded cyclodextrins

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ABSTRACT

Egg yolk (EY) and glycerol are common constituents of extenders used for sperm cryopreservation. It has been demonstrated that using cholesterol-loaded cyclodextrins (CLC) improves sperm cryosurvival in several species. However, standard freezing extenders could not be the most appropriate for CLC-treated sperm. This study evaluated the EY and glycerol requirements for freezing CLC-treated boar spermatozoa. Semen samples from 34 ejaculates coming from 4 boars were used. Each ejaculate was split into three aliquots: one was used untreated (control) and the other two were treated with 1 mg of CLC or methyl-β-cyclodextrin/120 x 10^6 sperm for 15 min at 22 ºC prior to cryopreservation. Our results indicated that reducing the concentration of EY was detrimental for sperm viability after thawing (31.57% ± 2 vs. 19.89 ± 2 for 20% and 10% EY, respectively; $P < 0.05$), even in semen treated with CLC. On the other hand, it was observed that traditional concentration of glycerol (3%) was not the appropriate for freezing CLC-treated sperm (61.10% ± 3 vs. 47.87% ± 3 viable sperm for control and CLC-treated sperm, respectively; $P < 0.05$). Thus, CLC-treated sperm showed a higher tolerance to high glycerol concentrations (5%) in terms of sperm viability (59.19% ± 3) than non-treated sperm (45.58% ± 3; $P<0.05$). Therefore, it could be necessary to adequate conventional freezing protocols for CLC-treated sperm. Nevertheless, additional studies will be needed to evaluate alternative cryoprotectants and to determine the effect of high glycerol concentrations on sperm functionality.

1. INTRODUCTION

Egg yolk (EY) and glycerol are nowadays indispensable ingredients of freezing extenders for boar sperm cryopreservation. The protective effect of EY, minimizing cold stress, is related with its content in cholesterol and phospholipids, which are in part moved to sperm membranes (Bathgate et al., 2006; Bergeron and Manjunath, 2006). Glycerol, as cryoprotectant, is essential since it lowers salt concentration and raises the percentage of unfrozen water at any given temperature (for a review, see Amann and Pickett, 1987). However, glycerol is also cytotoxic causing osmotic stress due to its low permeability through the membrane as a consequence of its high molecular weight (Guthrie et al., 2002).

Pre-freezing treatment with cholesterol-loaded cyclodextrins (CLC) adds cholesterol to sperm membranes improving cryosurvival in spermatozoa of several mammalian species (for a review, see Mocé et al., 2010a). Cholesterol plays an important role in the thermotropic behavior of the membranes (Johnson et al., 2000) by preventing the molecular packing of the phospholipids (which is required to form the gel phase) as temperature decreases (Parks, 1997) and minimizing cooling shock. Spermatozoa treated with CLC prior to cryopreservation are currently frozen using standard freezing extenders characterized by a high EY concentration. Under this freezing condition the effect of CLC-treatment could be understated. Since CLC-treatment increases the cholesterol amount of sperm membranes (Loomis and Graham, 2008; Mocé et al., 2010b; Moore et al. 2005; Purdy and Graham, 2004; Tomás et al., 2012), the EY of freezing extenders could be partially or even completely withdrawn, always considering that cryoprotective effect of EY is partially due to its high cholesterol content.

In addition, CLC-treatment could also modify the membrane permeability to penetrating cryoprotectants since cholesterol is one of the most important regulators of membrane fluidity and permeability (Zeng and Terada, 2000). In this way, CLC-treated stallion sperm showed changes in the membrane permeability to water and cryoprotectants (Glazar et al., 2009). Besides, CLC-treatment widened the sperm osmotic tolerance limits in all species studied, including pig (Tomás et al., 2011). Therefore, it is likely that the glycerol at the concentrations usually included in boar sperm freezing extenders could not be the most appropriate for CLC-treated sperm. In the light of this background, the aim of this study was to determine the most appropriate requirements of EY and glycerol for freezing CLC-treated boar spermatozoa.

2. MATERIALS AND METHODS

2.1. Reagents and media

All chemicals used were reagent grade and purchased from Sigma-Aldrich (Madrid, Spain) except flow cytometry fluorochromes, which were purchased from Invitrogen (Barcelona, Spain).

Beltsville Thawing Solution (BTS, Minitub ibérica, S.L., Tarragona, Spain) was used as basic semen extender; and supplemented with bovine serum albumin (BSA: 6 mg/mL) was used for post-thawing sperm dilution. The basic freezing extender (FE-1) was composed by lactose (321 mM) and EY (Westendorf et al., 1975). The concentrations of EY ranged from 10 to 20% according to the experiment (see Section Experimental design). The FE-1 was centrifuged (12,000g, 20 min, 5 ºC) and the supernatant was recovered and then filtered through 5-µm, 3-µm and 1.2-µm membrane filters (Millipore ibérica, Madrid,

Spain) in a three step process. The FE-1 supplemented with 1.5% of Orvus Es Paste (Equex STM®, Nova Chemical Sales Inc., Scituate, MA, USA) and glycerol was used for a second semen dilution at 5 °C before freezing (FE-2). The concentrations of glycerol also vary according to the experiment (see Section Experimental design). The final concentrations of EY and glycerol in the standard FE-2 were 20 and 9%, v/v, respectively. The pH of FE-1 and FE-2 was 6.2, and the osmolarity of the FE-1 ranged from 274 to 356 mOsmol/kg and the FE-2 ranged from 1200 to 3760 mOsmol/kg, according to the glycerol concentration used (from 2 to 7%, v/v). Phosphate buffered saline (PBS) was used as basic medium for mitochondrial membrane potential evaluation and was composed by NaCl (137mM), KCl (2.7mM), Na_2HPO_4 (0.86mM) and $Na_2HPO_4 \cdot 7H_2O$ (6.4mM) (pH 6.8 and 292 mOsmol/kg).

2.2. Preparation of cholesterol-loaded cyclodextrins (CLC)

Methyl-β-cyclodextrin (MBCD) was preloaded with cholesterol and working solutions of CLC and MBCD were prepared by mixing 50 mg of CLC or MBCD in 1 mL of BTS, using a vortex mixer, in accordance to the protocol developed by Purdy and Graham (Purdy and Graham, 2004), as described elsewhere (Blanch et al., 2012).

2.3. Semen collection and dilution

Mature Pietrain boars (n=4) were housed in the experimental farm of Centro de Tecnología Animal (CITA-IVIA, Segorbe, Castellón, Spain). Boars were fed with commercial diet (once/day) and provided water *ad libitum.* All boars were maintained under the guidelines approved by the Ethics Committee from the Instituto Valenciano de Investigaciones Agrarias and fulfilled the European regulations for the Care and Use of Animals for Scientific Purposes (EC Directive 2010/63/EU).

Sperm rich ejaculate fractions were collected weekly by the gloved-hand method and placed in pre-warmed thermo flasks covered with a gauze tissue to hold on the ejaculate gel fraction. Thereafter, sperm rich ejaculate fractions were diluted (1:1, v/v) with BTS at 37 ºC and slowly cooled to 22 ºC during 2 h.

2.4. Sperm Cryopreservation

Only those ejaculates with >75% motile sperm were cryopreserved. The ejaculates were split into 3 fractions: one fraction was untreated and the other two were treated with 1 mg/120 x 10^6 sperm of MBCD or CLC, respectively. The treated samples were incubated with MBCD or CLC for 15 min at 22 ºC (Blanch et al., 2012). Thereafter the three aliquots were cooled to 16 ºC during 2 h.

After cooling to 16 ºC, the 3 aliquots were processed using the straw-freezing procedure described by Westendorf et al. (1975) with some modifications (Blanch et al., 2012). The straws were thawed in a water bath at 39 ºC for 30 s. Post-thaw sperm quality was assessed in semen samples extended in BTS-BSA (25 x 10^6 sperm/mL) and incubated in a water bath at 39 ºC for 10 min.

2.5. Sperm quality evaluation

Sperm motility was objectively evaluated using a computer-aided sperm analysis system (ISAS® 1.0.17, Proiser R+D, Paterna, Spain) according to the protocol described by Blanch et al. (2012). The proportions of total motile sperm (%TMS, VAP>10 µm/s) and rapid progressively motile sperm (%RPM, VAP > 45 µm/s and STR ≥ 45%) were recorded.

Flow cytometry analyses were performed under dimmed light using either an EPICS XL flow cytometer (Coulter Corporation Inc., Miami, FL, USA) equipped with an

argon ion laser (Cyonics, Coherent, Santa Clara, CA, USA), with 15 mW of laser power at 488 nm or an Coulter FC500 flow cytometer (Coulter Corporation Inc., Miami, FL, USA) equipped with an argon ion and an Helium-Neon laser (Cyonics, Coherent, Santa Clara, CA, USA), with 15 mW of laser power at 488 and 630 nm, respectively. Data were analyzed using EXPO 32 ADC software (Beckman Coulter Inc., Miami, FL, USA). A total of 10,000 events with forward and side scatter properties similar to sperm cells were initially gated. From these events, those without reasonable DNA content after SYBR-14/ propidium iodide (PI) staining were gated out (double-gated sperm analysis). In the following cytometry test carried out, the percentages of these non-DNA events with scatter characteristics similar to sperm cells were taken into account to calculate the true sperm counts (Petrunkina et al., 2010).

Sperm viability (% SV) was evaluated by assessment of plasma membrane integrity using the LIVE/DEAD Sperm Viability Kit (L-7011; Molecular Probes Europe BV, Leiden, the Netherlands) and following the procedure described by Purdy and Graham (2004) and modified by Blanch et al. (2012). The samples were stained and incubated at 39 °C in the dark for 10 min. The fluorescence spectra of SYBR-14 and PI were detected using 505-545 and 605-635-nm band-pass filters, respectively. The analyzed sperm were categorized as follows: intact plasma membrane (SYBR-14+/PI−) and damaged plasma membrane (PI+). Only the sperm population that exhibited intact plasma membrane was showed in the results.

Mitochondrial membrane potential (MMP) was analyzed using a triple fluorescent procedure for flow cytometric analysis (SYBR 14- PI- Mitotracker Deep Red 633, MTDR) described by Januskauskas et al. (2005) and Martínez-Pastor et al. (2008) with modifications, as indicated below. Fifty µL aliquots of thawed sperm (25×10^6 sperm/mL)

were diluted in 450 µL of PBS containing 25 µL of SYBR-14 (100 nm in DMSO), 2.5 µL of PI (1.5 mM in purified water) and 3.75 µL of MTDR (6.6 µM in DMSO) and incubated at 37 ºC in the dark for 20 min. The fluorescence spectra of SYBR-14, PI and MTDR were detected using 505-545, 605-635, 600-650 -nm band-pass filters, respectively. The analyzed sperm were categorized as follows: viable sperm with high MMP (SYBR-14+ and high MTDR fluorescence), viable sperm with low MMP (SYBR-14+ and low MTDR fluorescence), and dead cells (PI+). Only viable sperm with low MMP were showed in the results.

