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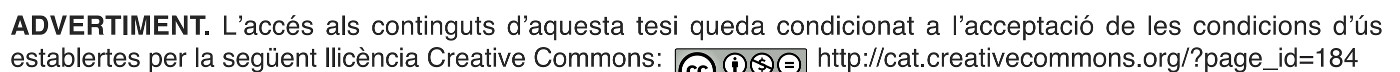
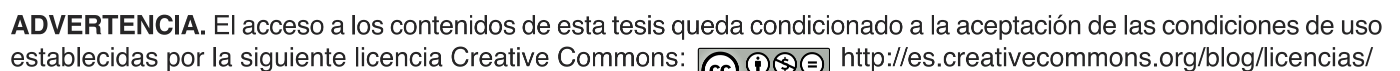
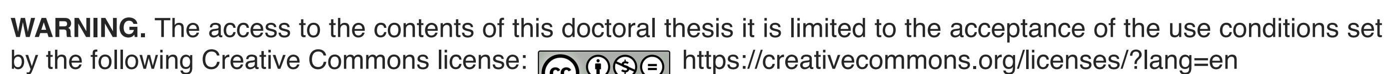
**ADVERTIMENT.** L’accés als continguts d’aquesta tesi queda condicionat a l’acceptació de les condicions d’ús establertes per la següent llicència Creative Commons:  http://cat.creativecommons.org/?page_id=184

**ADVERTENCIA.** El acceso a los contenidos de esta tesis queda condicionado a la aceptación de las condiciones de uso establecidas por la siguiente licencia Creative Commons:  http://es.creativecommons.org/blog/licencias/

**WARNING.** The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en

Universitat Autònoma de Barcelona

# OPTIMIZING VITRIFICATION OF IN VITRO MATURED BOVINE OOCYTES


TESI DOCTORAL PRESENTADA PER:

**Núria Arcarons Deseures**

SOTA LA DIRECCIÓ DE LA DOCTORA:

**Teresa Mogas Amorós**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Bellaterra, juny del 2017

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**Certifica:**

Que la memòria titulada **"Optimizing vitrification of in vitro matured bovine oocytes"**, presentada per Núria Arcarons Deseures amb la finalitat d'optar el grau de Doctor en Medicina i Sanitat Animals, ha estat realitzada sota la seva direcció, i considerant-la acabada, autoritzen la seva presentació perquè sigui jutjada per la comissió corresponent.

I perquè consti als efectes oportuns, signen la present a Bellaterra, 8 de juny de 2017.

Dra Teresa Mogas Amorós

La present memòria de tesi ha estat realitzada gràcies a una beca predoctoral de Formació de Professorat Universitari concedida pel *Ministerio de Educación Cultura y Deporte del Gobierno de España (Ref. FPU12/01419)* i al finançament proporcionat pel *Ministerio de Economia y Competitividad del Gobierno de España* (*Project AGL2013–46769).*

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*Als meus pares*

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## RESUM

La gran quantitat de beneficis que ofereix la crioconservació d'oòcits bovins, com ara la millora en l'aplicació pràctica de les tècniques de reproducció assistida en boví, la seguretat en el transport de material genètic a nivell internacional, el paper en la conservació de la biodiversitat i la comoditat per l'aprovisionament d'oòcits per la recerca bàsica, clonatge i programes de transferència nuclear, ha fet incrementar dràsticament la demanda d'oòcits bovins. En aquest context, la crioconservació exitosa d'oòcits bovins madurats in vitro resulta de vital importància per garantir l'abastiment d'oòcits bovins. No obstant això, encara no s'ha aconseguit un protocol de vitrificació d'oòcits bovins eficient i eficaç. L'objectiu d'aquesta tesis és, per tant, investigar la utilització d'elevades concentracions de NaCl o sucrosa abans de la vitrificació i escalfament, l'enriquiment de l'oolema amb colesterol abans de la vitrificació i l'addició d'un biopolímer sintetitzat per un bacteri de l' Antàrctica durant la vitrificació i l'escalfament, com a estratègies per millorar els protocols de vitrificació d'oòcits bovins.

S'ha descrit que l'exposició dels oòcits a concentracions elevades de clorur sòdic, sucrosa i trehalosa abans de la manipulació millora la criotolerància a la vitrificació i la capacitat de desenvolupament en l'espècie porcina. En el capítol IV d'aquesta tesis vam observar que el tractament amb solucions de 375 mOsmol de NaCl o sucrosa durant una hora abans de la vitrificació, no tenia efectes perjudicials per l'estat del fus meiòtic dels oòcits bovins madurats in vitro. Però, concentracions més altes donaven lloc a percentatges superiors de fusos meiòtics anormals. Concretament, el pretractament amb sucrosa abans de la vitrificació no va ser capaç de millorar el desenvolupament embrionari com s'havia observat en altres espècies.

L'enriquiment de la membrana amb colesterol podria incrementar la fluïdesa i la permeabilitat de la membrana i augmentar la criotolerància dels oòcits a la crioconservació. En el capítol V d'aquesta tesis vam utilitzar bodipy-colesterol per visualitzar el transport del colesterol per microscòpia confocal en oòcits bovins vius madurats in vitro. Aquest mètode ens va permetre determinar el temps d'incubació necessari per una incorporació òptima del colesterol a la membrana i evitar la no

desitjada penetració al citoplasma. Utilitzant diferents concentracions de metil-β-cyclodextrines carregades amb colesterol en medis amb diferents suplements. Tot i que no es va millorar la criotolerància en termes de supervivència i capacitat de desenvolupament, el tractament amb 2mg/ml de metil-β-cyclodextrines carregades amb colesterol va alterar l'expressió de gens relacionats amb el metabolisme lipídic, (CYP51), apoptosis (BAX) i metilació del DNA (DNMT3A) en mòrules bovines, sobretot quan els oòcits eren vitrificats a estadi de vesícula germinal.

Pseudomonas sp. ID1, un bacteri aïllat del sediment marí de l'Antàrtica, produeix un polisacàrid com a mecanisme de tolerància al refredament (M1 EPS). S'ha demostrat anteriorment que aquest exopolisacàrid també confereix crioprotecció per altres cèl·lules bacterianes, suggerint que pot ser utilitzat com a agent per la crioconservació cel·lular. En el capítol VI es va afegir M1 EPS a les solucions de vitrificació i escalfament com a agent bloquejant de la formació de gel, per limitar el dany dels oòcits bovins madurats in vitro i incrementar posteriorment la capacitat de desenvolupament embrionàri. Es van analitzar els efectes de la suplementació amb diferents concentracions de M1 EPS en l'organització dels fusos meiòtics, la capacitat de desenvolupament en termes de percentatges de blastocists i l'expressió gènica en blastocists de dia 8. La suplementació amb M1 EPS durant la vitrificació i l'escalfament dels oòcits de vedella prepúber madurats in vitro protegia el fus meiòtic contra la descondensació de cromosomes i microtúbuls causada per la vitrificació. Tot i que després del suplement amb M1 EPS no es va observar millora en els percentatges de blastocists, es van registrar diferents canvis en l'expressió d'alguns gens relacionats amb epigenètica (DNMT3A i KAT2A) i qualitat embrionària (BAX, BCL2) entre blastocists obtinguts a partir d'oòcits vitrificats amb diferents concentracions de M1 EPS.

En resum, basant-nos en els resultats d'aquesta tesis, podem concloure que la criotolerància dels oòcits bovins madurats in vitro no només depèn del dany que pateixin en l'organització del fus meiòtic. Sinó que també en els canvis en l'expressió gènica que condicionen el posterior desenvolupament embrionari.

# RESUMEN

La gran cantidad de beneficios que ofrece la crioconservación de ovocitos bovinos, tales como la mejora en la aplicación práctica de las técnicas de reproducción asistida en vacuno, la seguridad en el transporte de material genético a nivel internacional, el papel en la conservación de la biodiversidad y la comodidad para el abastecimiento de ovocitos para la investigación básica, clonación y programas de transferencia nuclear, ha incrementado drásticamente la demanda de ovocitos bovinos. En este contexto, la crioconservación exitosa de ovocitos bovinos madurados in vitro resulta de vital importancia para garantizar el abastecimiento de ovocitos bovinos. Sin embargo, aún no se ha logrado un protocolo de vitrificación de ovocitos bovinos eficiente y eficaz. El objetivo de esta tesis es, por tanto, investigar la utilización de elevadas concentraciones de NaCl o sacarosa antes de la vitrificación y calentamiento, el enriquecimiento del oolema con colesterol antes de la vitrificación y la adición de un biopolímero sintetizado por una bacteria de la Antárctica, que bloquea la formación de hielo, durante la vitrificación y el calentamiento, como estrategias para mejorar los protocolos de vitrificación de ovocitos bovinos.

Se ha descrito que la exposición de los ovocitos a concentraciones elevadas de cloruro sódico, sacarosa y trehalosa antes de la manipulación mejora la criotolerancia a la vitrificación y la capacidad de desarrollo en la especie porcina. En el capítulo IV de esta tesis observamos que el tratamiento con soluciones de 375 mOsmol de NaCl o sucrosa durante una hora antes de la vitrificación, no tenía efectos perjudiciales para el estado del huso meiótico de los ovocitos bovinos madurados in vitro. Pero concentraciones más altas daban lugar a porcentajes superiores de husos meióticos anormales. Concretamente, el pretratamiento con sacarosa antes de la vitrificación no fue capaz de mejorar el desarrollo embrionario como se había observado en otras especies.

El enriquecimiento de la membrana con colesterol podría incrementar la fluidez y la permeabilidad de la membrana y aumentar la criotolerancia de los ovocitos a la crioconservación. En el capítulo V de esta tesis utilizamos bodipy-colesterol para visualizar el transporte del colesterol por microscopía confocal en ovocitos bovinos vivos madurados in vitro. Este método nos permitió determinar el tiempo de incubación necesario para una incorporación óptima del colesterol en la membrana y evitar la no

deseada penetración en el citoplasma, utilizando diferentes concentraciones de metil-β-cyclodextrinas cargadas con colesterol. Aunque no se mejoró la criotolerancia en términos de supervivencia y capacidad de desarrollo, la adición de colesterol alteraba la expresión de genes relacionados con el metabolismo lipídico, (CYP51), apoptosis (BAX) y metilación del DNA (DNMT3A) en mórulas bovinas, sobre todo cuando los ovocitos eran vitrificados en estadio de vesícula germinal. Pseudomonas sp. ID1, una bacteria aislada del sedimento marino de la Antártica, produce un polisacárido como mecanismo de tolerancia al enfriamiento (M1 EPS). Se ha demostrado anteriormente que este exopolisacárido también confiere crioprotección a otras células bacterianas, sugiriendo que puede ser utilizado como agente para la crioconservación celular. En el capítulo VI de esta tesis, se añadió M1 EPS a las soluciones de vitrificación y calentamiento como agente bloqueante de la formación de hielo, para limitar el daño de los ovocitos bovinos madurados in vitro e incrementar posteriormente la capacidad de desarrollo embrionario. Se analizaron los efectos de la suplementación con diferentes concentraciones de M1 EPS en la organización de los husos meióticos, la capacidad de desarrollo en términos de porcentajes de blastocistos y la expresión génica en blastocistos de día 8. La suplementación con M1 EPS durante la vitrificación y el calentamiento de los ovocitos de ternera prepúber madurados in vitro protegía el huso meiótico contra la descondensación de cromosomas y microtúbulos causada por la vitrificación. Aunque después del suplemento con M1 EPS no se observó mejora en los porcentajes de blastocistos, se registraron diferentes cambios en la expresión de algunos genes relacionados con epigenética (DNMT3A y KAT2) y calidad embrionaria (BAX, Bcl2) entre blastocistos obtenidos a partir de ovocitos vitrificados con diferentes concentraciones de M1 EPS.

En resumen, basándonos en los resultados de esta tesis, podemos concluir que la criotolerancia los ovocitos bovinos madurados in vitro no sólo depende del daño que sufran en la organización del huso meiótico. Sino que también en los cambios en la expresión génica que condicionan el posterior desarrollo embrionario.

## SUMMARY

The numerous benefits of bovine oocyte cryopreservation, such as the enhancement of the practical application of assisted reproductive techniques in bovine, the safety in international transport of genetic material, the role in biodiversity preservation and the convenience for the oocyte source for basic research, cloning and nuclear transfer programs has dramatically increased the demand of bovine oocytes. In this context, successful preservation of in vitro matured bovine oocytes appears to be critical for guarantee the bovine oocyte supply. In spite of that, an efficient and efficacious vitrification protocol for in vitro matured bovine oocytes should be achieved soon.
The purpose of this thesis is, therefore, to investigate the use of high concentrations of NaCl or sucrose prior to vitrification/warming, the oolema enrichment with cholesterol prior to vitrification and the addition of an ice bocking biopolymer synthetized by a bacteria from Antarctica during vitrification and warming, as strategies to improve bovine oocyte vitrification protocols.

Some strategies are focused in modifying the oocyte itself to increase cryosurvival (chapter 4 and 5) and other in modify the cryopreservation procedure (chapter 6).

Exposure of oocytes to increased concentrations of sodium chloride, sucrose or trehalose prior to manipulation has been reported to improve both cryotolerance to vitrification and developmental competence in porcine specie. In the chapter IV of present thesis, we observed that treatment with 375 mOsmol NaCl or sucrose solution for 1 hour before vitrification had no detrimental effects on the meiotic spindle status of IVM bovine oocytes, but higher concentrations gave rise to higher abnormal spindle rates. In particular, sucrose pretreatment prior to vitrification was unable to improve embryo development as observed in other species.

Membrane cholesterol enrichment could increase the fluidity and permeability of the membrane and increase the cryotolerance of oocytes to cryopreservation. In chapter V of this thesis we used bodipy-cholesterol to image the cholesterol transport in live *in vitro* matured bovine oocytes incubated with cholesterol-loaded methyl-β-cyclodextrin by confocal microscopy. This method allowed us to determine the incubation time required for optimal cholesterol incorporation into membrane avoiding the non-desired penetration into cytoplasm, using different cholesterol-loaded methyl-β-cyclodextrin

concentrations in different supplemented media. However, cryotolerance in terms of survival and developmental competence was not improved, regardless of the application of the determined cholesterol-loaded methyl-β-cyclodextrin treatment or the holding medium used. However, the treatment of oocytes with 2mg/ml cholesterol loaded cyclodextrins before vitrification, altered the expression of genes related to lipid metabolism (CYP51), apoptosis (BAX) and DNA methylation (DNMT3A) in bovine morulae, mainly when oocytes were vitrified at germinal vesicle stage.