2.6. Experimental design

2.6.1. Experiment 1: Requirement of EY for freezing CLC- or MBCD-treated boar spermatozoa

Semen samples from 8 ejaculates were split each one into three aliquots and treated as described previously (control, MBCD-treated or CLC-treated). After seminal plasma removal, each aliquot was split each one into two subsamples. Subsample one was diluted with FE-1 containing 20% of EY, whereas subsample two with FE-1 containing 10% of EY. Thereafter, both subsamples were frozen (final glycerol concentration of 3%) and thawed following the protocol described above. In a preliminary experiment, EY concentrations lower than 10% (5 and 2.5%) were used, but sperm samples frozen with these freezing extenders exhibited extremely low viability and motility after thawing, therefore these percentages were discarded for subsequent experiments (results not shown).

2.6.2. Experiment 2: Requirement of glycerol for freezing CLC- or MBCD-treated boar spermatozoa

Semen samples from 13 ejaculates were split each one into three aliquots and treated as described previously (control, MBCD-treated, CLC-treated). After this, the aliquots were cooled to 16 ºC, centrifuged, resuspended with FE-1 containing 20% of EY and cooled to 5 ºC. Once at 5 ºC, each aliquot was split into four subsamples and diluted with FE-2 containing 6, 9, 12 or 15% of glycerol (final concentration of 2, 3, 4 and 5%, respectively). After, semen aliquots were frozen and thawed as described above.

2.6.3. Experiment 3: Effect of increasing glycerol concentration on cryosurvival of CLC-treated boar sperm

Consequent to the results obtained in the previous experiment, in this third experiment we evaluated if highest glycerol concentrations further improved the quality of cryopreserved CLC-treated sperm. Semen samples from 13 ejaculates were split each one into three aliquots and treated as described previously (control, MBCD-treated, CLC-treated). Thereafter, aliquots were cooled to 16 ºC, centrifuged, resuspended with FE-1 with 20% EY and cooled to 5 ºC. Then, CLC aliquots were split into two subsamples and diluted with FE-2 containing 9 or 21% of glycerol (final concentrations of 3 and 7%, respectively). The MBCD and control aliquots were diluted with FE-2 containing 9% of glycerol (final concentration of 3%). Samples were frozen and thawed as described above.

2.7. Statistical analyses

Statistical analyses were performed using SAS package (version 9.0, 2002, SAS Institute Inc., Cary, NC, USA). Data from the three experiments were analyzed using a mixed model analysis of variance (ANOVA). In the first experiment, semen treatment (three levels: control, MBCD, CLC), EY concentration (two levels: 20 and 10%) and their interaction were included as fixed effects. In the second experiment, semen treatment (three

levels: control, MBCD, CLC), glycerol concentration (four levels: 2, 3, 4 and 5%) and their interaction were included as fixed effects. In the third experiment, treatment (four levels: control, MBCD, CLC-glycerol 3%, CLC-glycerol 7%) was included as fixed effect. In all the experiments the ejaculate within boar was included in the model as random effect. When this analysis showed a significance effect, the means were compared using Tukey test. A P-value of < 0.05 was considered to be statistically significant. All of the data are shown as least squares mean ± standard error. The percentage of progressively motile sperm in the first experiment and the percentage of viable sperm with low mitochondrial membrane potential in the third experiment were normalized using the Freeman-Tukey arcsine transformation (Freeman and Tukey, 1950) before the analysis. Data are shown in tables in original form.

3. RESULTS

3.1. Experiment 1: Requirement of EY for freezing CLC- or MBCD-treatment boar spermatozoa

While motility parameters were not affected by CLC- and MBCD-treatment or EY concentration, the percentage of SV was negatively influenced ($P < 0.05$) by CLC- or MBCD-treatment and by lowest EY concentration (Table 1). The interaction between semen treatment and EY concentration was not significant.

Table 1. Post-thaw percentages of total motile sperm (TMS), rapid progressively motile sperm (RPM) and sperm with intact plasma membrane (sperm viability; SV) in boar semen samples treated with 1 mg/120 x 10^6 sperm of methyl-β-cyclodextrin (MBCD) or methyl-β-cyclodextrin pre-loaded with cholesterol (CLC) or no treated (control) and subsequently frozen in a extender with 20 or 10% of egg yolk. n= 8.

Semen treatment	Egg yolk concentration	TMS (%)	RPM (%)	SV (%)
Control		20.06 ± 3	17.25 ± 3	34.35 ± 3 [a]
MBCD		15.41 ± 3	13.18 ± 3	20.66 ± 3 [b]
CLC		13.44 ± 3	11.19 ± 3	22.17 ± 3 [b]
	20%	18.12 ± 3	15.87 ± 2	31.57 ± 2 [x]
	10%	14.48 ± 3	11.87 ± 2	19.89 ± 2 [y]

The results are presented as least square mean values ± standard error (LSM ± SE); [a,b]: Differences (P< 0.05) between semen treatments; [x,y]: Differences (P < 0.05) between egg yolk concentrations.

3.2. Experiment 2: Requirements of glycerol for freezing CLC- or MBCD-treatment boar spermatozoa

The interaction between glycerol concentration and semen treatments was significant (P < 0.05) for all the quality parameters (Table 2). On the one hand, increasing glycerol above 3% resulted in decreasing SV in control and samples treated with MBCD, but SV gradually improved with increased concentrations of glycerol in the sperm samples treated with CLC prior to cryopreservation. On the other hand, when the glycerol concentration exceeded 3 or 4%, a decrease in the percentages of TMS and RPM was observed in MBCD-treated and in control samples, respectively. By contrast, increasing

glycerol concentration from 3 to 4% exerted a positive effect on the percentages of TMS and RPM in CLC-treated samples ($P < 0.05$; Table 2).

Table 2. Percentages of total motile sperm (TMS), rapid progressively motile sperm (RPM) and plasma membrane intact sperm (sperm viability; SV) after thawing when boar spermatozoa were treated with 1 mg/120 x 10^6 sperm of methyl-β-cyclodextrin (MBCD), or methyl-β-cyclodextrin pre-loaded with cholesterol (CLC) or no treated (control) and then cryopreserved with different percentages of glycerol. Data of interaction between semen treatments and glycerol concentrations. n= 13.

Glycerol concentration	Semen treatment	TMS (%)	RPM (%)	SV (%)
2%	Control	50.46 ^{ab}	46.69 ^{ab}	56.15 ^{ab}
	MBCD	45.00 ^{abc}	40.23 ^{abc}	52.69 ^{bcd}
	CLC	32.46 ^{de}	28.61 ^{def}	47.31 ^{def}
3%	Control	53.18 ^{a}	49.29 ^{a}	61.10 ^{a}
	MBCD	36.58 ^{cde}	34.19 ^{cde}	46.59 ^{def}
	CLC	35.98 ^{cde}	33.19 ^{cde}	47.87 ^{cdef}
4%	Control	44.77 ^{abc}	41.46 ^{abc}	48.55 ^{cde}
	MBCD	26.61 ^{ef}	24.46 ^{ef}	41.43 ^{g}
	CLC	45.00 ^{abc}	41.08 ^{abc}	54.88 ^{abc}
5%	Control	37.23 ^{cd}	34.23 ^{cde}	45.58 ^{ef}
	MBCD	21.77 ^{f}	19.77 ^{f}	38.05 ^{fg}
	CLC	41.00 ^{bcd}	38.23 ^{bcd}	59.19 ^{ab}
s.e.		± 5	± 5	± 3

The results are presented as least square mean values ± standard error (LSM ± SE); $^{a-g}$: Column means with uncommon superscript letters are different ($P < 0.05$).

3.3. Experiment 3: Effect of increasing glycerol concentration on cryosurvival of CLC-treated boar sperm

The increment in the glycerol concentration in the CLC-treated sperm samples had contradictory effects on SV and sperm motility (Table 3). Similarly to the previous experiments, the concentration of 3% significantly decreased the percentage of SV, in comparison to control samples. However, this difference on SV between CLC-treated and control samples disappeared when the glycerol concentration was increased to 7% in the CLC-treated samples. An opposite trend was observed in sperm motility parameters, CLC-treated sperm samples frozen with 3% of glycerol exhibited similar percentages of TMS and RPM than control samples, while those frozen with 7% showed lower percentages of TMS and RPM ($P < 0.05$; Table 3) than control samples.

With respect to the mitochondrial membrane potential detected by Mitotracker Deep Red 633, when sperm were treated with CLC and frozen with 3 or 7% of glycerol, the percentages of viable spermatozoa showing low MMP were higher than for control and MBCD-treated sperm samples.

Table 3. Percentages of total motile sperm (TMS), rapid progressively motile sperm (RPM), plasma membrane intact sperm (sperm viability; SV) and viable sperm with low mitochondrial membrane potential (MMP) after thawing when boar spermatozoa were treated with 1 mg/120 x 10^6 sperm of methyl-β-cyclodextrin (MBCD) or no treated (control) and then cryopreserved with 3% of glycerol or treated with methyl-β-cyclodextrin pre-loaded with cholesterol (CLC) and then cyropreserved with different percentages of glycerol. n= 13.

Semen treatment	TMS (%)	RPM (%)	SV (%)	Viable sperm with low MMP (%)
Control-Glycerol 3%	51.40 ± 5 [a]	43.90 ± 5 [a]	57.94 ± 4 [a]	2.38 ± 1 [b]
MBCD-Glycerol 3%	39.70 ± 5 [b]	34.00 ± 5 [b]	56.00 ± 4 [ab]	1.62 ± 1 [b]
CLC-Glycerol 3%	49.20 ± 5 [a]	40.60 ± 5 [a]	51.15 ± 4 [b]	5.87 ± 1 [a]
CLC-Glycerol 7%	39.30 ± 5 [b]	31.90 ± 5 [b]	59.80 ± 4 [a]	7.29 ± 1 [a]

The results are presented as least square mean values ± standard error (LSM ± SE); [a-c]: Column means with uncommon superscript letters are different (P<0.05).

4. DISCUSSION

Since Phillips and Lardy (1940) reported that EY is beneficial for sperm preservation, it has been routinely included in most freezing extenders for semen from domestic species (Aboagla and Terada, 2004). However, using animal–derived additives such as EY in a semen extender implies sanitary risks and an extremely wide variability of

their composition (Bousseau et al., 1998). In that regard, there is increasing interest in finding a substitute for EY. Part of the positive effect of EY is likely due to the relatively high cholesterol content in the yolk (Bathgate et al., 2006). Therefore, treating the sperm prior to cryopreservation with CLC could be a useful strategy to reduce the EY concentration in the freezing extenders, and this could be a first step towards the development of chemically defined extenders.