Pseudomonas sp. ID1, a bacterium isolated from marine sediment from Antarctica, produces an exopolysaccharide as a cold adaptation mechanism (M1 EPS). It has already been demonstrated that it exopolysaccharide conferred cryoprotection for other bacteria cells, suggesting it can thus be applied as an agent for cell cryopreservation. In the Chapter VI the M1 EPS was added to vitrification and warming solutions as an ice blocking agent to limit the in vitro matured bovine oocyte damage and to increase further developmental competence. The effects of different concentrations of M1 EPS supplementation were examined on oocyte meiotic spindle organization, developmental competence in terms of blastocyst rates, and gene expression in day 8 blastocysts. M1 EPS supplementation during vitrification and warming of in vitro matured prepubertal heifer oocytes protected the meiotic spindle against chromosome and microtubule decondensation caused by vitrification. Although no improvement on blastocyst rates were observed after EPS supplementation, different changes in gene expression of some gens related with epigenetics (DNMT3A and KAT2A) and blastocyst quality (BAX, BCL2) were recorded between blastocysts derived from oocytes vitrified with different concentrations of M1 EPS.

In summary, on the basis of the results of the present Thesis, we can conclude that in vitro matured oocyte cryotolerance does not only depend on the damage on the meiotic spindle organization, but also on the gene expression changes that determine further embryonic development.

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## ABBREVIATIONS

| | |
|---|---|
| AFPs | antifreeze proteins |
| AFP III | antifreeze protein type three |
| AFGPs | antifreeze glycoproteins |
| AFGP8 | antifreeze glycoproteins  type eight….. |
| ATP | adenosine triphosphate |
| BAX | pro-apoptotic member of the Bcl-2 gene family. Apoptosis regulator |
| βCD | methyl-b-cyclodextrins |
| BCL2-1 | BCL2-like 1 gene is an antiapoptòtic member of the Bcl-2 family. |
| BSA | bovine serum albumin |
| BPY-Chol | bodipy-cholesterol: 23-(dipyrrometheneboron difluoride)-24-norcholesterol or TopFluor Cholesterol. |
| CB | cytochalasin B |
| CCs | cumulus cells |
| CLC | methyl-b-cyclodextrins loaded with cholesterol |
| COCs | cumulus-oocyte complexes |
| CO2 | carbon dioxide |
| CPA | cryoprotectant |
| CPS | closed pulled straw |
| CRs | partially denuded oocytes |
| CTL | cholestratienol |
| CYP51 | cytochrome P450 family 51 or lanosterol 14a-demethylase |
| DHE | dehydroergosterol |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| DNMT3A | de novo methyltransferase 3A |
| D2O | deuterium oxide |
| E. coli | escherichia coli |
| eqFMP | equilibrium freezing/melting point |
| EG | ethyleneglycol |

| | |
|---|---|
| EMG | electron microscope grid |
| EPS | exopolysaccharides |
| ES | equilibration solution |
| FfIBP | flavobacterium frigoris ice-binding protein |
| FCS | fetal calf serum |
| GADPH | glyceraldehyde-3-phosphate dehydrogenase (GAPDH) |
| GEE | glutathione ethyl ester |
| GSH | glutathione, L-$\gamma$-glutamyl-L-cysteinyl-glycine |
| GV | oocytes at germinal vesicle stage. |
| h | hours |
| H2AFZ | H2A histone family, member Z |
| HDAC-1 | histone deacetylase 1 |
| hFP | hysteretic freezing point |
| HHP | high hydrostatic pressure |
| HM | holding medium |
| HSP | heat shock proteins |
| H2O2 | hydrogen peroxide |
| IBPs | ice-binding proteins |
| ICSI | intracytoplasmic sperm injection |
| IGF2R | insulin-like growth factor type 2 receptor |
| INPs | ice-nucleating proteins |
| IRI | ice recrystallization inhibition |
| IVC | in vitro culture |
| IVF | in vitro fertilization |
| IVM | in vitro maturation |
| KAT2A | K(lysine) acetyltransferase 2A |
| LeIBP | glaciozyma sp. ice-binding protein |
| LN2 | liquid nitrogen |
| LPT | lipid phase transition |

| | |
|---|---|
| M1 EPS | exopolysaccharide produced by Pseudomonas sp. ID1 |
| MDS | minimum drop size |
| Min | minutes |
| MPF | maturation promoting factor |
| MII | oocytes at metaphase II stage |
| MTOC | microtubule organizing center |
| NaCl | sodium chloride |
| NBD | 22-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4yl) amino-23,24 bisnor-5-cholen-3β-ol |
| N2 | nitrogen |
| OPS | open-pulled straws |
| PA | parthenogenetic activation |
| PBS | phosphate-buffered saline |
| PEG | polyethylene glycol |
| PFA | paraformaldehyde |
| PGL | polyglycerol |
| PVA | polyvinyl alcohol |
| PVP | polyvinylpyrrolidone |
| PROH | propylene glycol or 1, 2-propanediol |
| ROS | reactive oxygen species |
| SSV | solid surface vitrification |
| SLC2A3 | solute carrier family 2 member 3 |
| TCM199 | tissue culture medium 199 |
| TH | thermal hysteresis |
| Tm | melting temperature |
| H-TCM199 | hepes buffered tissue culture medium 199 |
| Tg | glass transition temperature |
| UBE2A | ubiquitin-conjugating enzyme E2A |
| V-kim | vitrification straws. V-Kim is a closed vitrification system. |
| VS | vitrification solution |

# CHAPTER I

## General introduction

Bovine oocyte cryopreservation has numerous benefits, such as facilitate the practical application of assisted reproductive techniques, reducing the animal maintenance cost and allowing international movement of genetic material with the advantage that the disease dissemination risks are avoided. It also has a role in biodiversity preservation providing safeguards against catastrophic loss of rare or endangered animal genetic resources. Oocyte banking makes also easier the oocyte supply for basic research, cloning and nuclear transfer programs.

Traditionally, embryos and oocytes have been cryopreserved by slow freezing methods using low concentrations of cryoprotectants. During this procedure, osmotic shock and intracellular ice formation often cause cell damage. Today, vitrification is regarded as the best alternative to traditional slow freezing procedures for oocyte cryopreservation. Vitrification is an ice-free cryopreservation method that involves high concentrations of cryoprotectants combined with very rapid cooling by direct plunging in liquid nitrogen resulting in an ice crystal-free, solid glass-like structures [1].

Unfortunately, the practical application of this technique is still limited due to the reduced viability and decreased developmental capacity of oocytes after cryopreservation. The developmental competence of vitrified-warmed oocytes is restricted by the damage suffered at different cellular levels during cryopreservation processes, such as: zona pellucida structural and biochemical changes that lead to zona pellucida hardening [2-5], abnormal distribution of chromosomes, microtubules and actin microfilaments [6, 7], disorganization of the spindle apparatus with the consequent risk of chromosome loss and aneuploidy [8-12], changes to the lipid bilayer phase causing cytoplasmic membrane lysis [13] and nuclear fragmentation [14, 15], which is not readily repairable by the endogenous mechanisms within a haploid cell.

The most common strategy to deal with the lower success rates of cryopreservation of oocytes is to modify cryopreservation procedures, for example, by **using different cryoprotectants** (CPAs; [16, 17], **varying CPA concentrations** or **CPA exposure temperatures and times** [18-20] [21, 22], and **addition of macromolecular supplements** [16, 23], **ice blockers** [20] or **antifreeze proteins** [24-27] to vitrification solutions.

On the other hand, other procedures tested have been focused on **modifying the cells themselves** to make them more cryopreservable. For example some laboratories tested the **partial removal of cumulus cells** [20, 28-31], **modification of oocyte membrane constituents** [16, 32]**, polarization of cytoplasmic lipid droplets** by centrifugation [33], the **addition of cytoskeletal stabilizers** [17, 34-38] and **cytoskeletal relaxants** [19, 39-44] to vitrification solutions and the **application of a sublethal stress** for the oocytes in order to increase the subsequent stress tolerance [45-48]. Among the previous strategies, in this thesis we focused in both first papers (Chapter IV and V) on modifying the oocyte itself. In **chapter IV** by **inducing stress tolerance** mechanisms by preconditioning the oocyte **with osmotic stress** and, in **chapter V modifying the membrane cholesterol content** by **incubating oocytes with cholesterol loaded methyl-beta-cyclodextrins** prior to vitrification. Conversely, in the last paper **(Chapter VI**) the addition of a novel **ice blocker**, a biopolymer synthetized by a cold-adapted bacterium isolated from Antarctica was tested as a **macromolecular supplement for vitrification and warming solutions.**

# CHAPTER II

## Literature review

## 2.1 What is cryopreservation?

**Cryopreservation** is a process by which biological cells or tissues are preserved at subzero temperatures resulting in a radical decrease in the rate of metabolic processes and the ability to store samples for extended periods of time but maintaining the viability after warming [49]. The temperature that generally is used for storage of mammalian cells is -196°C, the temperature of liquid nitrogen. At these low temperatures, water exists only in a solid state, and no known biological reactions take place. Once, the cells are passed through the cryopreservation process and are stored in liquid nitrogen, the only potential damage that they might suffer is thought to be damage to DNA that is caused by background radiation. It further has been estimated that with the usual terrestrial background radiation levels of 0.1 cGy/y, mammalian cells may survive for hundreds or even thousands of years [50]. For a mammalian oocyte, a more sensitive cell, this time might be reduced to 200 years. The time when cells can suffer damage throughout the cryopreservation process is during transitions of temperature: during cooling to -196°C and during subsequent rewarming to the physiological temperature.

## 2.2. Applications of oocyte cryopreservation

In animal species the applications of cryopreservation of mammalian oocytes are:

- **Genetic selection.** In animals, oocyte preservation allows genetic material from a female to be stored unfertilized until she expresses her potential and the appropriate mate can be selected [51].

- **Assisted reproduction procedures.** Efficacious cryopreservation of oocytes would also facilitate many assisted reproduction procedures. Because of the relatively short life span of mammalian oocytes, storage of unfertilized oocytes would generate a continuous and readily available source of viable and developmentally competent oocytes which allows carry out in vitro embryo production, cloning and transgenesis at the convenient time.

- **International transport.** Oocyte cryopreservation is beneficial for international exchange of germplasm, as it avoids injury and sanitary risks involved in live animal transportation and reduces the associated costs.

- **Conservation of the livestock genetics**. Many domestic breeds of livestock are experiencing a gradual diminishment of genetic diversity mainly due to changing market demands and intensification of agriculture. Old small farming production systems are changing to modern large commercial systems, and as a result, selection goals are now very similar throughout the world. Improvements achieved in assisted reproductive technologies have facilitated that a single individual with high genetic value produce large number of progeny. In addition the to the breeding programs carried out by national and international companies, which bet on genetic selection of few breeds and also to the rapid and efficient transport of germplasm around the world, have caused all together livestock diversity diminishment[52].

  It is indispensable to conserve animal genetic resources in order to have the capacity to respond to selection plateaus, shifting market demands, but more importantly biosecurity, environmental, and food safety risks by keeping potentially useful genes and gene combinations available in gene banks, to take advantage of heterosis [51-53].

  Therefore, it is in the interest of the international community to **conserve the livestock genetics**. Ideally populations are saved as live animals; however, this approach is expensive, and unless these animals can be used for production, it is difficult to accomplish. Therefore, ex situ in vitro conservation strategies are developed to cryopreserve animal genetic resources in genome/gene banks to regenerate a particular population in future [52, 54, 55]. Oocyte cryopreservation has enormous potential for protecting and managing species and population integrity and heterozygoty [51].

- **Rescue of oocytes from died animals**. Oocyte cryopreservation also could be used to rescue gametes when livestock or wild females die unexpectedly or accidentally [23, 56].

- **Preservation of wild animal species** The preservation of endangered species from domestic and wild animals to maintain biodiversity could be undertaken by establishing oocyte and embryo banks [53, 57]. Fertility preservation strategies using oocyte cryopreservation have enormous potential for helping sustain and protect rare and endangered species, especially to assist managing the genomes of genetically valuable individuals. However, wide-scale applications are still limited by significant physiological variations among species and a lack of awareness of basic reproductive characteristics and of germplasm cryobiology. The benefits for wildlife include preserving genetic vigour, transporting valuable genes avoiding the stress associated to the transport of these animals and safeguarding the genetic diversity that protects fitness and species integrity [58].

- **Cloning**. Large amounts of oocytes are required for transgenesis and cloning by somatic cell nuclear transfer (SCNT). Successful oocyte cryopreservation would allow supplying with enough quantities of good quality oocytes [59, 60] . The cloning efficiency of vitrified oocytes are still lower compared that of fresh non-vitrified oocytes [60]. Although the blastocyst rates were still compromised, recipient cytoplasts were successfully cryopreserved and full-term development was achieved [61, 62]. This application may have considerable impact on the rapidly increasing cloning research and industry.

- **Animal models research.** Until recently, success in oocyte cryopreservation has been very limited mainly due to poor understanding of the complex physiological processes that lead to cell damage during cryopreservation. In the past three decades, however, a wealth of information has been collected using various different animal models, which has led to development of new technologies and optimization of existing ones. The use of these models has provided the opportunity for research that may not have been possible with human material. Today, results of these studies still continue to form the basis of oocyte cryobiology. Based on preliminary results of oocyte cryopreservation for SCNT in mice [63, 64], oocyte cryopreservation could be considered a great method to supply oocytes for research. It is still not clear whether cryopreserved human oocytes function like their fresh counterparts and whether cryopreserved oocytes retain their nuclear reprogramming activity in the cytoplasm. Therefore,

before application of therapeutic cloning in humans, it is important to test the validity and efficiency of embryonic stem cell derivation through SCNT by using cryopreserved oocytes in an animal model. Other applications, such as assessment of genotoxicity of the cryoprotectants or epigenetic modifications after oocyte cryopreservation, will likely be developed in the future as a result of the significant biomedical data that can be obtained through animal models.

## 2.3. Oocyte cryopreservation history

### 2.3.1. The beginning

Embryo cryopreservation research provided the basis for oocyte cryopreservation. Following the first successful freezing of mouse 8-cell stage embryos in 1972 [65], the first pregnancy from a cryopreserved cattle embryo was reported by [66]. The protocol most commonly used for successful embryo cryopreservation at that time required slow cooling from upper −7∘C to below −80∘C in phosphate-buffered saline supplemented with DMSO or glycerol as a permeable CPAs. During the slow cooling, embryonic blastomeres or oocytes were dehydrated in response to the osmotic pressure that gradually increases with the formation of extracellular ice crystals after ice seeding. The frozen embryos or oocytes were warmed very slowly to avoid the rapid influx of extracellular water into the dehydrated cells during warming. This earlier protocol was labor-intensive and time-consuming. Some years later, a two-step freezing method was reported using sheep and cattle embryos [67]. The slow cooling of embryos is interrupted at around −30∘C to −36∘C, followed by rapid cooling to −196∘C. The embryos in LN2 are believed to contain intracellular ice, although it is not detrimental at this point. But to survive, the frozen embryos must be warmed rapidly to avoid injury caused by recrystallization of the intracellular ice. This two-step freezing regimen allows the development of a temperature-controlled, programmable freezer and is still used widely for many mammalian species [67].

The first successful in vitro fertilization (IVF) and birth of live offspring using frozen-thawed mouse oocytes was reported in 1976 by Parkening et al. [68] and followed by Whittingham [69] and Leibo et al. [70]. Besides for mouse oocytes slow freezing procedure was acceptable for species whose oocytes are not sensitive to chilling, such as cat [71, 72] and human [73]. There are a few reports regarding successful pregnancies

from frozen-thawed bovine oocytes [33, 74]. However, oocytes from the large domestic species are rich in cytoplasmic lipid droplets and very sensitive to chilling, resulting in the poor revivability following the slow cooling [56].

#### 2.3.2. Development of vitrification

The turning point occurred in 1985 with the development of a novel cryopreservation procedure for mouse embryos [1]. This protocol involves dehydration of the embryos by exposing them to highly concentrated CPAs prior to cooling them to low temperature, rather than during the cooling process itself. The dehydrated embryos are rapidly cooled by being directly plunged into liquid nitrogen. Since the cryoprotective solution can be transformed into a stable glass without ice crystal formation during the rapid cooling process, this extremely rapid method of cryopreservation is referred to as "vitrification". The application of vitrification as an alternative to conventional freezing can reduce the time and equipment required, but technician-dependent performance of vitrification process is the limiting factor for its widespread use. After the publication of innovative results by Rall and Fahy [1], vitrification has been attempted to apply to oocytes. Seven years after the classic paper by Rall and Fahy [1], in 1992, appeared the earliest report on attempts to vitrify oocytes from cattle. Two research groups attempted to vitrify bovine oocytes in a relatively large volume inside a 0.25-mL straw by what now can be considered classical approaches [74, 75]. Each used a vitrification solution composed of DMSO, acetamide, and propylene glycol (PROH) in concentrations of 2, 1, and 3 M, respectively. Hamano, and colleagues [75] transferred embryos that developed from vitrified and inseminated oocytes, and pregnancies ensued, suggesting that vitrification can offer a means to cryopreserve bovine oocytes. Pregnancies or birth of live offspring have been published in mouse [76], human [77], and cattle [75], with an increased requirement for improving developmental competence of the vitrified-warmed oocytes. The main issue regarding success with vitrification was the necessity to balance the cryoprotectant solutions to reduce toxicity [78]. Critical to success was a very short duration of exposure to the vitrification solution [79, 80]. For many years, slow freezing and not vitrification was the method of choice for oocyte and embryo cryopreservation, because vitrification was not achieved easily owing to the need of high CPAs concentrations and relatively high volume samples.

Progress was rather slow during the initial stages of research. However, in 1996, Martino et al [81, 82] published two pioneer works opening the new window for oocyte cryobiology. After studying the kinetics of chilling injury in bovine oocytes, Martino and colleagues described their version of "ultrarapid" vitrification to overcome the chilling injury, using electron microscope grids as a support device for the oocytes. They were able to produce bovine blastocysts from 10% to 15% of the oocytes, a much greater rate of success than had been previously reported. That protocol was characterized by the extremely rapid cooling rate of oocytes suspended in <1 $\mu$L of a vitrification solution consisting of 30% EG plus 1 M sucrose placed onto electron microscope grids, a procedure derived from methods to cryopreserve Drosophila embryo [83]. The microgrids provide a cooling rate estimated to be <150,000∘C/min, in contrast to 2,500∘C/min with the conventionally used plastic straws.

These reports appear to have started a revolution in the field of oocyte cryobiology. After these reports in 1996, the vast majority of research efforts were directed at determining how to improve vitrification by ultrarapid cooling and warming of oocytes.

Another report appeared in 1998 that promulgated a different method for vitrification of bovine oocytes which also incorporated a rapid cooling and warming regimen [84]. They used a new device for vitrification, OPS that was simply a straw made by heating and pulling a standard freezing straw, resulting in a device with a finer wall thickness and smaller diameter (at the end where the cells are housed). This design allows faster heat transfer between the solution and the environment, and cooling/warming rates on the order of 20 000 °C/min could be achieved. When the oocytes were aspirated with 20% EG and 20% DMSO solution into open-pulled straws (OPS) and cooled by directly plunging into liquid nitrogen, 13% of the postwarm oocytes could develop into blastocysts after IVF and IVC. Such success seemed to have solved the significant problem of the poor success of bovine oocyte cryopreservation that have been bothering reproductive cryobiologists for years. Unfortunately, repeatability of this high success rate was a challenge  The OPS method has been improved to use open pulled glass capillaries [85, 86] or commercially available gel loading tips [87] using different CPA combinations. Other types of cryodevices so far reported for ultra-rapid cooling are the "Cryoloop" [88] and "Cryotop" [89]. Complete containerless methods, droplets and solid surface vitrification, have also been reported from two independent laboratories

[18, 90], respectively. Blastocyst yields from frozen-thawed or vitrified-warmed bovine metaphase-II oocytes, reported since 2000 onward are summarized in Table 2.1. There was no significant improvement on the blastocyst yield from cryopreserved bovine mature oocytes (commonly exceeding 10%), even after increased cleavage rates as >60% by using different cryodevices and vitrification protocols were obtained.

There does not seem to be a consensus regarding which vitrification solution and which device to achieve ultrarapid cooling and warming are the best, although this might not be of practical interest if any one of several can be made to work adequately in any given laboratory.

## 2.4. Principles of oocyte cryopreservation

### 2.4.1. Ice dynamics in pure water and in aqueous solutions containing solutes

When water is cooled to below its freezing point, it solidifies in a crystalline structure called **ice**. Ice formation starts when individual water molecules join to form microscopic ice nuclei. In pure water the highest temperature at which ice can form without changing the pressure is 0ºC, but nuclei do not usually form until the temperature falls below 0ºC.

Solutions those remain liquid, free of ice, even though they are below the temperature at which ice can form, are said to be in a supercooled state. The lowest temperature at which pure water can be held without freezing is thought to be -40ºC, but ice normally forms at temperatures between -5 and -15 ºC through spontaneous (due to homogeneous or heterogeneous nucleation) or induced (by seeding) ice nucleation. Once an ice nucleus has been formed, further water molecules can easily bond onto this surface, making the ice nucleus grow in size.

Adding salts and other solutes to pure water lowers the temperature at which ice forms/melt. Solutions with very high concentrations may never form ice rather they will solidify into an ice-free (vitreous) state. Molecules other than water molecules are not incorporated into the growing ice crystals. However, the formation and growth of ice crystals can be modified by compounds such as antifreeze proteins [91], ice blocking agents [92] and cryoprotectants.

### 2.4.2. Basic principles of oocyte cryopreservation (Understanding the principal goals for oocyte cryopreservation).

When water crystalizes, the space occupied by each individual water molecule increases (1 g of ice, density 0.92), then the volume that crystals occupy is higher than those of the liquid water (1g of liquid water, density 1). During cryopreservation, when the liquid water surrounding the cell solidifies, its expansion into ice causes **pressure and shearing forces** on intracellular organelles, which can suffer considerable damage. Therefore, cryopreservation procedures are designed to minimize damage caused by **intracellular ice crystal formation** and **growth** during cooling.

While water transitions from liquid to ice, the growing ice crystals bind only water molecules and the molecules other than water are physically excluded; then increase the **concentration of solutes** in the liquid phase. This lowers the freezing point of the remaining unfrozen solution (because adding salts and other solutes to pure water lowers the temperature at which ice forms/melt). As the temperature drops and the solid phase increase, the concentration of electrolytes and other solutes can reach very high [93] then can be toxic to intracellular proteins. Thus, the second goal for successful cryopreservation is avoidance of this **solution effect**.

During **rewarming**, cells can also suffer damage, because the solid ice melts and releases free water, resulting in **decreasing osmolarity** of the surrounding solution. When rewarming is **slow**, there is danger of free water **thawing and recrystallizing**, thus causing additional damage.

When rewarming is **rapid**, sudden **drops** in **extracellular osmotic pressure** (due to when ice is melted areas of pure water are formed) may lead to rapid movement of free water across and into the cell, leading to **swelling** and **cell damage** [50]. This is called **osmotic shock**, and its avoidance is a third major goal of successful cryopreservation.

Therefore, if you want to get successful cryopreservation, is not enough simply immersing oocytes into liquid nitrogen.

All successful methods for cell cryopreservation must try to avoid or minimize these three problems: ice crystal formation, solution effects, and osmotic shock. To overcome

these injuries, different cryopreservation methods play different strategies. We focused below on those strategies followed by vitrification methods.

## 2.5. Vitrification or non-equilibrium cooling method

Vitrification is a process of cryopreservation in which the solidification of a solution occurs during cooling without the formation of ice crystals, transforming it into a 'vitreous', amorphous state, solid glass-like form. This physical phenomenon requires either ultra-rapid cooling rates or the use of concentrated cryoprotectant solutions [94]. Non equilibrium protocols differ from slow protocols in that in non-equilibrium protocols most dehydration and cryoprotectant permeation takes place before cooling begins, and cooling is usually performed in a single step in which the sample is cooled directly from room or physiologic temperature to low subzero temperatures (<-130ºC).

Vitrification could be an alternative method to slow freezing that often causes cell damage by the osmotic shock and intracellular ice crystallization. Nevertheless, it is necessary to have into account the sudden exposure to high concentrations of cryoprotectants at room temperature is toxic to oocytes [37, 95, 96]. Therefore, the exposure time of oocytes and embryos to cryoprotectants is shortened, or they are often pre-equilibrated in a solution which contains a lower concentration of permeating cryoprotectants (equilibration solution or vitrification solution 1) than the vitrification solution (or vitrification solution 2) [97] after which the oocytes are plunged directly into liquid nitrogen.

To achieve successful vitrification, three important factors should be considered:

1. **Cooling and warming rate**: Vitrification occurs most readily at high cooling and warming rates. A high cooling rate is achieved with liquid nitrogen or liquid nitrogen slush and for warming is immersing the sample in a heated solution.
When using liquid nitrogen, the sample is plunged into liquid nitrogen resulting in cooling rates of hundreds to tens of thousands degrees Celsius per minute, depending on the container, the volume, the thermal conductivity, the solution composition, etc. [98]. To achieve liquid nitrogen slush formation, the liquid nitrogen needs to be cooled close

to its freezing point (-210ºC), for example, by applying a negative pressure above liquid nitrogen [83]. When liquid nitrogen slush is formed, the cooling rate is dramatically increased.

2. **Viscosity of the medium:** This is defined by the concentration and behavior of various CPAs and other additives during vitrification. If the concentration of CPAs is higher, the glass transition temperature (Tg) increases, and then the probability of ice nucleation decreases. The combination of different CPAs is often used to increase viscosity, increase the glass transition temperature (Tg) and reduce the toxicity. Some polymers (such as Ficoll, Dextran, polyvinylpyrrolidone, hyaluronic acid, polyvinyl alcohol, polyethylene glycol), proteins (such as bovine serum albumin (BSA) or sugars (such as sucrose or trehalose) can also be added to the vitrification and warming solutions in order to raise the total solute concentration of a solution without increasing the toxicity of the solution. For example, large polymers may markedly increase the viscosity of the solution and modify the formation of a stable glasslike state (vitrification) [23, 99].

3. **Volume**: smaller volumes allow better heat transfer that results in significant increase in the cooling rate, so enhancing the probability of vitrification [98]. Several cryodevices have been designed to use the minimum sample volume to achieve ultra-fast cooling rate. These techniques for oocyte and embryo vitrification can generally be divided into two categories, surface techniques and tubing techniques.

The **surface techniques** include: electron microscope (EM) grid [82, 83], minimum drop size (MDS; [100-102], cryotop [31, 103, 104], cryoloop [88, 105-107], hemi-straw [108, 109], solid surface [90, 110], nylon mesh [111, 112], cryoleaf [113], direct cover vitrification [114-116], fiber plug [117-119], vitrification spatula [120], and vitri-inga [121].

To the **tubing techniques** (Fig. 2) belong the plastic straw [1], OPS [84], closed pulled straw (CPS; [10, 122, 123], flexipet-denuding pipette [124], superfine OPS [125], CryoTip [126, 127], pipette tip [128, 129]), glass capillaries [85, 86], high-security vitrification device [130, 131], Rapid-i [132].

The surface methods let to perform small size drops (<0.1ml) and due to these systems are opened, high cooling and warming rates can be achieved because of the direct contact of the sample with the liquid nitrogen during cooling and with the warming solution during warming process. On the other hand, the tubing systems are safer and easier to handle because are closed and also achieve high cooling rates. All these small volume supports have a high surface-to-volume ratio that allows achieving high heat transfer.

The general equation that reflects the probability of vitrification, are formed by the combination of these three factors.

$$\text{Probability of vitrification} = \frac{\text{Cooling and warmig rates x Viscosity}}{\text{Volume}}$$

Decreasing the volume and increasing the cooling rate allow a moderate decrease in CPA concentration so as to minimize its toxic and osmotic hazardous effects [123]. This form of ultrarapid cooling must be followed by ultrarapid warming to prevent ice recrystallization.

If someone wanted to compare the slow freezing method with the physical process of vitrification, would be not correct define slow freezing as a method in which slow cooling rate and low CPA concentrations are used, and vitrification as high cooling rate and high CPA concentration are used. Successful vitrification can occur with a very low cooling rate [133] and very low concentration of CPAs [134, 135].

Briefly, cryopreservation by slow freezing is a process where extracellular water crystallizes, resulting in osmotic gradient that results in outward movement of osmotically active water from the intracellular compartment and their gradual dehydration until they reach the temperature at which intracellular vitrification occurs [136]. In cryopreservation by vitrification, both intra and extracellular compartments apparently vitrify after cellular dehydration has already occurred. For this reason the terms freezing and thawing are relevant to the slow freezing process while cooling and warming are relevant to vitrification. Both slow freezing and vitrification are currently

in use for oocyte cryopreservation. Several studies reported different outcomes using these methods (summarized in table 2.1).

Vitrification is time-saving and does not require special equipment like the programmed cryo-machine employed for manage the cooling rate on slow freezing methods. However, performing vitrification needs trained personnel with a good technical skill for loading the oocyte or the embryo properly into or onto the container.

Different cryopreservation protocols in different species provided different outcomes owing to several factors: the composition of the cryopreservation solution, equilibration and dilution strategies (volumes, times and temperatures), cooling rate warming rate, some examples are summarized in table 2.1.

## 2.6. Oocyte injuries during cryopreservation

Oocytes suffer considerable morphological and functional damage during cryopreservation. Injuries may occur at all phases of the cryopreservation procedure. Understanding the causes and mechanisms of damage may help the development of cryopreservation methods to avoid lethal or irreversible injuries.

Oocytes are among the largest mammalian cells, thus decreasing to a considerable extent the surface to volume ratio, making them more difficult to dehydrate and highly susceptible to intracellular ice formation [137]. However, it is not the single determinant factor that explain the damages that oocyte suffer during cryopreservation. For example, although zygotes have the same volume than oocytes, they are less sensitive to cryopreservation [138, 139]. The reason for oocyte susceptibility to cryopreservation is owing to their complex structure they suffer damage at different cellular levels during cryopreservation: the plasma membrane, zona pellucida, meiotic spindles, cytoskeleton, and so on.

### 2.6.1. Plasma membrane

Oocyte cryopreservation causes thermal [13] and chemical stress [140] to the plasma membrane and other intracellular organelles.