In a preliminary experiment, EY concentrations lower than 10% (5 and 2.5%) were used, but sperm samples frozen with these freezing extenders exhibited extremely low viability and motility after thawing, therefore these percentages were discarded for subsequent experiments (results not shown). Our results indicated that reducing the concentration of EY from 20 to 10% in a lactose-based diluent was detrimental for SV after thawing in all the sperm samples, even those treated with CLC prior freezing. However, and similarly to results achieved with ram (Marco-Jiménez et al., 2004) and rhesus monkey (Dong and Vandevoort, 2009) spermatozoa, the post-thaw motility of sperm samples did not decrease when the EY concentration was lowered. This detrimental effect in SV could be related to either the largest proportion of lactose in the extender or the poor cryoprotective ability of the lowered EY concentration with respect to the lactose proportion. It is unlikely that the raise in lactose (from 8.8% to 9.9% in the diluents containing 20 and 10% EY, respectively) could be responsible for the decrease observed in SV. In this regard, some authors did observe a negative effect of lactose on boar sperm motility but at concentrations of 10.8% (Wilmut and Polge, 1977) or 14% (Corcuera et al., 2007), all of which exceeded that used in our study. On the other hand, the EY protection at these low concentrations could not have been totally accomplished. Thus, low density lipoproteins (LDL) are also EY constituents responsible for sperm protection (for a review,

see Bergeron and Manjunath, 2006), and some authors observed that the LDL concentration that optimized boar sperm cryosurvival was 9% (w/v; Jiang et al., 2007). Considering that freezing extenders supplemented with 20% EY will contain approximately 6-7% (w/v) of LDL (Briand-Amirat et al., 2007), at concentrations of 10% EY, the concentration of LDL (3-3.5%) will be one third of that reported as optimal for boar sperm, which could explain the decrease in sperm quality at this EY concentration.

Cholesterol represents approximately 5% of total lipids composition of EY (Anton, 2007), and it contributes to the sperm cryoprotective effects of EY. In addition, cholesterol plays an important role in the thermotropic behavior of the sperm membranes (Johnson et al., 2000), and it is able to minimize the phase transition of the plasma membrane phospholipids (Holt, 2000) by reducing the transition temperatures of membranes and maintaining them in a fluid state at low temperatures (Graham et al, 2006). Thus, the detrimental effect of the MBCD-treatment for sperm freezing could be, a priori, expected since this treatment has been proven to decrease the cholesterol content of the sperm membranes (Van Gestel et al., 2005). For the same reasons, a beneficial effect of CLC-treatment for sperm freezing could be expected because it has been demonstrated that this treatment increases the cholesterol content of the sperm membranes (Loomis and Graham, 2008; Mocé et al., 2010b; Moore et al. 2005; Purdy and Graham, 2004; Tomás et al., 2012; Almlid and Johnson, 1988). Effectiveness should be even more relevant when spermatozoa were frozen using suboptimal EY concentrations. However, CLC-treatment was not effective for freezing boar sperm, irrespectively the EY concentration of the freezing extender. The reason why boar sperm did not respond to CLC, while spermatozoa of other cold-shock sensitive mammalian species did (for a review, see (Mocé et al., 2010a)) is not known. It could be related to the composition and the distribution of phospholipids in the

sperm plasma membrane, which differ among mammalian species (Watson, 1981). Alternatively, it could be also related to the specific mechanism by which sperm acquire the resistance to cooling, which also differ among mammalian species (Pursel et al., 1973). Otherwise, the concentration of glycerol used traditionally could not be the adequate for sperm treated with CLC as seen in our results discussed below.

Glycerol is the cryoprotectant most widely used for boar sperm cryopreservation. This cryoprotectant exerts both an extracellular effect by osmotic stimulation of cell dehydration and an intracellular effect, permeating the membrane and restricting the dehydration effect (Medeiros et al., 2002). The general view is that relatively low concentrations of glycerol (1 to 3%) are appropriate for boar sperm cryopreservation (Paquignon, 1985). The widening in the osmotic tolerance of CLC treated boar sperm (Tomás et al., 2011) indicates that their sperm permeability is altered and for this reason we studied if this type of sperm would have different requirements of glycerol concentrations than non-treated sperm. For this, we gradually increased the glycerol concentration of this cryoprotectant, starting from 2 % (one of the concentrations commonly used in boar sperm protocols) and increasing one by one percent. In our study, the glycerol concentration of 5% resulted detrimental for all the sperm quality parameters evaluated after thawing in the control samples, which is in agreement with previous reports (Almlid and Johnson, 1988; Graham and Crabo, 1972; Pursel et al., 1977). However, the effects of the glycerol concentrations on sperm cryosurvival differ among the semen treatments evaluated. Control and MBCD-treated sperm showed highest post-thaw sperm quality when cryopreserved with lowest glycerol concentrations (2-3%), while post-thaw quality of CLC-treated sperm was highest when frozen with the highest glycerol concentrations (4-5%) used in the second experiment. Consequently, a third experiment was carried out to evaluate the

putative effectiveness of highest glycerol concentrations further improved the quality of cryopreserved CLC-treated sperm. Increasing the final glycerol concentration of freezing extender to 7% in CLC-treated sperm samples increased the percentage of sperm with intact plasma membrane but decreased the percentage of motile spermatozoa after thawing. The dichotomy between the results observed for membrane integrity and motility corroborates previous results (Almlid and Johnson, 1988). Thus, boar sperm tolerate differently concentrations of glycerol, depending on the parameter evaluated. When the percentage of cryoprotectant is increased in the freezing extender, more deleterious effects of osmotic stress induced by the addition and the removal of the cryoprotectant is expected (Gao et al., 1993). Moreover, this effect is enhanced when the cryoprotectant used is the glycerol, since it permeates and equilibrates across the plasma membrane slowly; inducing a sudden cell volume change (Glazar et al., 2009). This could explain the negative effects of high glycerol concentrations on post-thaw sperm quality in the MBCD-treated and the control samples. However, the higher tolerance of CLC-treated sperm, in terms of sperm viability, to the highest glycerol concentrations, could be due to the changes on the permeability to glycerol and on the osmotic tolerance limits of the boar sperm membrane induced by the CLC-treatment. It has been demonstrated that CLC-treatment increases the osmotic tolerance limits of the boar sperm (Tomás et al., 2011), and it is then likely that cholesterol could increase the sperm membrane permeability to cryoprotectant lessening osmotic cell damage (Glazar et al., 2009; Mocé and Graham, 2006). However, the increase in the glycerol requirements in CLC-treated sperm to accomplish the quality (sperm viability) of non-treated boar sperm cryopreserved with lower glycerol concentrations (3%) could also indicate that the permeability of the membrane of CLC-treated sperm to this cryoprotectant is reduced and higher glycerol concentrations are needed to counteract this effect. The CLC treatment decreases the membrane permeability of bull sperm to water at subzero

temperatures (Li et al., 2006), although this was observed as a positive effect of this treatment (volume excursions due to the osmotic stress would decrease also). Nevertheless, cholesterol enrichment of human red blood cells inhibits several carrier-mediated transport pathways (Cooper, 1978) and the transport of small neutral molecules such as glycerol. Perhaps this phenomenon is predominating in boar sperm. Thus, the added cholesterol could be acting as a double-edged sword in boar sperm having a positive (widening the osmotic tolerance limits and reducing the sensitivity to cold-shock) and a negative effect (reducing the permeability of the membrane to the cryoprotectant). The reason why the high glycerol concentrations in CLC-treated samples did not induce at once an increase in the sperm motility is not known. Permeating cryoprotectants, depending on the concentrations used, can induce decreased motility (Medeiros et al., 2002). In our study, CLC-treated sperm samples showed the highest percentages of viable spermatozoa presenting low mitochondrial membrane potential post-thawing. However, these percentages were not high enough to fully explain the difference found between the data of viable and motile spermatozoa in the CLC-treated samples (20%). Perhaps this decrease in the motility as the glycerol concentration increased could be also related to the higher degree of dehydration of the sperm when a high concentration of glycerol is used. In this regard, some authors suggested that the reduction of intracellular water content could be the cause of the inhibition of sperm motility, since at lower intracellular water content the friction in tail would increase and this friction would in turn cause an inhibition of sliding of microtubules or other structural elements in the flagellum (Corcuera et al., 2007).

The improvement in sperm quality parameters after thawing observed in CLC-treated boar sperm samples as the glycerol concentration increased, suggests that the permeability of the membrane of these sperm to the glycerol is altered. Cryopreservation

protocols are not still optimized for CLC-treated boar spermatozoa, at least related to the concentrations of cryoprotectants. However, the post-thaw sperm quality of CLC-treated samples frozen with high glycerol concentration still did not exceed that showed by the control samples. Alternative cryoprotectants should be evaluated in future studies in order to optimize the cryopreservation protocol for boar spermatozoa.

In conclusion, the proportion of EY in the freezing extender cannot be reduced though the sperm samples are treated with CLC prior to freezing. As regards the optimal glycerol concentration of the extender for freezing CLC-treated sperm samples, those of 5-7% increased the percentages of viable sperm after thawing but decreased the percentage of motile sperm. Therefore, it seems clear that it would be necessary to adjust the freezing protocol for CLC-treated boar sperm and studies will be needed to evaluate alternative cryoprotectants or different freezing and thawing rates for CLC-treated sperm.

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CHAPTER 5

STUDY 3

The contradictory effect of amides on cryopreservation of boar semen treated with cholesterol-loaded cyclodextrin, methyl-β-cyclodextrin or non treated

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ABSTRACT

In this work, we studied the effectiveness of amides as cryoprotectants for boar sperm treated prior to cryopreservation with cholesterol-loaded cyclodextrins (CLC), methyl-β-cyclodextrin (MBCD) or non-treated. Semen samples from 26 ejaculates coming from 4 boars were used. The collected semen was split into three aliquots: one was used untreated (control) and the other two were treated with 1 mg of CLC or MBCD/120 x 10^6 sperm for 15 min at 22 ° C prior to cryopreservation. In the first experiment, the effect of different amides at a concentration of 5% (v:v or w:v) was determined in fresh semen incubated for 2 h at 22 ºC. Lactamide, Acetamide and Formamide had a marked detrimental effect ($P < 0.05$) on sperm viability. In the second experiment, we compared two concentrations (5 or 7%; v:v) of dimethylacetamide (DMA) for the cryopreservation of boar semen. Samples frozen with 5% DMA or 3% glycerol exhibited higher ($P < 0.05$) percentages of total and progressively motile sperm than samples frozen with 7% DMA, and DMA (5 and 7%) preserved the sperm viability better than glycerol, irrespective of the sperm treatment ($P<0.05$). In the third experiment, the cryoprotectants methylformamide, DMA, dimethylformamide (DMF) and glycerol were compared. Samples frozen with any of the amides (5%) exhibited higher sperm viability ($P < 0.05$) than samples frozen with glycerol (3%), although the opposite effect was observed for the percentages of total and progressively motile sperm. On the other hand, there was not an interaction ($P > 0.05$) between sperm samples and type of cryoprotectant, samples treated with CLC showed higher ($P < 0.05$) motility parameters than control or MBCD treated samples, and control samples showed the highest sperm viability ($P < 0.05$). In the last experiment, the sperm penetration ability of sow's oocytes in vitro of control or CLC treated samples frozen with glycerol or with DMF was compared. Samples frozen with glycerol achieved higher

penetration rate and a larger number of sperm per penetrated oocyte (P <0.05) than samples frozen with DMF. In addition, CLC-treated samples exhibited larger numbers of sperm per penetrated oocyte (P <0.05) compared to control samples. In conclusion, amides exerted a positive effect on sperm viability but negatively affected the sperm motility and the sperm fertilizing ability in vitro, irrespective of the sperm treatment. Moreover, CLC treated samples frozen with glycerol showed better in vitro fertilizing ability than control samples.