During oocyte cryopreservation, chilling injury occurs after exposure to low temperatures, but not freezing temperatures, and affects irreversibly the integrity of the membrane. Chilling injury takes place primarily at the temperature of lipid phase transition [141] the temperature in that membrane lipids change from the liquid crystalline phase to the gel phase. Unfortunately, changes that occur in this phase transition are usually not reversible, thus the cellular components are not reassembled correctly upon warming [142]. The phase transition temperature for bovine oocytes is thought to happen between 13 to 20ºC for GV oocytes and around 10ºC for MII ones [13]. Cells need membranes in a relatively fluid state to accomplish their functions.

The membrane composition, such as the cholesterol level, the phospholipid composition and the concentration of polyunsaturated fatty acids (PUFA) strongly influence the membrane fluidity at low temperatures changing its sensitivity to chilling injury, [141, 143]. One strategy to solve it might be lower the temperature when lipid phase transition occurs by modify the composition of the membrane [32, 144]

Another strategy to avoid the damage that chilling injury causes in the membrane, could be to use rapid cooling rates (as its used for vitrification) to cross the temperature at which the lipid phase transition happens, thus limiting the opportunity for damaging changes to occur [145].

At the same time that oocyte plasma membrane suffers damage during vitrification, the characteristics of plasma membrane can also impair the cryopreservation. In general, achieving a successful cryopreservation is more difficult in oocytes than in embryos, due partially to some differences in the characteristics of the plasma membrane among them. In bovine, the permeability of oocytes to water and CPAs is lower than that of morulae and blastocysts [146]. The plasma membranes of oocytes differ significantly from those of embryos, partially due to the lack of aquaporin expression which affects the movement of water and CPAs. In murine, bovine and porcine oocytes and early cleavage-stage embryos water and CPA move slowly, principally by simple diffusion, through the plasma membrane, for this reason flow rates are reduced and affected by the temperature [146-148]. Unlike in morulae, water and CPA move quickly, mainly by facilitated diffusion via channels [146-149], so the membrane permeability is higher and

has higher independence on the temperature. These factors allow embryos to be relatively more tolerant to vitrification and warming than oocytes [149].

The oocyte maturation stage determines some differences in oocyte membrane permeability. In bovine, GV oocytes present 2-fold lower hydraulic conductivity compared to matured MII oocytes [150, 151]. In other words, MII stage bovine oocytes have higher water and CPA permeability coefficients than GV stage oocytes. In this way, GV oocytes would be less able to tolerate potential water flux or shear force related damage during volume changes [152]. Conversely, at the same time this difference in plasma membrane permeability might make MII oocytes more sensitive, because changes of cell volume and intracellular CPA concentrations are more severe in MII than in GV bovine oocytes during CPA addition and dilution process [20]. It has to be into account when a vitrification-warming protocol is designed, in order to ensure the optimal CPA concentration, temperature and exposure time.

### 2.6.2. Zona pellucida hardening

Zona pellucida is a glycoproteic and lipidic envelopment [153, 154] that protects plasma membrane and cytoplasm from mechanic damage caused by possible ice formation during cryopreservation [155]. In vitrified oocytes it is particularly important during warming, because although ice crystal formation can be avoided with current vitrification methods, recrystallization often occurs during warming [155-157].

In addition, the oocyte cryopreservation process leads an increase in the intracellular calcium that triggers the premature cortical granules exocytosis [3, 158-160]. This rise in intracellular calcium concentration during the oocyte vitrification process may result from a cumulative effect of both CPAs [2, 3, 159, 161] and temperature drop [4, 162] Cortical granule fusion with the plasma membrane and the release of their proteolytic enzymes to the surrounding zona pellucida, causes zona pellucida hardening and this mean an obstacle for sperm penetration [2-4].

Vitrification causes zona pellucida deshydratation and alters the zona pellucida protein secondary structure, with a transformation to a more ordered structure [5, 163] and also causes changes in the lipid and carbohydrates configurations of the zona pellucida [5].

These biochemical and structural changes are believed to be related with cortical granule exocytosis and leads to zona pellucida hardening and the impairment of sperm penetration during in vitro fertilization [5, 163, 164].

The use of ICSI or subzonal sperm insertion can overcome the negative effect of zona hardening on the fertilization rates [4]. However, the fact that the oocytes have been object of the cortical reaction, indicate that the oocytes have been already artificially activated before to the real fertilization. So, this might partially explain that the oocytes do not follow a correct development after cryopreservation. Because when sperm is injected into a warmed oocyte during ICSI, due to the oocyte has already started its own course of development, the formation of a pronucleus from the sperm can be compromised [2, 165].

The origin of the CPA-induced calcium increase in oocytes seems to be different between different CPA. The source of the DMSO-induced calcium increase was from the intracellular calcium pool, whereas ethylene glycol caused an influx of calcium across the plasma membrane from the external medium Larman MG et al 2006. Larman MG et al 2006 suggested this after observing that removal of extracellular calcium from the medium failed to affect the response induced by DMSO but significantly reduced the ethylene glycol–induced calcium increase.

### 2.6.3. Meiotic spindles

Cryopreservation results in spindle disorganization with microtubule depolymerization and chromosomal dispersal [35, 166]. Meiotic spindles are dynamic bipolar structures composed of microtubules, chromosomes and associated structural proteins. The chromosomes align at the equatorial plane of the meiotic spindles. Microtubules are dynamic structures with continual polymerization and depolymerization of the tubulin dimmer at various stages of a cell cycle. Polymerized tubulin is in equilibrium with the free tubulin pool in the ooplasm.

Numerous reports have demonstrated that exposure of oocytes to **cooling** [11, 167, 168] and **cryoprotectants** [9, 11, 12, 169, 170], or the **cryopreservation process** [34, 40, 171, 172] can cause **depolymerization and disorganization of spindle microtubules**,

sometimes with attendant dispersal of chromosomes. Subsequent impaired repolymerization on rewarming may lead to scattering of chromosomes or lesions in the reformed spindle resulting in misaggregation of chromatids following resumption of meiosis [173] Thus, causing **chromosomal anomalies** after fertilization, such as aneuploidy and polyploidy [167, 174-180]. Spindle disorganization can also disrupt the sequence of events that lead to completion of meiosis and fertilization [181-183] and thus may contribute to the **low pregnancy** rates associated with some oocyte cryopreservation techniques [184, 185].

#### 2.6.3.1. Effects of the temperature on the meiotic spindle

The meiotic spindle has been shown to depolymerize when exposed to reduced temperatures in oocytes from several mammal species, such as mature bovine [186-188] porcine [11, 189], equine [190], mouse [168, 191-194]), and human oocytes [167, 195, 196].

In 1987, Pickering and Johnson described mouse spindle depolymerization that already occurred just reducing to room temperature; resulting in the majority (>75%) of spindles acquiring an abnormal configuration [192]. Further reduction in temperature (to 4°C) resulted in an abnormal spindle in all oocytes. The effects of cooling on the spindle appeared to be reversible in the mouse oocyte, with normal spindle formation occurring after step-wise re-warming. On returning to 37°C, repolymerization was observed, but numerous chromosomes were observed scattered throughout the cytoplasm [192, 193]. Depolymerization of the spindle following exposure to reduced temperature has been observed in oocytes from other species although the rate and the extent differ [189, 190] among species. Repeating their mouse study in human, Pickering *et al.* [197] observed a high proportion (50–75%) of oocytes with abnormal spindles (meiotic spindle completely disassembled, and this was accompanied by chromosomal dispersion in 60% of the oocytes) following brief exposure (30 min) at room temperature and a low frequency (25-50%) of normal repolymerization on return to 37°C. Similarly, Wang et al [167] confirmed by direct visualization of the human oocyte that human oocyte spindles undergo depolymerization between 27.1°C and 31.9°C, and that cytoplasmic organelles and granules in oocytes show reduced movement at room temperature (RT). In fact, even transient cooling to the room

temperature for only 10 min might cause irreversible disruption of the meiotic spindle in the human oocyte [167, 197, 198].

Complete disappearance of the spindle was observed in vitro-matured bovine oocytes after exposure to 4 degrees C for 10-20 min. When mature bovine oocytes were exposed to 25 degrees C for 30 min, 90% of spindle appeared abnormal or absent. This damage was irreversible in most oocytes regardless of cooling temperature or rewarming scheme [187].

Liu et al [11] reported that spindles in porcine oocytes began to depolymerize when the oocytes were exposed to 24ºC for 5 min, and when are maintained for 5 min at 4ºC the microtubules in the spindles of most oocytes becomes partially or completely disassembled. Furthermore, few spindles reassembled after re-warming for either cooling treatment. This observation contrasts with the finding that 89% of spindles in mouse oocytes reassembled after cooling to 4ºC for 1 hour and then re-warming at 37ºC [192].

#### 2.6.3.2. Effects of the cryoprotectants on the meiotic spindle

Cryoprotectants are known to cause depolymerization and disorganization of the spindle microtubules in several species, including the mouse [199, 200], rabbit [201], cow [169, 186], ovine [170], pig [202] and equine [190]. Differences in the rate and extent of microtubule depolymeritzation following exposure to CPA might be related to different sensitivity among oocytes from different species [203, 204] and stages [7] to different CPA concentrations [201] and exposure time and temperature [7].

Succu S et al [170] reported the only exposition of ovine prepubertal oocytes to vitrification solutions caused dramatic alterations of meiotic spindles which could not be restored afterwards and impaired developmental competence. In a parallel study performed for the same research group [205], using the same vitrification solutions and protocol, showed that in ovine adult oocyte spindle morphological configuration was altered after vitrification but not after the only exposition to vitrification solutions. They attributed this increased CPA toxicity observed in prepuberal oocytes to a deficiency of molecular and/or structural factors involved in spindle configuration restoring in prepubertal oocyte, which is consequently unable to bring the meiotic spindle altered by

the toxic effects of cryoprotectants back to the physiological structure. In bovine oocytes the sensitivity of the meiotic spindle to cryoprotectants was also different depending on the sexual maturity of the animal. Albarracin et al. [169] found that oocytes retrieved from adult cows were more sensitive to exposure to CPAs, while vitrification seemed to have worse effects on calf oocytes.

Rojas C et al [7] compared the effects of exposure to the CPA on porcine oocytes at two stages of maturation. Immature pig oocytes were found to be more sensitive to EG exposure than in vitro matured oocytes. In porcine, the exposure to EG at GV stage resulted in only 10 % of oocytes resumed meiosis and reached the MII stage and none of them presented a normal spindle structure [7]. The exposure to EG also altered the spindle organization in in vitro matured oocytes. A significant reduction in the percentages of normal spindle configuration was observed in mature oocytes exposed to EG (40.9%) compared to control oocytes (84.8%).  Wu C et al [189] confirmed that treatment with vitrification solution without freezing affected spindle organization in MII and GV porcine oocytes. Accordingly,  Shi et al [34] indicated that the cooling rate is not the only key factor to affect oocyte viability during vitrification based on they found that spindles were also damaged when the oocytes were treated by EG and DMSO only. The microtubule depolymerization caused by the exposure to cryoprotective agents (CPAs) depends on the concentration of CPAs. For example, at low concentrations (1 M), PROH was associated with meiotic spindle disruption in mouse oocytes, although it had a stabilizing effect at 1.5 and 2.0 M [206]. Chen *et al* [10] found that the spindles in mouse oocytes became disorganized or disappeared when they were exposed to VS containing 5.5 M EG and 1.0 M sucrose. Exposure of oocytes to 1.5 M DMSO caused depolymerization of microtubules in mouse oocytes [9] but showed stabilizing effects on human oocyte spindles during cooling [204].

However, due to in some of these experiments the exposure to CPA had been performed at room temperature, it is difficult to discern the effect of cryoprotectants in meiotic spindle from the effect that could exert the reduction of the physiological temperature to room temperature.

In contrast, other papers reported no effect of cryoprotectants on meiotic spindle or even a protective effect. In MII porcine oocytes, Shi et al [207] observed no differences in mitochondria distribution and spindle organization after CPA exposure compared to

fresh oocytes. However, CPA exposure lead to lower blastocyst rates (11.4% vs 23%) Similarly, simple incubation of metaphase II human oocytes in cryoprotective solutions without freezing has been shown previously to have no effect on the structure of the meiotic spindle [208]. Metaphase II mouse oocytes exposed to vitrification solutions, composed of EG and DMSO, and warming solutions didn't show microtubule depolymeryzation either immediately after exposure or after 2 hours of incubation after washing [172]. Gook et al [173] conjectured that cryoprotectants protects against the temperature-dependent depolymerization of the spindle, reducing the proportion of cooled mouse oocytes with an abnormal spindle based on observations from other papers [206, 209-211] that did not show an intrinsic cause-effect connection.

**2.6.3.3. Spindle recovery after warming**

In order to recover spindle and cytoskeletal defects caused by vitrification and warming processes and thus, avoid the associated chromosomal anomalies and fertilization problems, some studies suggested that is necessary a post-thawing incubation at physiological temperature for sufficient time before its subsequent treatment [10, 172, 212]. The duration of this time interval, which is mainly a reflection of the time needed for tubulin repolymerization, may vary depending on cryopreservation method and the species (table 2.1). The repolimerization capacity of microtubules during the post-thawing incubation was related to the microtubular organizing centers, the concentration of free tubulins, the presence of chromosomes, and the associated kinetochores [213]. Most of the studies revealed that oocytes analyzed immediately after thawing displayed severe disorganization or disappearance of spindles using slow, rapid, ultra-rapid, or vitrification methods [8, 9]. In general, incubation for 1-3 h after thawing/warming at 37 ºC resulted in recovery of spindles in diverse degrees that may be dependent on time intervals after thawing, methods of freezing, and species [10, 127, 172]

In vitrified mouse oocytes, Chen et al [10]observed that post-thawing incubation for 1 hour allowed recovery of normal spindle and chromosomes to diverse degrees, but incubation for 2 or 3 hours resulted in higher percentages of normal spindles than 1 hour. Consequently percentages of fertilization and blastocyst formation of vitrified mouse oocytes improved when inseminated at 2 or 3 h of incubation [10]. Gomes CM et al [172] experimentally demonstrated that providing an environment of 37°C for at least

2 hours in vitrified metaphase II mouse oocytes, β-tubulin repolymerize into microtubules that reorganize into normal-appearing MII spindles.

Gomes et al. [172] demonstrated that depolymerization of the mouse oocyte spindle also occurs in oocytes vitrified in straws in response to the cooling and warming process in the observations of Pol-scope and immunocytochemistry. After 37ºC culture for 2 hours, β-tubulin repolymerized and oocytes vitrified/warmed had normal-appearing MII spindles [172]. In contrast Chang et al [214] found that all mouse oocytes vitrified by Cryotop presented an intact spindle immediately after warming. After performing sequential observations, using immunocytochemistry with confocal microscopy, they indicated that the chromosome alignment was maintained throughout the complete course of the vitrification, warming and post-warming stage. However, the fixative used in the study contains deuterium oxide (D2O) and Taxol [214]. These two agents are known to promote microtubule polymerization, [215-217] and indeed induced formation of spindle-like microtubule bundles in oocytes, where microtubules had been depolymerized by cold treatment [212]. Thus, it is likely that inclusion of D2O and Taxol in the fixative may have caused aberrant polymerization of microtubules during fixation in the previous study [214]. Oocyte vitrification may have less impact on spindle structure than slow freezing method [9, 171, 218].