1. INTRODUCTION

Glycerol is the penetrating cryoprotectant most frequently used for boar sperm cryopreservation. Despite its benefits, glycerol is also cytotoxic and causes osmotic stress due to its low permeability through the membrane as a consequence of its high molecular weight (Guthrie et al., 2002).

The search for alternative cryoprotectants to glycerol has increased in the last years, with the objective of minimizing osmotic damage inflicted to the sperm. Thus, the amides are a group of cryoprotectants that present lower molecular weights and greater membrane permeability than glycerol and they have been increasingly used in the last decade for semen freezing protocols (stallions, Alvarenga et al., 2005; bull, Forero-Gonzalez et al., 2012; Pukazhenthi et al., 2014; dog, Futino et al., 2010; goat, Bezerra et al., 2011; ram, Moustacas et al., 2011; fish, Varela Junior et al., 2012; kangaroo, McClean et al., 2008; and koala, Zee et al., 2008). Some studies have also addressed the effect of acetamide (A), dimethylacetamide (DMA), methylformamide (MF) and dimethylformamide (DMF) on boar semen cryopreservation (Wilmut and Polge, 1977; Bianchi et al., 2008; Kim et al., 2011; Buranaamnuay et al., 2011; Malo et al., 2012) with contradictory results. Moreover,

to the best of our knowledge, studies to evaluate the use of lactamide (L), formamide (F) or methylacetamide (MA) on boar sperm cryopreservation have not been conducted, and the effect amides may have on the fertilizing ability of cryopreserved boar sperm has never been determined.

The pre-freezing treatment of sperm with cholesterol-loaded cyclodextrins (CLC) improved the sperm cryosurvival of several mammalian species (reviewed by Mocé et al., 2010), although the response of boar sperm to this treatment is highly variable when glycerol is used as cryoprotectant (Zeng and Terada, 2001, Bailey et al., 2008, Torres et al., 2009, Tomás et al., 2011, Blanch et al., 2012). This treatment modifies the permeability of the sperm membrane in bulls and stallions (Li et al., 2006; Moore et al., 2005), and widens the osmotic tolerance limits of the sperm from several species (reviewed by Mocé et al., 2010), including boars (Tomás et al., 2011). Perhaps combining this treatment with other cryoprotectants different than glycerol in the cryopreservation protocols, the quality of the samples after cryopreservation will increase further. To our knowledge, there is only a recent study where CLC treatment was combined with the use of DMF in the freezing extender for stallion sperm (Mesa and Henao, 2012), but these studies have not been conducted in any other species.

The aim of the present study is to determine the effectiveness of amides as cryoprotectants for boar sperm treated prior to cryopreservation with cholesterol-loaded cyclodextrins (CLC), methyl-β-cyclodextrin (MBCD) or untreated (control). Four experiments were conducted to determine: 1) the effect of different amides on fresh semen; 2) the effect of two concentrations of DMA on boar sperm cryosurvival; 3) the effectiveness of different amides as cryoprotectants for boar semen; 4) the in vitro fertilizing ability of boar sperm cryopreserved with DMF.

2. MATERIALS AND METHODS

2.1. Reagents and extenders

All chemicals used were reagent grade and purchased from Sigma-Aldrich (Madrid, Spain) except for the fluorochromes, which were purchased from Invitrogen (Barcelona, Spain).

Beltsville Thawing Solution (BTS, Minitub ibérica, S.L., Tarragona, Spain) was used as the basic semen extender; and supplemented with bovine serum albumin (BSA: 6 mg/mL) was used for post-thawing sperm dilution. The basic freezing extender (FE-1) was composed by lactose (11% w:v) and egg yolk (20% v:v; Westendorf et al., 1975). The FE-1 was centrifuged (12,000 g, 20 min, 5 ºC) and the supernatant was recovered and then filtered through 5-µm, 3-µm and 1.2-µm membrane filters (Millipore ibérica, Madrid, Spain) in a three step process. The FE-1 supplemented with 1.5% (v:v) of Orvus Es Paste (Equex STM®, Nova Chemical Sales Inc., Scituate, MA, USA) and 9% (v:v) of glycerol or 15 or 21% (v:v) (see experimental design) of the different amides evaluated (L, A, MA, DMA, F, MF, DMF) was used for a second semen dilution at 5 °C before freezing (FE-2). The final concentrations of cryoprotectants in the cryopreserved samples were 3% (v:v) for glycerol and 5 or 7% for the amides (w:v for L and A and v:v for MA, DMA, F, MF and DMF).

To evaluate the in vitro fertilizing ability of the sperm, a homologous in vitro penetration assay was performed according to the method described by Martínez et al. (1993). The composition of all the extenders used [NaCl solution, modified Dulbecco´s phosphate-buffered saline (mDPBS), modified M-199 with Earle´s salts and sodium

hydrogen carbonate (mTCM-199) and the co-incubation medium (IVF-TCM199)] is detailed in Tomás et al. (2011).

2.2. Preparation of cholesterol-loaded cyclodextrins (CLC)

Methyl-β-cyclodextrin was preloaded with cholesterol in accordance to the protocol developed by Purdy and Graham (2004), as described elsewhere (Blanch et al., 2012). Working solutions of CLC and MBCD were freshly prepared by mixing 50 mg of CLC or MBCD in 1 mL of BTS using a vortex mixer on the day of use.

2.3. Semen collection and dilution

Mature Pietrain boars (n=4) were housed in the experimental farm of Centro de Tecnología Animal (CITA-IVIA, Segorbe, Castellón, Spain). Boars were fed with commercial diet (once/day) and provided water *ad libitum.* All boars were maintained under the guidelines approved by the Ethics Committee from the Instituto Valenciano de Investigaciones Agrarias and fulfilled the European regulations for the Care and Use of Animals for Scientific Purposes (EC Directive 2010/63/EU).

Sperm rich ejaculate fractions were collected weekly by the gloved-hand method and placed in pre-warmed thermo flasks covered with a gauze tissue to hold on the ejaculate gel fraction. Thereafter, sperm rich ejaculate fractions were diluted (1:1, v:v) with BTS at 37 ºC and slowly cooled to 22 ºC during 2 h.

2.4. Sperm cryopreservation

Only those ejaculates with >75% motile sperm were cryopreserved. The ejaculates were split into 3 fractions: one fraction was used untreated and the other two were treated with 1 mg/120 x 10^6 sperm of MBCD or CLC, respectively. The treated samples were

incubated with MBCD or CLC for 15 min at 22 ºC (Blanch et al., 2012). Thereafter the three aliquots were cooled to 16 ºC during 2 h. After cooling to 16 ºC, the semen was processed using the straw-freezing procedure described by Westendorf et al. (1975) with some modifications (Blanch et al., 2012), except for sperm final concentration that was 800 x 10^6 sperm/mL. Thawing of straws was performed in a water bath at 39 ºC for 30 s. Post-thaw sperm quality was assessed in semen samples extended in BTS-BSA (25 x 10^6 sperm/mL) and incubated in a water bath at 39 ºC for 10 min.

2.5. Sperm quality evaluation

Sperm motility was objectively evaluated using a computer-aided sperm analysis system (ISAS® 1.0.17, Proiser R+D, Paterna, Spain) according to the protocol described by Blanch et al. (2012). The percentages of total motile sperm (% TMS, VAP>10 μm/s) and rapid progressively motile sperm (% RPM, VAP > 45 μm/s and STR ≥ 45%) were recorded.

Flow cytometry analyses were performed under dimmed light using either an EPICS XL flow cytometer (Coulter Corporation Inc., Miami, FL, USA) equipped with an argon ion laser (Cyonics, Coherent, Santa Clara, CA, USA), with 15 mW of laser power at 488 nm or an Coulter FC500 flow cytometer (Coulter Corporation Inc., Miami, FL, USA) equipped with an argon ion and an Helium-Neon laser (Cyonics, Coherent, Santa Clara, CA, USA), with 15 mW of laser power at 488 and 630 nm, respectively. Data were analyzed using Expo 32 ADC software in EPICS XL and CXP Analysis in Coulter FC500 (Beckman Coulter Inc, Miami, FL, USA). A total of 10,000 events with forward and side scatter properties similar to sperm cells were initially gated. From these events, those without reasonable DNA content after SYBR-14/ propidium iodide (PI) staining were gated out (double-gated sperm analysis). In the following cytometry test carried out, the

percentages of these non-DNA events with scatter characteristics similar to sperm cells were taken into account to calculate the true sperm counts (Petrunkina et al., 2010).

Sperm viability (% SV) was evaluated by assessment of plasma membrane integrity using the LIVE/DEAD Sperm Viability Kit (L-7011; Molecular Probes Europe BV, Leiden, the Netherlands) and following the procedure described by Purdy and Graham (2004) and modified by Blanch et al. (2012). The samples were stained and incubated at 39 °C in the dark for 10 min. The fluorescence spectra of SYBR-14 and PI were detected using 505-545 and 605-635-nm band-pass filters, respectively. The analyzed sperm were categorized as follows: intact plasma membrane (SYBR-14+/PI−) and damaged plasma membrane (PI+). Only the sperm population that exhibited intact plasma membrane was showed in the results.

Mitochondrial membrane potential was analyzed using a triple fluorescent procedure for flow cytometric analysis (SYBR 14- PI- Mitotracker Deep Red 633, MTDR) described by Januskauskas et al. (2005) and Martinez-Pastor et al. (2008) with modifications, as indicated below. Fifty µL aliquots of thawed sperm (25×10^6 sperm/mL) were diluted in 450 µL of PBS containing 25 µL of SYBR-14 (100 nm in DMSO), 2.5 µL of PI (1.5mM in purified water) and 3.75 µL of MTDR (6.6 µM in DMSO) and incubated at 37 ºC in the dark for 20 min. The fluorescence spectra of SYBR-14, PI and MTDR were detected using 505-545, 605-635, 600-650 -nm band-pass filters, respectively. The analyzed sperm were categorized as follows: viable sperm with high mitochondrial membrane potential (SYBR-14+ and high MTDR fluorescence), viable sperm with low mitochondrial membrane potential (SYBR-14+ and low MTDR fluorescence), and dead cells (PI+). Only viable sperm with low mitochondrial membrane potential were showed in the results.