Theorically, small volume supports, such as OPS, CPS, and electron microscopic grids, preserved the spindle morphology and chromosomal pattern better than conventional straws [9, 165]. The rapid cooling and warming rates achieved by minimum volume methods may quickly traverse the temperature that is damaging to the spindle [9, 10], assumed to be 15ºC to −15ºC [81]. Moreover, oocytes of minimum volume methods are directly warmed in the dilution solution and immediately diluted. This reduces exposure of oocytes to inappropriate temperatures and concentrated cryoprotectants [219].

Morato et al [37] found that cryotops achieved better spindle preservation than OPS for vitrification of bovine oocytes. After 1h of recovery post warming Chen et al [9] found oocytes vitrified in OPS had more normal spindles restored (78%) than did those in conventional straws (21%). Coticchio et al [176]found that spindles were significantly affected after the slow-freezing method using 0.1M sucrose concentration, while they were unchanged using the 0.3M sucrose protocol. Ciotti et al [171] reported that spindle

recovery was faster in vitrification than in slow freezing. In contrast, Cobo et al [127] found comparable spindle recovery from vitrification and slow freezing after 3 hours of incubation.

Asgari et al [220] recommended instead resting vitrified-warmed matured oocytes for 1–3 h to recover spindle and cytoskeleton before starting a further treatment, as is generally accepted, warmed oocytes should be used for the subsequent treatments as soon as possible. Because vitrified-warmed matured oocytes may resume meiosis spontaneously during this recommended rest time. They observed after 2 h postwarming most of the oocytes reorganized the spindle in a characteristic configuration of the anaphase II stage. Therefore, it was concluded that during the rest period, although tubulin can polymerize to restore microtubule structure, the oocyte can resume meiosis and dictate spindles to reorganize in anaphase II structure and not MII. A potential problem of matured oocyte cryobiology is precocious calcium release which results in a sharp decrease in maturation promoting factor (MPF), the central kinase of mitosis and meiosis resumption [3]. Therefore, there seems to be a conflict between the presumed advantages and possible disadvantages of post-warming rest time.

**2.6.3.4. Differences in spindle injury among maturation stages**

To circumvent the problem of microtubule depolymerization observed in the meiotic spindle of MII oocytes, oocytes could be cryopreserved at GV stage, when meiosis is arrested at the prophase I stage and the chromosomes are protected within the membrane of the germinal vesicle and when no microtubular structures have formed yet [221, 222]. Due to the absence of spindle at the GV stage, it would be expected that oocytes cryopreserved at the GV stage would show more stability regarding cryoinjury to the meiotic spindle than those cryopreserved at the MII stage.

On the contrary Boiso et al [208] found that only 5.2% of the analyzed oocytes cryopreserved at the GV stage show a well-structured spindle with the chromosomes aligned on the metaphase plate after in vitro maturation. They observed absence of detectable microtubules in most of the oocytes analyzed (76.3%), while the number of abnormal detectable spindles was relatively low (18.4%). Oocytes cryopreserved at the GV stage that displayed abnormal configurations had either disorganized chromosomes or chromosomes clustered in a discrete group with an aberrant, less condensed

appearance (a morphological feature not found in abnormal cryopreserved MII oocytes) as previously described [223]. Coincident with the high rate of spindle and metaphase plate abnormalities observed in cryopreserved immature oocytes, they found a high rate of aneuploidy and polyploidy afterwards [224]. This indicated that meiotic progression does not occur normally in oocytes cryopreserved at the GV stage. Gomes C et al [168] hypothesized that there are temperature-dependent differences in the depolymerization/repolymerization equilibrium of oocyte spindles according to meiotic stage and this temperature could influence the response of oocytes to cryopreservation.

Comparing porcine oocyte vitrification at diferent maturation stages, Wu c et al [189] also reported irreversible damage to the cytoskeleton of porcine GV- and MII-oocytes after vitrification, which could be an important factor affecting developmental competence. After in vitro maturation, none of the porcine oocytes vitrified with OPS at GV stage showed normal spindle pattern, compared to the 25.6% normal spindle morphology observed in oocytes vitrified at MII after 2h recovery post-warming. However the maturation rate after GV vitrification in this study is quite low (6%) compared to 22% reported for Isachenko et al [225].

Although GV oocytes don't suffer damage to the spindle because it is not formed yet, GV oocytes can suffer damage to cytoplasmatic organelles that impair subsequent maturation and further development.

**Table 2.1. Effects of different oocyte vitrification protocols and post-warming incubation time on meiotic spindle of oocytes from different species**

| Sp | Cryopreservation Method | Recovery | Results | Ref. |
|---|---|---|---|---|
| H | SF vs VIT (cryotip) | 3h | After 3 h of incubation, comparable spindle recovery from vitrification and slow freezing | [127] |
| H | SF | 0h vs 3h | Spinde disappeared after thawing, but all reappeared after 3h incubation | [177] |
| H | VIT (cryotop) | 2h | Transient hydrostatic pressure did not damage meiotic spindle | [226] |
| H | VIT (cryoleaf) | 2h | Pre-equilibrium at 37°C may reduce the incidence of defective spindle configuration | [227] |
| H | SF | 3h | 0.3M sucrose in the freezing solution preserves better meiotc spindle than 0.1M | [228] |
| H | SF vs VIT (cryoleaf) | 2-3h | Vitrification preserves better spindle than slow freezing (60.9% vs 82.3%) | [211] |
| H | SF vs VIT (HSV) | 0-300min | Meiotic spindle recovery is faster in vitrification compared to slow freezing | [171] |
| M | VIT (OPS vs straw) | 1h | OPS preserves better spindles than conventional straw | [9] |
| M | VIT (CPS, OPS, straw, EMG) | 1h, 2h, 3h | After 2 and 3h incubation, significantly better spindle patterns than 1h. Better spindle in OPS, CPS, EMG than straw | [10] |
| B | VIT (OPS) | 2h | CPA had a drastic effect on spindle organization in cow oocytes, while VIT more severly affected calf spindle | [169] |
| B | VIT (OPS vs cryotop) | 2h | Cryotops preserved better spindle than OPS | [37] |
| B | VIT (FDP vs OPS) | 2h | OPS preserved better spindle than FDP | [229] |
| B | VIT (cryotop) | 2H | Oocytes matured with Lc+R showed normal spindle configuration rates after vitrification similar to fresh counterparts | [230] |
| B | VIT (OPS) | 2h | Calf oocytes treated with taxol prior to vitrification had similar normal spindle rates than control | [17] |
| P | VIT (OPS) | 2h | MII stage porcine oocytes had better resistance to cryopreservation than GV stage oocytes. | [7] |

| | | | | |
|---|---|---|---|---|
| P | VIT (OPS) | 30h/2h | Irreversible damage to the cytoskeleton of porcine GV- and MII-oocytes after vitrification, | [189] |
| P | VIT (0.25ml straws) | 2h | Spindle morphology was affected by vitrification but not for CPA exposure | [207] |
| P | VIT (OPS) | 30m | Exposure to VS/WS and vitrification/warming procedures can cause spindle disassembly and chromosome misalignment | [34] |
| O | VIT (cryoloops) | 2h | Vitrification and cryoprotectant exposure altered spindle morphological configuration in prepubertal ovine oocytes | [170] |
| O | VIT (cryotop) | 0h vs 2h | In sheep, chemical activation without having to await microtubule reorganization improves embryonic development | [220] |
| E | VIT (OPS) | 30h+45' | COCs horse oocytes preserved better the spindle morphology after vitrification than cumulus-free oocytes | [231] |
| E | SF vs VIT (OPS) | 30h/0h+45' | SF of MII oocytes yielded higher prop of MIIs with a normal spindle (35%) than SF of GV oocytes ( 1%), OPS of GV oocytes ( 15%) or OPS of MII oocytes ( 20%) | [190] |
| R | VIT (cryotop) | 2h | Taxol or Cytochalasin B treatment prior to vitrification did not improve the spindle morphology | [44] |
| R | VIT (cryoloop) | 2h | Spindles were severely injured in both vitrification protocols | [201] |
| R | VIT (cryoloop vs Vit Master) | 2h | Vit Master vitrification preserved better spindle morphology than cryoloop. | [201] |

Sp: species; H: human; M: mouse; B: bovine; O: ovine; P: porcine; E: equine; R: rabbit. SF: slow freezing; VIT: vitrification; HSV: high security vitrification straw; CPS: closed pulled straws; EMG: electron microscopy grids FDP: flexipet denuding pipettes; OPS: open pulled straws. Recovery: time interval after warming while oocytes are maintained to physiological temperature before being submitted to further treatments. CPA: cryoprotectants

### 2.6.4. Gene expression

Stress suffered during oocyte vitrification and warming might have an effect on oocyte gene expression and it can remain affected during the subsequent stages of development. Then, oocyte vitrification can alter blastocyst gene expression because, although during embryonic genome activation most of oocyte proteins and RNA are degraded, some maternal factors persist due to they have a key role for the successful subsequent embryonic cell cleavage and development [232]. Maternal factors, stored in the oocyte and encoded by maternal-effect genes, have been proven to play crucial roles in regulating epigenetic marks and maintain DNA imprints during preimplantation development [233]. These maternal factors participate in physiological important functions during early preimplantational embryo development such as, processing of the male genome after fertilization, removal of maternal detritus, activation of the embryonic genome and the formation of the blastocyst. However, if maternal genes impose epigenetic marks that are stably inherited (e.g. DNA methylation) or modify structures important for development, these maternal effects might become manifest only at later developmental stages [234].

Recent studies have shown that oocyte vitrification could cause epigenetic modifications of preimplantational mouse embryos such as histone acetylation [235] and DNA methylation [236]. Cryopreservation also altered the expression profile of human MII oocytes [237] and vitrification decreased the expression of oocyte methyltransferase 1 (DNMT1o) mRNA in mouse MII oocytes [238]. Cheng KR et al [239] reported that after mouse oocyte vitrification DNA methylation of differentially methylated regions of three imprinted genes were lower in blastocysts obtained from vitrified oocytes than those from the fresh oocytes. Moreover, the relative expression levels of methyl transferases (DNMTs) were significantly decreased after oocyte vitrification, but in blastocysts no significant differences were observed in expression levels of DNMT1, DNMT3A and DNMT3L; only DNMT3b expression was lower in vitrified group. Bovine oocyte vitrification caused loss of DNA methylation in oocytes and embryos that severly affected the epigenetic of oocytes and embryos and might contribute to the impaired potential of embryonic development after oocyte vitrification [240].

Thus, it can be generally accepted that mRNA abundance decreased in oocytes after thawing, causing potential disturbance of the imprinting in the preimplantation embryo [240].

## 2.7. Approaches to circumvent cryopreservation injuries

### 2.7.1. Ice blockers

In recent decades, numerous attempts have been made to improve cryopreservation outcomes by limiting the oocyte damage. During freezing and thawing, physical damage, such as ice formation and recrystallization, is one of the main causes of cryoinjury. Because ice binding proteins and synthetic ice blockers can inhibit ice formation and recrystallization, treatment with ice binding proteins or synthetic ice blockers have been used to increase the survival and development after oocyte cryopreservation [20, 24-27, 241, 242].

**Ice binding proteins**

Ice-binding proteins (IBPs) produced by polar organisms inhibit the growth of ice crystals to protect themselves from freezing damage. These proteins include antifreeze proteins (AFPs), ice-recrystallization inhibition proteins and ice nucleation proteins [243-245]. IBPs have the property of binding to ice crystals, thereby inhibiting the growth of extracellular ice crystals and protecting the organism against osmotic shock and physical damage.

AFPs were first discovered by DeVries in Antarctic marine fish ([246, 247]; which, as their name implies, they function to prevent freezing. *AFPs are groups of proteins that bind to ice crystals and inhibit ice growth, lowering the freezing point below the equilibrium melting point. This phenomenon is quantitatively described as thermal hysteresis (TH)* [248]). AFPs also protect cell membranes from cryoinjury by inhibiting the ice recrystallization [249, 250]. Since then, AFPs have been identified in numerous other organisms, such as freeze avoiding insects (those that must prevent freezing, as they die if frozen) and other terrestrial arthropods. However, proteins with similar, but considerably lesser, antifreeze (thermal hysteresis or TH) activities have been found in freeze-tolerant species (those able to survive extracellular freezing), where they do not prevent organismal freezing, but instead function to control the site and temperature of

ice formation, ice crystal structure and rate of freezing. Other proteins, ice-nucleating proteins (INPs), actually promote ice formation by limiting supercooling and induce freezing in the extracellular fluid at high subzero temperatures; thereby allowing them to control the location and temperature of ice nucleation, and the rate of ice growth. The term 'ice-binding proteins' (IBPs) is now used to include both true AFPs in freeze-avoiding species and the proteins in freeze-tolerant species (low TH activity proteins and IBPs) [251]. IBPs have the property of binding to ice crystals, thereby inhibiting the growth of extracellular ice crystals and protecting the organism against osmotic shock and physical damage

Rubinsky et al [241] discovered that antifreeze glycoproteins (AFGPs) from Antarctic fishes produce dramatic improvements in the morphological integrity in immature pig oocytes and 2-cell stage mouse embryos, upon vitrification, and suggest that antifreeze proteins (AFPs) have the ability to inhibit ice formation and stabilize the plasma membrane. Since then, different studies have been performed to assess the effect of AFP on oocyte vitrification [24-27, 242].

Fish antifreeze protein III (AFP III) added as a non-permeable cryoprotectant during oocyte vitrification demonstrated to protect mature [24, 26, 242] and immature mouse oocytes [27] from most current damage suffered during cryopreservation and enhance embryo development after warming.

On bovine specie, antifreeze glycoprotein 8 (AFGP8) supplementation during vitrification effectively protects MII-stage oocytes against chilling injury, as well as improves their cryosurvival and subsequent embryonic development [25]. Recently, Chaves DF et al [252] reported different concentrations (500 and 1000 ng/mL) AFP III into vitrification media did not preserve in vitro matured bovine oocyte meiotic spindle organization against the cryoinjuries and did not improve blastocyst formation.

Lee HH, et al., [24] compared the effect of adding three AFP from different source on murine oocyte vitrification-warming solutions and concluded that AFPs from bacteria (Flavobacterium frigoris ice-binding protein (FfIBP)), yeast (Glaciozyma sp. ice-binding protein (LeIBP)) and fish (Type III AFP) added to the vitrification and warming solutions individually, improve murine oocyte quality and embryo development.

Theoretically, no ice is formed during vitrification; however, AFPs may prevent potential ice formation during vitrification and, especially, recrystallization during warming. In addition, another possible explanation is that AFPs may bind to the cell membrane and, as a result, stabilize the oocyte membrane [253-255].