2.6. In vitro fertilizing ability evaluation

The in vitro fertilizing ability of frozen-thawed boar sperm was evaluated in immature pig oocytes using a homologous in vitro penetration test conducted in accordance to the protocol described by Martínez et al. (1993), detailed in Tomás et al. (2011).

The straws were thawed as described previously. After thawing, an aliquot of 250 µL of the thawed sperm (200 x 10^6 sperm) was washed in 10 mL of mDPBS and centrifuged at 1,200g for 3 min at 30 ºC. The supernatant was removed, and the sperm pellet was resuspended in mTCM-199.

The oocyte preparation and oocyte-sperm co-incubation were performed according to the method described by Tomás et al. (2011). Briefly, ovaries were washed in a NaCl solution, the cumulus oocyte complexes (COCs) were collected by slicing the ovaries and the COCs were transferred to a Petri dish. The oocytes surrounded by a compact cumulus mass and containing a uniform ooplasm were selected and transferred in groups of 20 into Petri dishes containing 2 mL IVF-TCM199. The sperm (4 x 10^6 spermatozoa at a final concentration of 2 x 10^6 sperm/mL) were added to each Petri dish and the gametes were co-incubated for 16-18h at 39 ºC in an atmosphere of 5% CO_2 and 100% humidity. A the end of the co-incubation period, the oocytes were denuded by pipetting, placed onto glass slides and fixed and stained with a solution of 1% (w:v) lacmoid (diluted with 45% (v:v) acetic acid in ultrapure water).

The oocytes were examined for evidence of sperm penetration under a phase-contrast microscope at 400 x 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 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. Experimental design

2.7.1. Experiment 1: The effect of different amides on fresh semen

This experiment was performed to determine the effect of the amides on fresh boar semen. The amides were used at a concentration of 5% because this concentration resulted in the best post-thaw results in previous studies (Bianchi et al., 2008). Fresh ejaculates (n = 6) were centrifuged at 800 x g for 10 min at 22 ºC (Eppendorf Ibérica, S.L., Madrid, Spain), the seminal plasma was removed and the pellet was resuspended with BTS and split into three aliquots. Aliquots one and two were treated with 1 mg of MBCD or CLC/ 120 x 10^6 sperm and the third aliquot was used as control sample (non-treated). The aliquots were incubated for 15 min at 22 ºC and then they were resuspended with FE-1 to a concentration of 1200 x 10^6 sperm/mL and split each one in 8 subsamples. Then, the subsamples were re-diluted (2:1, v:v) with the different FE-2 extender (9% glycerol, or 15% of L, A, MA, DMA, F, MF, or DMF) to a final concentration of 800 x 10^6 sperm/mL and equilibrated for 15 min at 22 ºC. The sperm viability was evaluated at time 0 and after incubation at 22 ºC for 4h following the protocol previously described.

2.7.2. Experiment 2: Effect of two concentrations of DMA on boar sperm cryosurvival

This experiment was performed to determine the effect of two concentrations of DMA on the cryosurvival of boar sperm. The DMA was chosen based on the results obtained in the first experiment and in previous studies (Bianchi et al., 2008). Ejaculates were collected (n = 10), each was split into three aliquots and they were treated with CLC or MBCD as described previously. After this, all the samples were cooled to 16 ºC, centrifuged, resuspended with FE-1 and cooled to 5 ºC. Once at 5 ºC, the control aliquot

was split into three subsamples and MBCD and CLC aliquots into two subsamples. Then, control samples were diluted with FE-2 (2:1, v:v) containing 9% of glycerol, 15% DMA or 21% DMA (final concentrations of 3, 5 and 7%, respectively). MBCD and CLC samples were diluted with FE-2 containing 15 or 21% of DMA (final concentrations of 5 and 7%, respectively). After, semen aliquots were frozen and thawed as described above. Motility and sperm viability were evaluated after thawing in accordance to the protocols previously described.

2.7.3. Experiment 3: Determining the effectiveness of different amides as cryoprotectants for boar semen

In this experiment we determined the cryoprotective effect of DMA, MF and DMF for boar sperm. Consequent to the results obtained in the first experiment, the amides A, L and F were not used. In addition, MA was not used due to its low cryoprotective effect (Blanch et al., 2010). For this experiment ejaculates were collected (n = 10) and each was split into three aliquots that were treated in the same way as in the previous experiments (control, MBCD, CLC). Aliquots were then cooled to 16 ºC, centrifuged, resuspended with FE-1 and cooled to 5 ºC. After cooling to 5 ºC, each aliquot was split into four subsamples and diluted with FE-2 containing 9% of glycerol or 15% of DMA, MF or DMF (final concentrations of 3 and 5%, respectively). Samples were frozen and thawed as described above. After thawing the sperm motility, sperm viability and mitochondrial membrane potential were evaluated in accordance to the protocols previously described.

2.7.4. Experiment 4: Determining the in vitro fertilizing ability of boar sperm cryopreserved with DMF or glycerol

Consequent to the results obtained in the experiment 3, the fertilizing ability of CLC-treated and control sperm frozen with glycerol or DMF was compared in an in vitro assay. Nine of the ten ejaculates cryopreserved in the experiment 3 were used. After thawing, 3 straws per treatment from 3 different ejaculates were mixed to constitute a pool. Thus, the 9 ejaculates resulted in 3 different pools per treatment. Then, the sperm and the oocytes were prepared as described previously. The sperm were added to three Petri dishes containing 20 oocytes/dish/treatment, thus, 180 oocytes were used per treatment. From these 180 oocytes, those considered as degenerated after the co-incubation were not evaluated.

2.8. Statistical analyses

Statistical analyses were performed using SAS package (version 9.0, 2002, SAS Institute Inc., Cary, NC, USA). The data for the SV, TMS and RPM (experiments 1, 2 and 3) and the data for sperm per penetrated oocyte (experiment 4) were analyzed using a mixed model analysis of variance (ANOVA) including the ejaculate within male as a random effect. In the first experiment, semen treatment (three levels: control, MBCD, CLC), cryoprotectant (eight levels: glycerol, L, A, MA, DMA, F, MF, DMF) and their interaction were included as fixed effects. In the second experiment, treatment (seven levels: control-glycerol, control-5% DMA, control-7% DMA, MBCD-5% DMA, MBCD-7% DMA, CLC-5% DMA, CLC-7% DMA) was included as fixed effect. In the third experiment, semen treatment (three levels: control, MBCD, CLC), cryoprotectant (four levels: glycerol, DMA, MF, DMF) and their interaction were included as fixed effects. In the fourth experiment, semen treatment (two levels: control, CLC), cryoprotectant (two levels: glycerol, DMF) and their interaction were included as fixed effects. The Tukey adjustment was used to test the differences of the least square means at a fixed 5% error

level and the results are presented as the least square mean values (LSM) ± the standard error (SE).

The binomial data of oocyte penetration rate were analyzed using logistic regression models, including the pool of ejaculates as a random effect and the semen treatment (two levels: control, CLC), cryoprotectant (two levels: glycerol, DMF) and their interaction as fixed effects. The results are presented as predictions on the scale of the data ± the associated standard error.

3. RESULTS

3.1. Experiment 1: The effect of different amides on fresh semen

Lactamide, acetamide and formamide negatively affected the sperm viability at both 0 and 2 h of incubation at 22 ºC (Table 1; $P < 0.05$), while the other amides did not affect the sperm quality.

With respect to the sperm treatment, MBCD samples exhibited the worst SV at times 0 and 4 h (Table 1; $P < 0.05$), whereas CLC samples showed similar SV than control samples at time 0. In addition, CLC samples exhibited higher SV than control or MBCD samples after 4 h of incubation at 22 ºC (Table 1; $P < 0.05$).

The interaction between semen treatment and type of amide was not significant (data not shown in tables).

Table 1. Molecular weights (MW) of the cryoprotectants and percentages of sperm with intact plasma membrane (sperm viability; SV) in boar semen samples treated with 1 mg/120 x 10^6 sperm of methyl-β-cyclodextrin (MBCD) or methyl-β-cyclodextrin pre-loaded with cholesterol (CLC) and subsequently diluted in a extender with 3% of glycerol or 5% of different amides. Evaluation was performed before (0h) and after incubation at 22 ºC for 4h (n = 6 ejaculates).

Semen treatment	Cryoprotectant	MW (g/mol)	SV 0h (%)	SV 4h (%)
Control			70.57 ± 3 ^{x}	68.29 ± 3 ^{y}
MBCD			57.00 ± 3 ^{y}	59.00 ± 3 ^{z}
CLC			74.48 ± 3 ^{x}	74.69 ± 3 ^{x}
	Glycerol	92.09	78.63 ± 4 ^{a}	78.80 ± 3 ^{a}
	Lactamide	89.09	40.31 ± 4 ^{c}	39.59 ± 3 ^{c}
	Acetamide	59.07	45.74 ± 4 ^{bc}	44.56 ± 3 ^{c}
	Methylacetamide	73.10	79.82 ± 4 ^{a}	78.58 ± 3 ^{a}
	Dimethylacetamide	87.12	81.40 ± 4 ^{a}	79.80 ± 3 ^{a}
	Formamide	45.05	50.54 ± 4 ^{b}	58.55 ± 3 ^{b}
	Methylformamide	59.07	81.05 ± 4 ^{a}	79.63 ± 3 ^{a}
	Dimethylformamide	73.10	81.28 ± 4 ^{a}	78.84 ± 3 ^{a}

The results are presented as least square mean values ± standard error (LSM ± SE); ^{x,y,z}: uncommon superscripted letters indicate differences ($P < 0.05$) between semen treatments within a column; ^{a,b,c}: uncommon superscripted letters indicate differences ($P < 0.05$) between cryoprotectants within a column.

3.2. **Experiment 2:** Effect of two concentrations of DMA on boar sperm cryosurvival

The samples cryopreserved with DMA exhibited higher SV ($P<0.05$; Table 2) than the control samples cryopreserved with glycerol, regardless of the concentration of DMA (5 or 7%) or semen treatment (control, MBCD or CLC). However, samples frozen with 7% of DMA showed percentages of TMS or RPM significantly lower than samples frozen with glycerol (Table 2; $P < 0.05$) in all semen treatments.

Table 2. Post-thaw percentages of total motile sperm (TMS), rapid progressively motile sperm (RPM) and sperm with intact plasma membrane (sperm viability; SV) when boar spermatozoa (n = 10 ejaculates) were treated with 1 mg/120 x 10^6 sperm of methyl-β-cyclodextrin (MBCD), or methyl-β-cyclodextrin pre-loaded with cholesterol (CLC) and then cyropreserved with 3% of glycerol or different percentages (5 or 7%) of dimethylacetamide (DMA).