#### 2.7.1.1.Ice-binding polysaccharides

Aside from AFP, other molecular compounds such as lipoproteins or polysaccharides may exert a cryoprotectant role.

Other types of thermal hysteresis-producing biomolecules (biomolecules endowed with antifreeze properties) such as lipoproteins and polysaccharides from bacteria, insects, lichen and plants have been known for several decades [256-259]. Polysaccharides constitute a common class of molecules that interact with ice in nature either by triggering ice nucleation or by inhibition of ice nucleation and growth. For example, a high molecular weight polysaccharide from *Bacillus thuringiensis* was found to inhibit ice nucleation [260]. Another polysaccharide with a fatty acid component from the Alaskan beetle *Upis ceramboides* exhibited a pronounced thermal hysteresis due to ice binding [261]. Boreal birch pollen polysaccharides showed ice-nucleating as well as ice-binding ‘antifreeze’ activities [262].

The extracellular exudates, composed primarily of exopolysaccharides (EPS), that are produced by psychrophilic and ice-dwelling microorganisms have reached a prominent position among the cryoprotectants [263-265]. Recently Carrillo S et al [266] discovered an extracellular polysaccharides synthetized from the psychrophilic marine bacterium Colwellia psychrerythraea 34H that mimics antifreeze glycoproteins since in its purified form is endowed with ice recrystallization inhibitory activity.

EPS produced by Pseudomonas sp. ID1, a cold-adapted bacterium isolated from marine sediment collected from South Shetland Islands (Antarctica), is a high molecular weight heteropolysaccharide (>2 × $10^6$Da) composed mainly of glucose, galactose and fucose. This biopolymer also contains small amounts of uronic acid, and aminoacids (glutamic acid, aspartic acid, glycine and alanine) [267]. Similar to extracellular polysaccharides from Colwellia psychrerythraea 34H, the results of chemical and spectroscopic analyses

of the purified capsular material from Pseudomonas ID1 revealed the presence of amino acids in the composition. In fact, the presence of amino acids is crucial for the interaction with ice, in several different kinds of AFP [265, 268] and is quite uncommon among the composition of several EPS produced by many marine bacteria.

EPS from Pseudomonas sp. ID1 is able to preserve the cell structure of these bacteria and maintain its viability after being submitted to freezing temperatures of −20◦C and −80◦C, demonstrating that EPS production is an adaptation mechanism for survival in cold marine environments such as the Antarctica [267]. Pseudomonas sp. ID1 EPS, not only conferred cryoprotection for the strain itself, but also for E. coli cells, suggesting it can thus be applied as an agent for cell cryopreservation, alone or in combination with other cryoprotectants currently in use. Several studies have demonstrated that EPS production in bacteria at low temperatures is a cold temperature adaptation mechanism [265, 269, 270]. Other authors have reported that the EPS cryoprotective activity not only benefits the cold-adapted bacterial producer, but also non-producing cells, such as E. coli, suggesting a universal cryoprotectant role for these biopolymers [271, 272]. There were no studies that have investigated the effect of EPS on the outcome of oocyte vitrification.

#### 2.7.1.2.Synthetic ice blockers

Short time after the discovery of polar fish proteins (AFPs), Fahy and co-workers [92] proposed creating synthetic ice blocker specifically designed to bind to nucleators and nascent ice crystals in a manner similar to that of natural antifreeze proteins. Up to now, several specific molecules and polymers were proposed and added to vitrification solutions. SuperCool X-1000 and SuperCool Z-1000 are the most used products among them, which have been reported as an additive in solution developed for kidney and tissue- engineered bone vitrification [273, 274]. Unlike conventional cryoprotectants that inhibit freezing by interacting with water, ice blockers are believed to bind to the surface of growing ice crystals and inhibit the addition of any further water molecules in specific planes of growth [92] This selective attraction to surfaces of ice growth permits ice blockers to exert significant effects even while present at very low concentrations. Small quantities of ice blocker can therefore modify the number and size of ice crystals and thereby change the vitrification tendency of a solution without adding additional

toxicity [275]. The commercially available ice blockers are SuperCool X-1000 and SuperCool Z-1000. The SuperCool X-1000 is a copolymer of polyvinyl alcohol (PVA) of mean molecular mass 2000 Da, with 20% of the hydroxyls replaced by acetate groups.

The Super- Cool Z-1000 is a copolymer of polyglycerol (PGL) of mean molecular mass 750 Da [276]. The combination of SuperCool X-1000 and Super- Cool Z-1000 has been used as an additive in solutions developed for kidney vitrification [274].

Zhou XL et al [20] reported ice blockers X-1000 and/or Z-1000 in vitrification solution did not affect the survival rate and developmental competence of immature bovine oocytes vitrified and warmed by cryotop method. Similarly Badrzadeh et [277], studying the effect of different ice blocker media (X-1000 and/or Z-1000) on vitrification/ warming of mouse embryos using V-kim closed system, found that combination of ice blocker X-1000 and Z-1000 resulted in significant higher survival rate of mouse embryos after vitrification/thawing process, but did not affect the blastocyst formation rate.

Zhou et al [20] attributed the different results obtained in oocytes compared to organs, on the function of ice blockers during vitrification. In large volume vitrification systems, such as organs and engineered tissues, ice blockers can suppress nucleation and recrystallization by binding to nucleators in solutions during vitrification and warming. Therefore, the ice growth was inhibited and damage to the systems was reduced. Nevertheless, in small volume vitrification systems, such as Cryotop and V-kim, the reduced amount of liquid and ultra-fast cooling rate make the extent of vitrification high enough, the main obstacle in these systems is not nucleators. Ice blockers cannot exert effects to vitrification process.

### 2.7.2. Stress tolerance, induced resistance

Other approach to improve the tolerance of oocytes to cryopreservation is the application of **sublethal stress** rightly defined and **applied appropriately** before manipulation in order to induce **a general adaptation** that permits increase the subsequent **stress tolerance** [46]. Recently have been reported that treatment of gametes or embryos with several different kinds of stress-inducing mechanisms, such

as high hydrostatic pressure (HHP), or osmotic, heat or oxidative stress resulted in increased morphological intactness, fertilizing ability or developmental competence after various in vitro or in vivo procedures, such as cryopreservation, parthenogenetic activation, or somatic cell nuclear transfer [46, 278].

In stress response participates an amount of chaperones that were first detected after heat shock, for this reason were called heat shock proteins (HSP), although later found to be involved in responses to several abrupt changes like temperature, high hydrostatic pressure (HHP), pH, osmotic stress and starvation. HSP participate in various cellular functions, including protein, DNA, and chromatin stabilization and repair; cell cycle control; redox regulation; energy metabolism; fatty acid/lipid metabolism; and the elimination of damaged proteins [279, 280]. The production of **heat shock proteins** might explain the improvement in survival after vitrification and developmental competence of oocytes after stress treatment induced by different stress factors [48]. Proteins produced as a result of a response to HHP, heat or osmotic stress can give protection towards damage produced by vitrification, because different signals of stress can trigger similar responses due to the similar effect on macromolecular disruption (membrane lipids, proteins and DNA) [281]. So, common set of stress-inducible proteins are expressed and might be the basis for cross-protection [46].

If the stress impact is over the limit of tolerance, programmed cell death (apoptosis) or necrosis occurs [282]. Stress-induced proteins may either reduce or facilitate the activation of the apoptotic cascade [283, 284].

**High hydrostatic pressure** (HHP) is a technique that consist in apply high pressures to a sample, generated generally by a pressurizing device, and cause stress for the cells in a magnitude determined by the pressure and the duration time of application. Initially HHP was chosen as a sublethal stressor, to increase the subsequent stress tolerance because of its unique and outstanding features: (i) acts instantly and uniformly at every point of the cell, (ii) presents zero penetration problems or gradient effects, (iii) can be applied with the highest precision, consistency, reliability and safety, (iv) functions with an extremely high safety margin and wide therapeutic range for the cells and (v) has no or minimal cell-to-cell variation [46]. In porcine oocytes 20-MPa pressure for 60 min treatment increases the stress tolerance, as it induced more than tenfold increase in blastocyst rates after vitrification and parthenogenetic activation [45, 283]. Similarly,

**sublethal HHP stress** treatment of immature porcine oocytes resulted in an increased blastocyst rate and higher blastocyst cell numbers following in vitro maturation, parthenogenetic activation, and in vitro culture [285]. The stress tolerance of mouse oocytes was comparable to that of porcine ones. Treatment of MII phase mouse eggs with 20 MPa pressure for 60 min before vitrification resulted in increased survival after warming and intracytoplasmic sperm injection and increased pregnancy rate and litter size [46].

**Osmotic stress** is a physiologic dysfunction caused by a sudden change in the solute concentration around a cell, which causes a rapid change in the movement of water across its cell membrane. It represents another method to induce sublethal injury and increase subsequent stress tolerance. Lin et al [47] reported that **osmotic stress** of oocytes resulted in higher developmental rates after cryopreservation, parthenogenic activation, and somatic cell nuclear transfer [47]. Exposure of porcine oocytes to elevated NaCl concentration improves their cryotolerance and developmental competence [47]. In another study they observed the same effect when sucrose, trehalose and NaCl were compared [48].

Another way to induce a stress response into the cells is the application of oxidative stress. **Oxidative stress** is caused by the intracellular accumulation of reactive oxygen species (ROS) or a disturbance of the cellular redox state. Short term exposure of bovine in vitro matured cumulus-oocyte complexes (COCs) to hydrogen peroxide (H2O2) (50–100 µmol/L) resulted in a significantly higher blastocyst yield (47.3%) than the one achieved without **oxidative stress** treatment (31.8%), whereas apoptotic cell ratio was inversely related with H2O2 concentration [286].

**Heat stress** occurs when the endogenous cellular mechanisms to control the cell temperature fails, due to a sudden or to a higher increase in the environmental temperature. Heat stress can be used as another sublethal stress treatment, applied appropriately at a given temperature, during a determined time interval of time, to generate an induced cell resistance to further stress. Isom et al. [287] found increased developmental competence of parthenogenetically activated porcine oocytes when a 42ºC **heat stress** was applied for 9 hours immediately after activation. However, the same effect was not observed on in vitro fertilized or cloned embryos.

Although the expression kinetics of stress-related proteins revealed that the time of the peak expression varied between cells and stress impacts [288], those studies concurred on the highest protective effect of sublethal HHP or osmotic treatment was achieved if the second manipulation (cryopreservation, parthenogenetic activation, or enucleation) started 1 to 2 h after the end of the initial stress [45, 289].

### 2.7.3. Modification membrane composition

Cooling [13] and osmotic stress [140] during oocyte cryopreservation might cause irreversible damage to membrane integrity. Oocytes undergo huge volume changes due to water and cryoprotectant movement during cryopreservation processes [140]. For this reason, cells with more flexible and permeable membranes to water and cryoprotectants in general will suffer less damage that those cells with rigid and non-permeable membranes [290].

The function and integrity of cell membranes are affected by the lipid phase transition (LPT). The phase transition temperature is defined as the temperature required to induce a change in the lipid physical state from the ordered gel phase, where the hydrocarbon chains are fully extended and closely packed, to the disordered liquid crystalline phase, where the hydrocarbon chains are randomly oriented and fluid [291].

When temperature decreases bellow lipid phase transition, cell membranes undergo a transition from the liquid phase to the gel phase [141].The tendency for phase changes in membranes during cooling is highly dependent on membrane composition [292]. LPT profile of cellular membranes is influenced by the fatty acid composition of phospholipids, the protein concentration and the presence of sterols. Cellular membranes with short chain polyunsaturated fatty acids are more fluid at low temperatures and are more resistant to chilling stress [292-295]

Lowering the temperature results in profound changes in the phase transitions of lipids of the oocytes of several species and influence the degree of cryoinjury in oocytes from humans [141], cattle [13] and sheep [144]. Due to a low LPT temperature is associated with resistance to chilling stress [13, 141] oocytes with greater degrees of cell membrane fluidity will more likely survive the cryopreservation process.

Several groups have attempted to change plasma membrane composition during in vitro procedures to improve cryopreservation of embryos and gametes [144, 296-298]

The addition of **phosphatidylcholine** or **dipalmitoylphosphatidylcholine,** unsaturated fatty acids, to mature bovine oocytes by **electrofusion of liposome** with their plasma membrane decreased their sensitivity to chilling through the modification of the lipid phase transition temperature [299]. In other studies, the addition of **linoleic acid–albumin** to culture media, to change oocyte membrane lipid constituents, improved cryosurvival of bovine enucleated oocytes; the proportion of development to blastocysts of bovine embryos produced by nuclear transfer into frozen-thawed cytoplasts was improved from 4% to 14% [300].

**Cholesterol** is a major structural lipid constituent of the membrane and regulates its function. Therefore, the cholesterol/phospholipid ratio is a vital determinant of plasma membrane fluidity and stability during cryopreservation [143, 290]. Membranes with high concentrations of cholesterol are more fluid at low temperatures and consequently more resistant to damage during cooling [141].

When cholesterol is inserted into a phospholipid bilayer acts as a normalizing agent, creating disorder in highly ordered bilayers and bringing order to those that are disordered [301]. The effect of cholesterol on membrane fluidity depends on the cholesterol concentration and the temperature. Cholesterol induces the liquid-ordered phase, which increases the fluidity of the phospholipid hydrocarbon chains above the gel to liquid-crystalline phase transition temperature and decreases it below the phase transition.

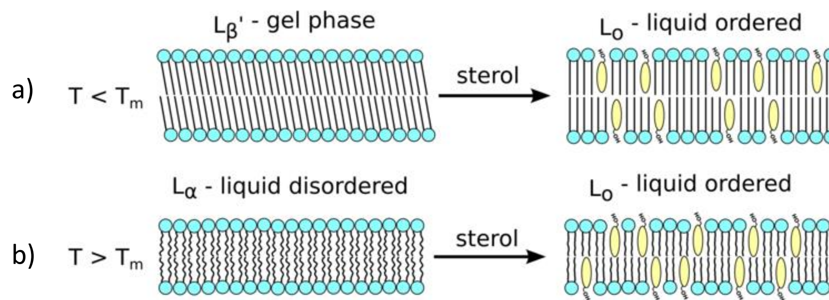


**Figure 2.1.** This diagram represents the changes in membrane phases caused by cholesterol addition depending on the temperature. (a) When cholesterol is incorporated into membrane bellow the lipid phase transition temperature, the membrane that was in a gel phase change to liquid ordered phase (the fluidity increase). (b) While cholesterol addition above the melting temperature, when the membrane is in a liquid disordered phase, makes it change to liquid ordered phase (the fluidity decreases). *Image from Physics LibreTexts*

Specifically, below the lipid phase transition (gel phase), the increase in cholesterol concentration leads to a decrease in bilayer thickness, lipid tail order parameters and an increase in area per lipid component indicating the induction of local disorder [291], increasing the fluidity. Yet, when increasing cholesterol concentration in the temperatures above the lipid phase transition (liquid phase), this trend is reversed [291], decreasing the fluidity.