Semen treatment		TMS (%)	RPM (%)	SV (%)
Control	3% Glycerol	30.90 ± 4 [a]	26.40 ± 4 [a]	48.93 ± 3 [b]
Control	5% DMA	26.20 ± 4 [ab]	21.90 ± 4 [ab]	63.94 ± 3 [a]
Control	7% DMA	22.60 ± 4 [b]	17.90 ± 4 [b]	62.69 ± 3 [a]
MBCD	5% DMA	27.80 ± 4 [ab]	21.80 ± 4 [ab]	62.10 ± 3 [a]
MBCD	7% DMA	22.20 ± 4 [b]	17.30 ± 4 [b]	57.30 ± 3 [a]
CLC	5% DMA	33.20 ± 4 [a]	27.20 ± 4 [a]	61.93 ± 3 [a]
CLC	7% DMA	22.30 ± 4 [b]	17.80 ± 4 [b]	64.45 ± 3 [a]

The results are presented as least square mean values ± standard error (LSM ± SE); [a,b]: uncommon superscripted letters indicate differences ($P < 0.05$) between treatments within a column.

3.3. Experiment 3: Determining the effectiveness of different amides as cryoprotectants for boar semen

With respect to the semen treatment, there were contradictory results between the percentages of viable sperm and the percentages of motile sperm. Whereas the SV was significantly lower for CLC samples than for control samples, the opposite was observed

for the percentages of TMS and RPM (Table 3; $P < 0.05$). In addition, MBCD samples showed the lowest percentages in all the parameters evaluated.

As in the previous experiment, important differences between SV and motility arose in those samples cryopreserved with amides. All the amides significantly enhanced the percentages of SV compared to glycerol, especially MF and DMF. However, these samples showed significantly lower percentages of TMS and RPM than samples frozen with glycerol, mainly samples cryopreserved with MF (Table 3; $P < 0.05$).

The mitochondrial membrane potential was similar for all the semen treatments and cryoprotectants (Table 3; $P > 0.05$).

In addition, interactions between semen treatments and cryoprotectants were not observed for any of the parameters evaluated (data not shown in tables).

Table 3. Post-thaw percentages of total motile sperm (TMS), rapid progressively motile sperm (RPM), sperm with intact plasma membrane (sperm viability; SV) and viable sperm with low mitochondrial membrane potential (VS-LMMP) when boar spermatozoa (n = 10 ejaculates) were treated with 1 mg/120 x 10^6 sperm of methyl-β-cyclodextrin (MBCD), or methyl-β-cyclodextrin pre-loaded with cholesterol (CLC) and then cryopreserved with 3% of glycerol, 5% of methylformamide (MF), 5% of dimethylacetamide (DMA) or 5% of dimethylformamide (DMF).

Semen treatment	**Cryoprotectant**	**TMS (%)**	**RPM (%)**	**SV (%)**	**VS-LMMP (%)**
Control		24.14 ± 5 ^{y}	20.59 ± 5 ^{y}	52.69 ± 4 ^{x}	4.21 ± 1
MBCD		21.74 ± 5 ^{y}	18.83 ± 5 ^{y}	44.59 ± 4 ^{y}	2.94 ± 1
CLC		34.24 ± 5 ^{x}	29.17 ± 5 ^{x}	47.46 ± 4 ^{y}	3.62 ± 1
	Glycerol 3%	35.90 ± 5 ^{a}	32.40 ± 5 ^{a}	41.22 ± 4 ^{c}	3.02 ± 1
	MF 5%	20.00 ± 5 ^{c}	16.23 ± 5 ^{c}	53.24 ± 4 ^{a}	4.02 ± 1
	DMA 5%	25.73 ± 5 ^{b}	22.10 ± 5 ^{b}	46.01 ± 4 ^{b}	3.66 ± 1
	DMF 5%	25.20 ± 5 ^{bc}	20.73 ± 5 ^{bc}	52.51 ± 4 ^{a}	3.65 ± 1

The results are presented as least square mean values ± standard error (LSM ± SE); ^{x,y}: uncommon superscripted letters indicate differences ($P < 0.05$) between semen treatments within a column; ^{a,b,c}: uncommon superscripted letters indicate differences ($P < 0.05$) between cryoprotectants within a column.

3.4. Experiment 4: Determining the in vitro fertilizing ability of boar sperm cryopreserved with DMF or glycerol

The sperm treated with CLC exhibited greater numbers of sperm per penetrated oocyte than the control samples (Table 4; $P < 0.05$). Besides samples frozen with DMF showed lower penetration ability and fewer number of sperm per penetrated oocyte than samples frozen with glycerol (Table 4; $P < 0.05$).

An interaction between semen treatment and cryoprotectant was observed for the number of sperm per penetrated oocyte (Table 4; $P < 0.05$). While the number of sperm per penetrated oocyte was similar for CLC and control samples frozen with DMF, CLC samples exhibited greater numbers of sperm per penetrated oocyte than control samples when the glycerol was used.

Table 4. Sperm penetration ability of sow´s immature oocytes in vitro (penetration rate (%) and number of sperm per oocytes) when boar spermatozoa (n = 3 pools of semen) were treated with 1 mg/120 x 10^6 sperm of methyl-β-cyclodextrin (MBCD), or methyl-β-cyclodextrin pre-loaded with cholesterol (CLC) and then cryopreserved with 3% of glycerol or 5% of dimethylformamide (DMF).

Semen treatment	Cryoprotectant	Penetration rate (%)[1]	No. of sperm/oocyte[2]
Control		43.63 ± 4 (118/301)	2.70 ± 0.3 ^{y}
CLC		50.09 ± 4 (172/347)	3.74 ± 0.3 ^{x}
	Glycerol	80.09 ± 2* (238/296)	4.87 ± 0.3 *
	DMF	16.19 ± 2 (52/352)	1.58 ± 0.3
Control	Glycerol	76.00 ± 4 (95/125)	3.81 ± 0.4 ^{b}
	DMF	15.91 ± 3 (23/176)	1.59 ± 0.4 ^{c}
CLC	Glycerol	83.63 ± 3 (143/171)	5.91 ± 0.4 ^{a}
	DMF	16.48 ± 3 (29/176)	1.57 ± 0.4 ^{c}

[1]: the results are presented as predictions on the scale of the data ± the associated standard error. [2]: results are presented as least square mean values ± standard error (LSM ± SE); ^{x,y}: uncommon superscripted letters indicate differences (P < 0.05) between semen treatments within a column; *: Indicate differences (P < 0.05) between cryoprotectants within a column; ^{a,b,c}: uncommon superscripted letters indicate differences (P < 0.05) between "semen treatment x cryoprotectants" within a column.

4. DISCUSSION

One factor that directly influences the quality of frozen-thawed sperm is the cryoprotective agent (Forero-Gonzalez et al., 2012). The ability of a compound to act as an effective cryoprotectant depends upon its capacity to protect cells from freezing damage and its non-toxicity to the cells (Squires et al., 2004). Glycerol has been the major penetrating cryoprotectant routinely used to freeze boar semen. However, glycerol causes injury to cells during the freezing process, partly due to the osmotic stress caused when glycerol permeates the cell membrane (Gilmore et al., 1998). Amides have lower molecular weights (MW) and will penetrate more readily and induce less osmotic damage than the glycerol (Alvarenga et al., 2005).

From the amides evaluated, L, A and F were detrimental for boar sperm at the concentration of 5%. Sperm viability was similar among the other treatments, indicating that MA, DMA, MF and DMF do not produce immediate detrimental effects on sperm. Our findings were similar to the results found in dog (Futino et al., 2010) and ram (Moustacas et al., 2011) where MF and DMF were tested, but differ from previous studies in boar where A at concentrations ≤ 5.9% (w:v) did not affect the percentage of motile sperm (Wilmut and Polge, 1977). It is likely that this disagreement is due to the differences in the protocol followed to treat and evaluate the sperm after diluting with the freezing extenders (dilution of the samples or not in cryoprotectant-free diluents before evaluating the sperm quality). A priori, osmotic shock is discarded based on the MW of the amides (Table 1) which will permeate more readily the membrane than the glycerol. However, permeability does not exclusively depend on MW but also on some other factors (such as membrane lipid composition, molecule composition and temperature; Finkelstein, 1976). Thus, MA, DMA, MF and DMF are highly lipophilic due to incorporation of methyl (CH_3) into the amide

molecule, which increases their permeability through the sperm membrane (Bianchi et al., 2008). Whether the detrimental effect of A, F and L on the sperm is due to osmotic shock, toxicity or a combination or both was not determined.

Compared to the control samples cryopreserved with glycerol (3%), DMA provided greater post-thaw SV irrespective of its concentration (5 or 7%) and of the sperm treatment (control, MBCD or CLC). The cryoprotective effect of DMA on SV can be attributed to its ability to permeate the cell membrane, that will decrease the possibility of cellular damage caused by osmotic stress. These results are in agreement with those reported by Bianchi et al. (2008) and in disagreement with those observed by Kim et al. (2011) who found no differences between 5% DMA and 3% glycerol. The reason for this dissimilarity may be due to differences in the methods used for the evaluation of sperm plasma membrane integrity. Nevertheless, in our study, this improvement was not reflected in motility parameters and those samples frozen with 7% DMA showed the worst results and samples frozen with 5% DMA exhibited similar motility than those frozen with glycerol. These results were in agreement with those observed by other authors (Buranaamnuay et al., 2011; Kim et al., 2011) and in disagreement with the results reported by Bianchi et al. (2008). This unexpected effect of amides on sperm motility remains unclear and will be discussed below. For a global view, control samples frozen with glycerol and CLC samples frozen with 5 % DMA showed the highest ratios (63 and 53 %, respectively; data not shown in Tables) of functioning sperm (expressed as (% TMS x 100)/ % SV) .

The other amides evaluated (MF and DMF) behaved similarly to the DMA. Sperm frozen with amides showed higher post-thaw SV than samples frozen with glycerol, and in particular, MF and DMF achieved the highest percentages of SV. Besides, amides provided

lower motility rates after cryopreservation than glycerol, providing the DMF the best combination of motility and SV after cryopreservation. Again, this increased cryoprotective effect of amides on SV can be ascribed to their highly lipophilic nature and their low molecular weight and viscosity in comparison with glycerol which will increase their permeability through the sperm cell membrane. These results are in agreement with previous reports (Bianchi et al., 2008) but are in disagreement with other studies where 3% DMF (Malo et al., 2012) or 5% DMA or DMF (Buranaamnuay et al., 2011) were tested.

Samples frozen with amides showed substantial differences between the percentages of sperm viability and sperm motility, in accordance with previous studies (Malo et al., 2012; Buranaamnuay et al., 2011). Since all the samples exhibited similar percentages of live sperm with low MMP, we can discard the malfunction of the mitochondria as the cause of the difference between the parameters in the samples cryopreserved with amides. Whether this decline in motility is reversible or not once inseminated in the female is unknown. There is evidence that the cryoprotectants exert biochemical injury apart from the osmotic damages (Arakawa et al., 1990). Undesirable effects of glycerol on the sperm have been reported (reviewed by Hammersted et al., 1990) but similar studies have not been conducted yet for the amides. Some authors suggested that glycerol damage is related to an osmotic effect but is also due to the intrinsic toxicity upon the actin cytoskeleton that may compromise the capacity of the spermatozoa to cope with osmotic stress (Macías-García et al., 2012). Based on the low MW of the amides, they will induce less osmotic damage on the membrane than glycerol (Alvarenga et al., 2005), and for this reason they might improve the post-thaw SV. Nonetheless, the reduction in the motility could be due to a direct effect of these compounds on the cytoskeleton which would compromise the motion ability of the spermatozoa, without changing the amount of

ATP generated by the mitochondria. Alternatively, the freezing velocities routinely used for the cryopreservation of samples processed with glycerol could not be optimal for the samples frozen with amides. Some authors suggested that the samples frozen with amides would benefit from faster freezing rates because of the differences in permeability and molecular weight between these compounds and glycerol (Alvarenga et al., 2005).

Treating boar sperm with MBCD was detrimental for the SV in fresh sperm while the CLC treatment significantly enhanced the SV after 4 h of incubation at 22 ºC. MBCD removes cholesterol from the membrane (Tomás, 2012) and reduces the osmotic tolerance limits of boar sperm (Tomás et al., 2011), and for these reasons their membrane will not be able to cope well with the volume excursions produced during the addition and the removal (when sperm were diluted for the evaluation of the SV) of the cryoprotectants. Contrary CLC adds cholesterol to the membrane (Tomás, 2012) and enhances the osmotic tolerance limits of boar sperm (Tomás et al., 2011), rendering a membrane more resistant to the volume excursions produced during the addition and the removal of the cryoprotectants. According to these results, it was expected that MBCD samples would present lower quality and CLC samples would present similar or higher quality after freezing-thawing than control samples.

Nevertheless, the response of boar sperm to the treatment with CLC after cryopreservation was variable, results in accordance with previous observations where glycerol was used (Bailey et al., 2008, Torres et al., 2009, Tomás et al., 2011; 2012; Blanch et al., 2012). In addition, the treatment of boar sperm with MBCD did not affect the sperm quality after thawing, which agrees with the results of previous studies that used the same protocol to treat the sperm (Tomás et al., 2011; 2013; Blanch et al., 2012). This lack of effect may be due to the presence of egg yolk in the freezing extender. Thus, when an egg

yolk-free extender was used, this treatment resulted in decreased sperm viability after cold shocking the sperm (Galantino-Homer et al., 2006).

Moreover, we did not observe interactions between the sperm treatments (control, MBCD, CLC) and the type of cryoprotectant. Our results are similar to those observed in a previous study with Colombian creole stallion sperm using glycerol and DMF (Mesa and Henao, 2012). Since CLC treatment enhanced the osmotic tolerance limits of boar sperm (Tomás et al., 2011), it is possible that the difference in MW between cryoprotectants were not big enough to influence sperm cryosurvival. The percentage of SV was significantly lower in samples treated with MBCD or CLC than in the control samples. However, CLC treated samples showed the highest ratio of functioning sperm (72%) compared to control and MBCD (< 50%; data not shown in Tables). These results are in accordance with previous studies in our laboratory (Blanch et al., 2012; Blanch et al., 2014; Tomás et al., 2011) where the response to the treatment with CLC was highly variable. The reasons for this variability remain unclear and have been extensively discussed previously (Blanch et al., 2012, Blanch et al., 2014; Tomás et al., 2011).

The gamete interaction assays evaluate the effectiveness of the sperm receptors to bind to the oocyte and to the oolemma and to initiate fertilisation (Graham and Mocé, 2005). 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). Indeed, a positive correlation between the in vitro penetration rate and the in vivo boar sperm fertilising ability has been observed (Martínez et al., 1998).

CLC sperm exhibited greater numbers of sperm per penetrated oocyte than control samples, result in agreement with previous reports in boar sperm frozen with glycerol (Tomás et al., 2011). With respect to the cryoprotectant, sperm frozen with glycerol showed higher penetration ability and greater numbers of sperm per penetrated oocyte than the sperm frozen with DMF. To the best of our knowledge, this is the first study where the in vitro fertilizing ability of boar sperm frozen with amides is reported. The lower fertility of the samples frozen with DMF could be due to the lower motility that these samples exhibited or to the lower percentage of functioning sperm (48%), compared to the samples frozen with glycerol (87%; data not shown in Tables). Albeit the interaction between the cryoprotectant and the sperm treatment indicates that the effects of DMF on the sperm are not only limited to the motility. Indeed, DMF abolishes the effects that CLC treatment has on the sperm penetration ability. For this reason, it is likely that DMF is affecting some sperm structures involved in the fertilization process or is interacting with the normal progress of the mechanisms that lead to the fertilization.

In conclusion, lactamide, acetamide and formamide negatively affected the sperm viability immediately after addition to fresh semen. The other amides efficiently improved the sperm viability after cryopreservation but negatively affected the sperm motility (MF, DMA and DMF) and the sperm fertilizing ability in vitro (DMF), irrespective of the sperm treatment. Besides, CLC treated samples frozen with glycerol showed better in vitro fertilizing ability than control samples. According to our results, the substitution of glycerol for amides in the boar sperm cryopreservation diluents is a priori not recommended but the effect that these compounds have on in vivo fertilizing ability should be studied also in order to draw definitive conclusions.

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CHAPTER 6

GENERAL DISCUSSION

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1. RESPONSE OF BOAR SPERM TO THE TREATMENT WITH CHOLESTEROL-LOADED CYCLODEXTRINS (CLC) OR CYCLODEXTRINS ALONE (MBCD) USING A CONVENTIONAL FREEZING EXTENDER

In order to develop a protocol for adding CLCs to boar sperm, preliminary experiments were conducted to determine the optimal type of cyclodextrin (methyl-β-cyclodextrin) and the optimal step of the cryopreservation protocol for treating the sperm with CLCs (after the incubation of boar sperm with seminal plasma at 16 ºC) (Blanch, 2007). Taking these results as our starting point, the next step was to determine the adequate concentration of CLC for treating boar sperm prior to cryopreservation (Chapter 3). The CLC concentration that improved the percentage of plasma membrane intact sperm both immediately after thawing and 1 h after incubation and the percentage of total motile sperm 1 h after thawing was 1 mg/120 x 10^6 sperm. This is similar to the optimal CLC concentration reported by others for boar sperm (1.5 mg; Lee et al., 2015) and for other species sperm (1-3 mg CLC/ 120 x 10^6 sperm) including bull, ram, goat, stallion, and donkey sperm (Mocé et al., 2010). However, this CLC-treatment improved boar sperm motility and viability only 6 to 8 percentage points after thawing. This benefit is smaller than the increment observed in other studies with boar sperm, which reported increases in sperm viability rates after CLC-treatment of 18 percentage points (Lee et al., 2015) or in subjective sperm motility rates of 12 percentage points (Torres et al., 2009). These different results may be due to differences between the breeds or males used, different protocols used to treat the sperm with CLCs (in presence or absence of egg yolk) or the type of cyclodextrin used. In other species most studies reported increments in cryosurvival rates by 10 to 20 percentage points after CLC-treatment (Mocé et al., 2010). The differences between boar sperm and sperm from other

cold-shock sensitive species in their response to CLC-treatment may be due to differences in the specific phospholipids that compose the sperm plasma membranes (Parks and Lynch, 1992), since the phospholipid composition of boar sperm membrane is more similar to sperm from some of the more cold-shock resistant species such as human and rabbit (Watson, 1981). These results suggested that adjustments in the freezing extender for CLC-treated boar sperm would be necessary. Therefore, different concentrations of EY and glycerol as well as alternative cryoprotectants were evaluated (Chapters 4 and 5).

Unexpectedly, treating boar sperm with cyclodextrins alone (a treatment that decreases the cholesterol content of the sperm membrane; Companyó et al., 2007) did not decrease sperm quality after thawing. Treating boar sperm with MBCD was neither beneficial nor detrimental to boar sperm, as it was confirmed later by other authors (Lee et al., 2015). However, this response was contrary to the one observed in previous reports in which the addition of cyclodextrin alone improved (Zeng and Terada, 2000; 2001) or decreased (Bailey et al., 2008) the boar sperm quality after cryopreservation. The differences observed between the results of different studies may be due to differences in the protocols used to treat the sperm with the cyclodextrins, since some added cyclodextrin to the sperm in the presence or absence of egg yolk during the incubation (it is likely that cyclodextrins capture cholesterol from egg yolk), or to the amount of egg yolk added in the freezing extender.

Differences in the ability of sperm from individual sires to survive cryopreservation have been well documented (Holt 2000; Roca et al., 2006). One reason for this phenomenon may be due to differences in the amount of cholesterol composing the sperm plasma membrane from different boars. Thus, it is reasonable to assume that CLC treatment will be more effective for freezing sperm from boars classified as “poor freezers” than for sperm from boars which cryopreserved well. This, however, was not the case, as although

CLC treatment slightly improved the percentage of viable, total and progressively motile sperm, these differences were not significant. It would be important to study the effect of CLCs in a larger number of males to draw definitive conclusions on this subject. In addition, studying the composition of the sperm plasma membranes from males classified as good or poor freezers would also be interesting to determine the role of cholesterol in boar sperm cryopreservation.

2. RESPONSE OF BOAR SPERM TO THE TREATMENT WITH CHOLESTEROL-LOADED CYCLODEXTRINS (CLC) OR CYCLODEXTRINS ALONE (MBCD) USING ALTERNATIVE GLYCEROL AND EGG YOLK CONCENTRATIONS IN THE FREEZING EXTENDER

2.1. Egg yolk

The protective effect of egg yolk (EY), minimizing cold stress, is related with its content in cholesterol and phospholipids, which are in part moved to sperm membranes (Bathgate et al., 2006; Bergeron and Manjunath, 2006). Since CLC-treatment increased the cholesterol amount of sperm membranes (Mocé et al., 2010), the EY of freezing extenders could be partially or even completely withdrawn, always considering that the cryoprotective effect of EY is totally or partially due to its high cholesterol content. Therefore, treating the sperm prior to cryopreservation with CLC could be a useful strategy to reduce the EY concentration in the freezing extenders, and this could be a first step towards the development of chemically defined extenders.