Then, to increase membrane fluidity and permeability at low temperatures, cholesterol can be added to the plasma membrane, thereby providing an alternative method for increasing oocyte tolerance for cryopreservation.

The beneficial effect of adding cholesterol has already been demonstrated to increase bull [302, 303] as well as stallion [304] sperm cryosurvival. In these studies, enriching the plasma membrane with cholesterol was done by incubating the cells with cholesterol previously loaded into methyl-b-cyclodextrins (βCD). Cyclodextrins, which are cyclic oligosaccharides consisting of five or more α-D-glucopyranose residues linked by type α-1,4 glucosidic bonds, contain a hydrophobic center capable of integrating lipids.

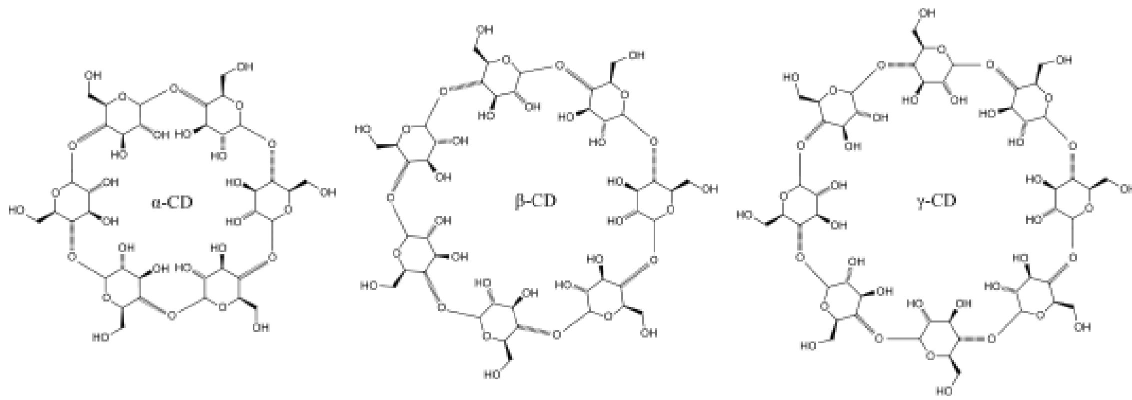


**Figure 2.2. Chemical structure of the three main types of cyclodextrins**

Among them, βCDs have the highest affinity for inclusion of cholesterol and are the most efficient in extracting cholesterol from isolated or intact membranes from a variety of cells (reviewed in:[305]. However, the high affinity of βCDs for cholesterol can be used not only to remove cholesterol from the biological membranes but also to generate cholesterol inclusion complexes that donate cholesterol to the membrane and increase membrane cholesterol level. βCD-cholesterol inclusion complexes are typically generated by mixing cholesterol suspension with a cyclodextrin solution. The efficiency of cholesterol transfer from βCD inclusion complex to biological membranes depends on βCD:cholesterol molar ratio, βCD-cholesterol concentration, and duration of the exposure [306, 307].

In oocytes, co-incubation of bovine immature [188] or *in vitro* matured oocytes [32] with βCDs loaded with cholesterol (CLC) improved nuclear maturation of oocytes after vitrification but failed to improve their embryo development up to the blastocyst stage.

When examining the incorporation of cholesterol in the oocyte plasma membrane after incubation with CLC, Horvath and Seidel [32] and Jiménez-Trigos et al [308] observed that cholesterol was transported through the cumulus layers and the zona pellucida in oocytes incubated for 1 hour with cholesterol labeled with 22-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4yl) amino-23,24 bisnor-5-cholen-3β-ol (NBD). However, little information was provided regarding the exact localization of cholesterol within the oocyte or how much cholesterol entered the cell. So, the lack of effect on developmental competence reported by Horvath and Seidel [32], Sprícigo [188] and Jímenez-trigos [308] could be

due to a non-optimal cholesterol concentration into membrane, either to the cholesterol diffused throughout the plasma membrane and it accumulated into the cytoplasm.

Imaging cholesterol inclusion to plasma membrane by live confocal microscopy could be useful to determine the incubation time required for optimal cholesterol incorporation into membrane at a given concentration. Analysis of the intracellular distribution of cholesterol requires using appropriate cholesterol analogs. An ideal cholesterol analog would have essentially the same molecular properties like cholesterol but would bear an intrinsic label, which allows its detection by fluorescence techniques or other methods [309]. One particular complication when examining the transport of cholesterol or other lipids by microscopy is the observation that coupling to a fluorophore can dramatically change the structural and biochemical properties of a molecule and its interactions with other membrane components [310]. Some studies reported NBD-labeled analogs of cholesterol could not be suitable for following cholesterol trafficking due to it can be substrate for sterol modifying enzymes, and having opposite membrane behavior or completely different intracellular itineraries than cholesterol [309]. These problems could be avoided using natural sterol with intrinsic fluorescence properties such as dehydroergosterol (DHE) or cholestratienol (CTL), that act as close analogs of cholesterol. However, its disadvantage is that DHE and CTL emits light in the UV region of the spectrum making detection by fluorescence microscopy for analysis of intracellular sterol transport very difficult [309].

Boron-dipyrromethene (BODIPY)-cholesterol closely mimics the membrane partitioning and trafficking actions of cholesterol and because of its excellent fluorescent properties, enables the direct monitoring of sterol movement by time-lapse imaging using trace amounts of the probe [311]. So far, BODIPY-cholesterol has been used as a cholesterol probe in model membranes and in trafficking studies in living cells [312, 313].

# CHAPTER III:

Objectives and experimental design

The objective of the present thesis was to optimize the current protocols for bovine oocyte vitrification in order to improve the subsequent developmental competence by:

1. According to earlier investigations, sublethal stress, properly defined and applied appropriately before manipulation, could improve the cryotolerance of oocytes through adaptation mechanisms enhancing their subsequent stress tolerance (Pribenszky et al. 2010). Several controlled stress treatment protocols for oocytes have been reported using hydrostatic pressure, osmotic, oxidative or mechanic impacts and seems to improve their survival and subsequent in vitro development during in vitro maturation, cryopreservation, enucleation or somatic cell nuclear transfer (Du Y et al 2008; Lin 2009a,b, Lin L et al 2010, Pribenszky *et al.* 2010, 2012). In the first paper of this PhD dissertation **(chapter IV)** we hypothesized that the temporarily exposure of *in vitro*-matured (IVM) bovine oocytes to high concentrations of NaCl or sucrose before vitrification–warming could improve their cryotolerance. To assess this objective, in the first experiment, microtubule and chromosome configurations were analysed after exposure of oocytes to various concentrations of NaCl and sucrose. In the second experiment, oocytes were treated with selected concentrations of NaCl or sucrose, vitrified–warmed and microtubule and chromosome configurations evaluated. Finally, and based on results obtained in the previous experiment, oocytes were exposed to 375 mOsm sucrose, vitrified–warmed, fertilized and cultured up to the blastocyst stage.

2. Oocytes undergo huge volume changes due to water and cryoprotectant movement during cryopreservation processes [140]. To increase membrane fluidity and permeability at low temperatures, cholesterol can be added to the plasma membrane, thereby providing an alternative method for increasing oocyte tolerance for cryopreservation (Seidel 2006; Varghese *et al.* 2009; Ghetler *et al.* 2005). Previous studies reported co-incubation of bovine immature (Spricigo et al. 2012) or in vitro matured oocytes (Horvath and Seidel 2006) with methyl-β-cyclodextrins (MβCD) loaded with cholesterol (CLC) improved nuclear maturation of oocytes after vitrification but failed to improve their embryo development up to the blastocyst stage. As these studies did not determine the cholesterol inclusion into membrane, we hypothesized that

following cholesterol incorporation by in vivo confocal microscopy during CLC co-incubation we could determine the best combination of CLC concentration and time to ensure the optimal cholesterol inclusion in the membrane to reduce the plasma membrane damage suffered during vitrification and thus, to enhance the oocyte cryosurvival. So, the main objective of the second paper of this PhD dissertation **(chapter V)** was to examine whether the exposure of bovine oocytes to CLC before vitrification–warming could improve their cryotolerance and embryo development after in vitro fertilization. To fulfil this goal, the first set of experiments characterized intracellular trafficking and localization of fluorescently-labeled cholesterol in *in vitro* matured oocytes incubated with CLC either in a FCS or PVA supplemented medium and its effect on early embryo development after vitrification/warming. In a second set of experiments three different concentrations of CLC were compared in terms of live subcellular localization of fluorescently-labeled cholesterol. Subsequently, the effects of a previous co-incubation with the resulting CLC concentrations and incubation times prior to vitrification on early embryo development were assessed. Finally, in the third experiment, bovine immature or in vitro matured oocytes were vitrified after 30 min incubation with 2mg/ml CLC, fertilized and cultured to evaluate early embryo development and quality in terms of gene expression. The expression pattern of genes involved in apoptosis (BAX), lipid metabolism (CYP51), imprinting (DNMT3A, IGF2R, UBEA2), heat (HSP70.1) and oxidative stress (MnSOD) were determined on Day 5 morulae by qRT-PCR.

3. In order to survive extremes of temperature some organisms (such as fishes, insects, plants, bacteria and yeasts) have developed unique defenses against their environment, leading to the biosynthesis of large diversity of molecular compounds to sustain life at extreme conditions (Wilson, Z. E. & Brimble, M. A 2009, Yamashita, Y., Kawahara, H. & Obata, H. 2002, Walters, K. R., et al 2009, Dreischmeier k et al 2016). Polysaccharides constitute a common class of molecules that interact with ice in nature either by triggering ice nucleation or by inhibition of ice nucleation and growth. Extracellular polysaccharides synthetized from the psychrotolerant marine bacterium Pseudomonas sp ID1 (M1 EPS) from Antartica allow their survival in subzero environments. As Pseudomonas sp. ID1 EPS, not only conferred cryoprotection for the strain itself, but also for E. coli cells, it

suggested that it could be applied as an agent for cell cryopreservation (Carrión O et al 2015). Other authors also suggested a universal cryoprotectant role for these biopolymers (Kim SJ, Yim JH. 2007.; Liu et al., 2013). Although several different studies have been performed to assess the effect of AFP on oocyte vitrification (Jo JW et al 2011, Jo JW et al 2012, Wen Y et al 2014, Lee HH et al 2015 , Liang s et al. 2016), there were no studies that have investigated the effect of EPS on the outcome of oocyte vitrification. In the third study of this thesis **(chapter VI),** we hypothesized that EPS synthetized by Pseudomonas sp ID1 could protect in vitro matured bovine oocytes from most current damage suffered during cryopreservation and enhance embryo development after warming. Thus, this third study was designed to examine whether the addition of EPS M1, produced by Pseudomonas sp. ID1, to vitrification and warming solutions for in vitro matured bovine oocytes would improve their cryotolerance. In the first study microtubule and chromosome configurations were analyzed by immunochemistry after exposure of oocytes to various concentrations of EPS M1 in vitrification and warming solutions. In the second experiment oocytes were treated with different concentrations of EPS M1 in vitrification and warming solutions fertilized and cultured up to the blastocyst stage. Finally, differences in terms of gene expression on blastocysts obtained from oocytes exposed to different concentrations of EPS M1 during vitrification-warming process were analyzed. The expression pattern of genes involved in apoptosis (BAX, BLC2-like 1), epigenetic modifications (DNA methylation (DNMT3A), histone modification (KAT2A, HDAC1) and chromatin remodeling (UBEA2)) and glucose metabolism (SLC2A3) were determined on Day 8 blastocysts by qRT-PCR.

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# CHAPTER IV

## Spindle configuration and developmental competence of *in vitro*-matured bovine oocytes exposed to NaCl or sucrose prior to cryotop vitrification

**Spindle configuration and developmental competence of *in vitro*-matured bovine oocytes exposed to NaCl or sucrose prior to cryotop vitrification**

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Abridged title: Cryotolerance of osmotically-stressed IVM bovine oocytes

**Keywords:** chromosomes; microtubule configuration; embryo development; blastocyst; osmotic stress

## 4.1 Abstract

This study examines whether exposure to high concentrations of NaCl or sucrose prior to vitrification improves the cryotolerance of *in vitro*-matured bovine oocytes. In Experiment 1, oocytes were exposed to different concentrations of NaCl or sucrose for 1 h. According to the results obtained, in Experiment 2, oocytes were exposed to 375mOsm NaCl or sucrose solution, vitrified and warmed. Microtubule and chromosome configurations were examined by immunocytochemistry. In Experiment 3, *in vitro* embryo development was assessed after vitrification of oocytes subjected or not to sucrose (375mOsm) pretreatment. Similar percentages of oocytes showing normal spindle configurations were observed in the 375mOsm pretreatment and control groups. Groups treated with NaCl or sucrose concentrations higher than 375mOsm gave rise to higher abnormal spindle rates. After vitrification/warming, significantly higher percentages of oocytes with normal chromosome configurations were recorded when oocytes were exposed to 375mOsm sucrose prior to vitrification compared to control vitrified oocytes. However, these percentages were significantly lower than those recorded in untreated controls. Cleavage and blastocyst rates were higher in the non-vitrified oocytes than vitrified oocytes. In conclusion, pretreatment with 375mOsm NaCl or sucrose had no adverse effects on the spindle status of vitrified/warmed cow oocytes. However, sucrose pretreatment offered no benefits for embryo development.

## 4.2. Introduction

The freezing of sperm and embryos is technical feasible and is today a widely used practical procedure. In contrast, the cryopreservation of oocytes has proved much more difficult and has so far led to low survival rates. The long term cryopreservation of oocytes is an important strategy to maintain genetic diversity, and is essential for research and development in the fields of biotechnology, biomedicine and assisted reproduction including infertility treatment, cloning and gene banking.

Traditionally, embryos and oocytes have been cryopreserved by slow freezing methods using low concentrations of cryoprotectants. During this procedure, osmotic shock and intracellular ice formation often cause cell damage. Today, vitrification is regarded as the best alternative to traditional slow freezing procedures. Vitrification is an ice-free cryopreservation method that involves high concentrations of cryoprotectants combined with very rapid cooling by direct plunging in liquid nitrogen resulting in an ice-crystal free solid glass-like structure [1].

In the past 2 years, successful survival rates of over 90% after vitrification and warming and live-birth rates of over 50% have been reported for human oocytes [314] However, despite many recent advances, the ideal protocol for the cryopreservation of cow oocytes has not yet been developed. Thus, to date the practical use of cow oocyte vitrification is limited because of the high sensitivity of these oocytes to cooling and cryopreservation, and low rates of blastocyst production have been observed after warming compared to non-vitrified controls [96].

Vitrified/warmed oocytes usually show several ultrastructural [315] and structural modifications including: abnormal distributions of chromosomes, microtubules and actin microfilaments [7, 169], disorganization of the spindle apparatus with the consequent risk of chromosome loss and aneuploidy [11], lipid bilayer phase changes causing cytoplasm membrane lysis [13], and nuclear fragmentation [14, 15], which is not readily repairable by the endogenous mechanisms of a haploid cell. Thus, it seems that the sensitivity to chilling of cow oocytes is determined by their complex structure.