In Chapter 4, reducing the concentration of EY from 20 to 10% (v:v) in lactose-based diluent was detrimental for sperm viability after thawing irrespective of the treatment that the sperm received. In our study, we decreased the level of EY by increasing the presence of lactose solution in the freezing extender, and it is likely that lactose was detrimental for sperm cryosurvival when used at concentrations above 8.8 % (w/v; concentration of lactose in diluent containing 20 % EY). In this regard some authors did observe a negative effect of lactose on boar sperm motility at concentrations exceeding the one used in our study (Wilmut and Polge, 1977; Corcuera et al., 2007).

Thus, CLC treatment was not effective for freezing boar sperm, irrespective of the EY concentration of the freezing extender. It is possible that at the low EY concentrations the effect of CLC treatment could be understated because of the detrimental effect of high lactose concentration. Otherwise, the concentration of glycerol used traditionally might not be adequate for sperm treated with CLC, as seen in section below.

2.2. Glycerol

The treatment of boar sperm with cholesterol-loaded cyclodextrins widens the sperm osmotic tolerance limits (Tomás et al., 2011). This fact suggests that ~~s~~perm permeability is altered. Thus, it is reasonable to expect that this type of sperm would have different requirements for glycerol concentrations than non-treated sperm.

The general view is that relatively low concentrations of glycerol (1-3%; v:v) are suitable for boar sperm cryopreservation (Paquignon, 1985). In Chapter 4, we gradually increased the glycerol concentration from 2 to 5%, and we observed that these concentrations produced different effects on sperm cryosurvival depending on the treatment the sperm had received (control, MBCD and CLC). Non-treated sperm and sperm treated with MBCD showed the highest cryosurvival with concentrations of glycerol traditionally

used (2-3%) whereas the post-thaw quality of CLC-treated sperm was highest when frozen with the highest glycerol concentrations (4-5%). Increasing the final glycerol concentration of the freezing extender to 7% in CLC-treated sperm samples increased the percentage of sperm with an intact plasma membrane. However, this benefit did not surpass the results obtained for non-treated samples cryopreserved with traditional glycerol concentrations. The increase in the glycerol requirements in CLC-treated sperm to achieve the quality of non-treated boar sperm cryopreserved with lower glycerol concentrations could also indicate that the permeability of the membrane of CLC-treated sperm to this cryoprotectant is reduced and that higher glycerol concentrations would be needed to counteract this effect. In previous studies, CLC-treatment decreased the membrane permeability to water at subzero temperatures (Li et al., 2006) and inhibited the transport of small neutral molecules such as glycerol (Cooper, 1978). The presence of cholesterol significantly increases the orientational order of the phospholipid hydrocarbon chains inducing a condensation effect of the phospholipids resulting in an increase in the thickness and the order of the bilayer. This cholesterol-induced membrane condensation decreases the permeability of the phospholipid bilayer in the liquid crystalline phase (Amann and Pickett, 1987; Johnson et al., 2000; Mocé et al. 2010). Thus, the added cholesterol could become a double edged-sword. The added cholesterol would widen the osmotic tolerance limits and reduce the sensitivity to cold shock (positive effects) but it would also reduce the permeability of the membrane to the cryoprotectant (negative effect). Taking into account this hypothesis, we considered that the substitution of glycerol for low molecular weight cryoprotectants (such as amides) was worth of evaluation (Chapter 5).

Otherwise, the highest concentrations of glycerol that improved the percentage of sperm viability in CLC-treated sperm were detrimental for the percentage of motile spermatozoa after thawing. This decrease in motility might also be due to the higher degree of

dehydration of the sperm when a high concentration of glycerol is used. Since the friction in the tail at a lower intracellular content of water would increase, this friction would in turn cause an inhibition of sliding of microtubules or other structural elements in the flagellum involved in the motility process (Corcuera et al., 2007).

3. RESPONSE OF BOAR SPERM TO THE TREATMENT WITH CHOLESTEROL-LOADED CYCLODEXTRINS (CLC) OR CYCLODEXTRINS ALONE (MBCD) USING ALTERNATIVE CRYOPROTECTANTS (AMIDES) IN THE FREEZING EXTENDER

The amides are a group of cryoprotectants that present lower molecular weights and greater membrane permeability than glycerol and for this reason these compounds will penetrate more readily and induce less osmotic damage than glycerol (Alvarenga et al., 2005). Amides have been increasingly used in the last decade for semen freezing protocols from different species including boar (see Chapter 5), with contradictory results.

The use of Lactamide, Acetamide and Formamide at a concentration of 5% (v:v) are contraindicated for boar sperm, since they were detrimental for the sperm right after diluting the fresh semen with the freezing extender. A priori, osmotic shock could be discarded since the molecular weights of the amides are lower than the molecular weight of glycerol. However, permeability does not exclusively depend on MW (Finkelstein, 1976). Thus, methylacetamide (MA), dimethylacetamide (DMA), methylformamide (MF) and dimethylformamide (DMF) are highly lipophilic due to incorporation of methyl (CH_3) into the amide molecule, which increases their permeability through the sperm membrane (Bianchi et al., 2008).

In order to develop a protocol for freezing boar sperm using amides as cryoprotectants, it is necessary to determine the more appropiate amide concentration and type of amide to use. For this reason, we tested two concentrations (5 and 7%; v:v) of DMA. These concentrations were chosen as a consequence of the results obtained in previous studies (Bianchi et al., 2008; Kim et al., 2011). DMA (5 and 7%) provided greater post-thaw sperm viability than glycerol (3%) irrespective of the sperm treatment (control, MBCD and CLC). The cryoprotective effect of DMA on SV can be attributed to its ability to permeate the cell membrane, that will decrease the possibility of cellular damage caused by osmotic stress. Nevertheless, this improvement was not reflected in motility parameters, and the samples frozen with 7% DMA presented the lowest motility. For a global view, control samples frozen with glycerol and CLC samples frozen with 5 % DMA showed the highest ratios (63 and 53 %, respectively) of functioning sperm (expressed as (% TMS x 100)/ % SV) .

The behavior of the other amides evaluated (MF and DMF) was similar to that exhibited by the DMA. On the one hand, sperm frozen with amides showed higher post-thaw sperm viability than samples frozen with glycerol, in particular MF and DMF. Again, this increased cryoprotectant effect of amides would be due to their highly lipophilic nature and their low molecular weight and viscosity in comparison with glycerol which increase their permeability through the sperm cell membrane. On the other hand, sperm frozen with any of the amides showed lower post-thaw motility parameters than the samples frozen with glycerol. This unexpected effect of amides on sperm motility remains unclear and will be discussed below. From the amides evaluated, DMF was the compound that provided the

best combination of motility and sperm viability after cryopreservation and for this reason it was chosen for the in vitro fertility trial.

Our results for sperm viability are in general agreement with those observed by Bianchi et al. (2008). In addition, our results for sperm motility were in accordance with previous studies (Malo et al., 2012; Buranaamnuay et al., 2011), including the substantial differences observed between the percentages of sperm viability and sperm motility. The malfunction of the mitochondria can be a priori discarded as being responsible for the difference between these parameters since all the samples exhibited similar percentages of live sperm with low mitochondrial membrane potential. Due to the low molecular weight of the amides, these compounds will induce less osmotic damage on the membrane than glycerol (Alvarenga et al., 2005), and for this reason they might improve the post-thaw sperm viability. Thus, the reduced motility could be due to a direct effect of these compounds on the cytoskeleton which would compromise the motion ability of the spermatozoa.

The responses of fresh and cryopreserved boar sperm to the treatment with CLC or MBCD were very different. Thus, treating boar sperm with MBCD was clearly detrimental for the sperm viability in fresh sperm while CLC treatment significantly enhanced the sperm viability after 4 h of incubation at 22 ºC. It has been previously demonstrated that CLC treatment widened the osmotic tolerance limits of boar sperm (Tomás et al., 2011) and it is possible that they would resist better than the other treatments the volume excursions produced during the addition and the removal of the cryoprotectants. Nonetheless, CLC-treated sperm exhibited a variable response after cryopreservation and the pre-freezing treatment of boar sperm with MBCD did not affect the sperm quality after thawing. These results were in accordance with previous studies using glycerol (Tomás et al., 2011; 2013; Chapter 3 and 4). The percentage of sperm viability was significantly lower in samples

treated with MBCD or CLC than in the control samples. However, sperm treated with CLC showed the highest percentages of total motile sperm and rapid progressively motile sperm. Thus, CLC treated samples showed the highest ratio of functioning sperm (72%) compared to control and MBCD (< 50%). Nevertheless, the reasons for this variability remain unclear.

Although the CLC treatment did not improve the motility and viability of the sperm after thawing, this treatment enhanced the penetration ability of immature oocytes in vitro compared to the control samples, provided that glycerol was used as cryoprotectant. These results are in agreement with previous reports (Tomás et al., 2011). A positive correlation between the in vitro penetration rate and the in vivo boar sperm fertilising ability has been observed (Martínez et al., 1998). Thus, a priori, it is expected that the CLC-treated sperm will also exhibit a higher fertilizing ability in vivo.

On the other hand, this is the first study where the in vitro fertilizing ability of boar sperm frozen with amides is reported. DMF negatively affected the fertilizing ability of boar sperm in vitro. This result could be partially explained by the lower motility or the lower percentage of functioning sperm (48%) exhibited by the samples frozen with DMF. Interestingly, DMF abolished the beneficial effect of CLC on the sperm penetration ability, which could indicate that this cryoprotectant produces some effects in some other sperm structures. Thus, this cryoprotectant could alter some sperm attributes necessary for the interaction with the oocytes. Taking into account these results, a priori the use of amides for boar sperm cryopreservation is not recommended, although in vivo fertility trials should be conducted to draw definitive conclusions.

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CHAPTER 7

CONCLUSIONS

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The conclusions obtained in this Thesis are detailed next:

1. Treating boar sperm with 1 mg of cholesterol-loaded methyl-β-cyclodextrin (CLC) or methyl-β-cyclodextrin alone/ 120 x 10^6 sperm prior to cryopreservation provided either slight or no benefit, respectively, to post-thaw sperm plasma membrane integrity and motility, using a conventional freezing extender. In addition, this response is not dependant on the original ability of the sperm to survive cryopreservation.

2. The concentration of egg yolk in the freezing extender for boar sperm (20%; v:v) cannot be reduced even if the samples are treated with CLC prior to freezing. Besides, the traditional glycerol concentration (3%; v:v) was not the appropriate for CLC treated sperm, increasing the percentage of viable sperm at higher glycerol concentrations (5-7%; v:v).

3. Three of the amides (lactamide, acetamide and formamide) produced deleterious effects in fresh boar sperm. The other amides (methylformamide, dimethylacetamide and dimethylformamide) efficiently improved the sperm viability after cryopreservation buy negatively affected the sperm motility and the sperm fertilizing ability in vitro (dimethylformamide), irrespective of the sperm treatment. On the other hand, the CLC treated samples showed better in vitro fertilizing ability than the control samples when glycerol was used as cryoprotectant.