Several measures designed to optimize bovine oocyte vitrification protocols have been tested such as the use of different cryoprotectants (CPAs) [16, 17], CPA concentrations

or CPA exposure temperatures and times [18-21], along with the addition of macromolecular supplements [23, 32]. Other procedures tested have been the partial removal of cumulus cells [20], modification of oocyte membrane constituents [16, 32], polarization of cytoplasmic lipid droplets by centrifugation [33], and the addition of cytoskeleton relaxants [17, 39, 40] or ice blockers to vitrification solutions [20].

The results of earlier investigations suggest that sublethal stress, properly defined and applied appropriately before manipulation, could improve the cryotolerance of oocytes through adaptation mechanisms enhancing their subsequent stress tolerance [46]. Thus, pretreatment with high hydrostatic pressure, or osmotic, heat or oxidative stress seems to improve morphological intactness, fertilizing ability and developmental competence after various *in vitro* and *in vivo* procedures, such as cryopreservation, parthenogenic activation, or somatic cell nuclear transfer [46, 278]. In the pig, exposure of oocytes to increased concentrations of sodium chloride, sucrose or trehalose prior to manipulation has been reported to improve both cryotolerance to vitrification and developmental competence [47, 48]. However, to the best of our knowledge, how the effect of temporarily increased NaCl or sucrose concentration may influence on cryotolerance and developmental competence of bovine oocytes has not been yet examined.

In this study, we examined whether the exposure of *in vitro*-matured (IVM) bovine oocytes to high concentrations of NaCl or sucrose prior to vitrification/warming would improve their cryotolerance. In a first experiment, microtubule and chromosome configurations were analyzed after exposure of oocytes to various concentrations of NaCl and sucrose. In a second experiment, oocytes were treated with selected concentrations of NaCl or sucrose, vitrified/warmed and microtubule and chromosome configurations evaluated. Finally, oocytes were exposed to the most efficient concentration of sucrose, vitrified/warmed, fertilized and cultured up to the blastocyst stage.

## 4.3. Materials and methods

### 4.3.1. Chemicals and supplies

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) unless otherwise stated.

### 4.3.2.. Bovine oocyte collection and *in vitro* maturation

The methods used for the *in vitro* maturation of oocytes have been described elsewhere [316]. Briefly, ovaries from slaughtered cows (>24 months old) were transported from a local abattoir to the laboratory in phosphate buffered saline (PBS) at 35-37ºC. Cumulus oocyte complexes (COCs) were obtained by aspirating 2 to10 mm follicles. Only COCs with three or more layers of cumulus cells and a homogeneous cytoplasm were selected for maturation. After three washes in modified PBS (PBS supplemented with 36 mg/mL pyruvate, 50 mg/mL gentamicin and 0.5 mg/mL cow serum albumin (BSA)), groups of up to 50 COCs were placed in 500 μL of maturation medium in four-well plates, and cultured for 24 h at 38.5ºC in a 5% $CO_2$ humidified air atmosphere. The maturation medium (MM1) was TCM-199 supplemented with 10% (v/v) fetal calf serum (FCS), 10 ng/mL epidermal growth factor and 50 mg/mL gentamicin.

**4.3.3. NaCl and sucrose treatment**

Twenty one (Experiments 2 and 3) or twenty two hours (Experiment 1) after placement in the maturation medium, oocytes were randomly transferred to 500 μL of MM2 (TCM-199 HEPES buffered containing 2% (v/v) FCS) supplemented with the corresponding amount of NaCl or sucrose (see details below) in four well dishes and incubated for 1 h at 38.5ºC in a 5% $CO_2$ humidified air atmosphere. As controls, oocytes were incubated in MM2. After recovery for 1 h in MM1, oocyte spindle configuration was evaluated in Experiment 1. Oocytes in Experiments 2 and 3 were vitrified/warmed (see below) and oocyte spindle configuration (Experiment 2) and embryo development (Experiment 3) were checked.

**4.3.4. Oocyte vitrification and warming**

**Vitrification protocol**

Oocytes were vitrified using the cryotop device and vitrification and warming solutions described by Kuwayama et al. [89]. The holding medium (HM) for formulating all vitrification-warming solutions was TCM-199 Hepes buffered with 20% (v/v) FCS. Partially denuded oocytes were transferred to an equilibration solution (ES) consisting of 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethylsulfoxide (DMSO) in HM for 10 min and then transferred to the vitrification solution (VS) containing 15% (v/v) DMSO, 15% (v/v) EG and 0.5 M sucrose dissolved in HM. After incubating for 30–40 s, the oocytes were loaded onto the cryotop, almost all the solution removed to leave

only a thin layer covering the oocytes, and the device plunged into liquid nitrogen. The entire process from exposure in VS to plunging was completed within 90 seconds.

**Warming protocol**

All warming steps were performed at 38.5°C. Vitrified oocytes were warmed by directly immersing the cryotop into the warming solution containing 1 M sucrose dissolved in HM for 1 min. The recovered oocytes were then transferred to 0.5 M sucrose dissolved in HM for 3 min and incubated in HM for 5 min. After a final rinse again in HM for 1 min, the oocytes were transferred to MM1 to recover for a further hour.

### 4.3.5. *In vitro* fertilization and embryo culture

*In vitro* matured oocytes were *in vitro* fertilized at 38.5ºC in a 5% $CO_2$ atmosphere. Briefly, the oocytes were washed once in fertilization medium before being transferred, in groups of up to 25, to four-well plates containing 250 µL of fertilization medium per well (Tyrode's medium supplemented with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/mL fatty acid-free BSA and 10 mg/mL heparin–sodium salt (Calbiochem, Darmstadt, Germany)). Frozen–thawed spermatozoa from bulls of proven fertility were used in all the experimental procedures. Motile spermatozoa were obtained by centrifuging frozen–thawed sperm from Asturian bulls (ASEAVA, Llanera, Asturias, Spain) on a discontinuous gradient (Bovipure, Nidacon International, Gothenburg, Sweden) for 10 min at 100 x g at room temperature. Viable spermatozoa collected from the bottom were washed (Boviwash, Nidacon International, Gothenburg, Sweden) and pelleted by centrifugation at 100 x g for 5 min. Spermatozoa were counted in a Neubauer chamber and diluted in an appropriate volume of fertilization medium to give a final concentration of $2 \times 10^6$ spermatozoa/mL. A 250 µL aliquot of this suspension was then added to each fertilization well to obtain a final concentration of $1 \times 10^6$ spermatozoa/mL.

Plates were incubated at 38.5ºC in a 5% $CO_2$ humidified air atmosphere. At approximately 22 h post-insemination (pi), presumptive zygotes were denuded by gentle pipetting and transferred to 25 µL culture droplets of synthetic oviductal fluid (SOF) [317] (1 embryo/mL) containing FCS (5%, v/v) under mineral oil. Embryos were incubated for 6 days at 38.5ºC in a humidified 5% $CO_2$/5% $O_2$/90% $N_2$ atmosphere.

Cleavage rates were recorded at 48 hpi and the number of blastocysts determined on Day 7 post-insemination.

### 4.3.6. MII spindle status

After *in vitro* maturation, oocytes were denuded of cumulus cells by gentle pipetting in PBS before immunostaining for tubulin and chromatin. Fresh *in vitro*-matured oocytes were used as controls to determine normal patterns of microtubule and chromatin organization. Samples of oocytes from the experimental groups were fixed in 4% (w/v) formaldehyde PBS (30 min, 38.5ºC), permeabilized using Triton X-100 (2.5% (v/v) in PBS) for 15 min, and simultaneously immunostained for tubulin and chromatin as described elsewhere [224]. The fixed oocytes were incubated with the anti-α-tubulin monoclonal antibody (Molecular Probes, Paisley, UK) (1:250) overnight, followed by incubation with the antimouse IgG antibody Alexa Fluor 488 (Molecular Probes, Paisley, UK) (1:5000) for 1 h. Between incubations, the oocytes were washed three times in pre-warmed PBS for 5 min. Fixed and stained oocytes were mounted on poly-L-lysine treated coverslips fitted with a self-adhesive reinforcement ring in a 3-µl drop of Vectashield containing 125 ng/ml of 4',6-diamidino-2-phenylindole (DAPI) (Vectorlabs, Burlingame, CA), and flattened with a coverslip. The preparation was sealed with nail varnish and stored at 4ºC protected from light until observation within the following 2 days. Meiotic spindle configuration was assessed under an epifluorescence microscope (Axioscop 40FL, Carl Zeiss, Germany). The criteria used to classify chromosome and microtubule distributions have been described elsewhere [169]. Briefly, the meiotic spindle was defined as normal when the classic symmetrical barrel shape was observed, with chromosomes aligned regularly in a compact group along the equatorial plane. In contrast, abnormal spindles showed disorganized, clumped, dispersed, or unidentifiable spindle elements and chromatin was abnormally organized (showing clustering or dispersal from the spindle centre). Detailed images of these normal and abnormal patterns are shown in Fig. 1.

### 4.3.7. Statistical analysis

All statistical tests were performed using the software package IBM SPSS 19 for Windows (IBM corp.; Chicago, Illinois). ANOVA was performed to analyze differences in meiotic spindle configuration after treatment, cleavage rates and blastocyst yield. All data were checked for normality using the Levene and

Kolmogorov-Smimov tests, and non-normally distributed data compared using the Kruskall-Wallis test. Significance was set at $p < 0.05$.

#### 4.3.8. Experimental design

Three experiments were conducted as four (Experiments 1 and 2) or seven (Experiment 3) replicates.

##### 4.3.8.1. Experiment 1: Effects of transient exposure of *in vitro*-matured bovine oocytes to elevated NaCl or sucrose concentrations on meiotic spindle status

To examine the effects of NaCl, 307 oocytes, *in vitro*-matured for 22 h, were randomly allocated to eight groups (A1–A8). Oocytes from groups A2–A8 were incubated for 1 h in MM2 supplemented with 0.25%, 0.5%, 1%, 1.5%, 2%, 3% and 4% (w/v) NaCl corresponding to osmolalities of 375, 443, 602, 749, 901, 1206 and 1517 mOsm, respectively, as measured using an osmometer (Osmomat 030, Gonotec). Oocytes in group A1 incubated in MM2 (294 mOsm) for 1 h served as controls.

To examine the effects of sucrose, 392 oocytes, *in vitro*-matured for 22 h were randomly allocated to six groups (B1–B6). Oocytes from groups B2–B5 were incubated for 1 h in MM2 supplemented with 2.77%, 5.10%, 10.54% and 15.57% (w/v) sucrose corresponding to osmolalities of 375, 455, 631 and 812 mOsm, respectively (as measured using an osmometer (Osmomat 030, Gonotec). A group of oocytes (B6) treated with 0.25% NaCl (375 mOsm) was also included to allow comparisons with the previous experiment. Oocytes in B1 incubated in MM2 (294 mOsm) for 1 h served as controls.

After treatment, oocytes were left to recover for 1 h in MM1. Subsequently, oocytes from the two sets of experiments were fixed in 4% (v/v) PFA and immunostained to examine meiotic spindle status.

##### 4.3.8.2. Experiment 2: Cryotolerance of in vitro-matured bovine oocytes exposed to NaCl or sucrose prior to vitrification

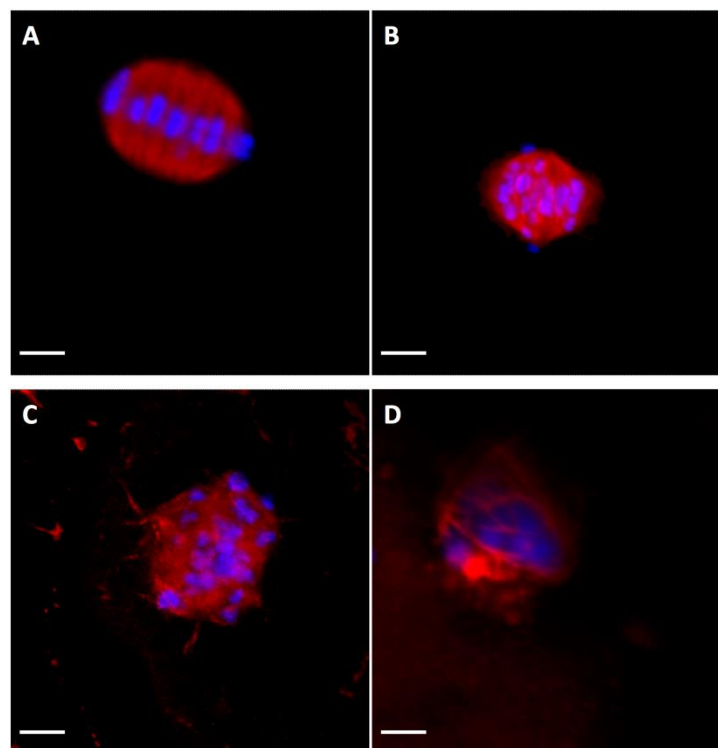
After 21 h of *in vitro* maturation, sets of 253 and 370 oocytes were randomly distributed into three groups (C1-C3 and D1-D3). Oocytes in C1 and D1 were incubated in MM2 for 2 h as controls. According to the results of Experiment 1, oocytes in C2-C3 and D2-

D3 were pre-treated with 0% and 0.25% NaCl (375 mOsmol) or 0% and 2.77% sucrose (375 mOsmol) for 1 h, respectively, left to recover for 1 h in MM2 and then vitrified and warmed by the cryotop method. After 1 h of recovery in MM1, all oocytes were fixed in 4% (v/v) PFA and immunostained to examine meiotic spindle status.

#### 4.3.8.3. Experiment 3: Effects of sucrose pre-treatment on the developmental competence of vitrified/warmed bovine oocytes

747 oocytes were randomly allocated to four groups: E1, comprised of oocytes matured *in vitro* for 24 h; E2, of oocytes exposed to 2.77% (375 mOsmol) sucrose for 1 h and left to recover for 1 h in MM2; E3, of oocytes vitrified/warmed by the cryotop method; and E4, of oocytes exposed to 2.77% (375 mOsmol) sucrose for 1 h, left to recover for 1 h in MM2 and vitrified/warmed by the cryotop method. Oocytes in groups E2, E3 and E4 were allowed to mature for an additional 1 h in MM1 after treatment. After maturation, the oocytes were fertilized and at 22 hpi, presumptive zygotes were placed in culture drops. Cleavage rates were determined at 48 hpi and blastocyst rates recorded on Day 7.

**Fig.4.1. Microtubule and chromosome configurations examined by immunocytochemistry of *in vitro* matured bovine oocytes after vitrification with or without NaCl or sucrose pretreatment.** (*a*) Normal barrel-shaped MII spindle morpho logy. Scale bar = 3.1 mm. (*b–d*) Vitrified–warmed oocytes treated with NaCl or sucrose before the vitrification procedure exhibiting abnormal spindle structures. (*b*) Abnormal spindle morphology showing partly disorganised chromosomes. Note that chromosomes are not aligned Scale bar = 4.2 mm. (*c*) Completely disorganised microtubules and chromosomes. Scale bar = 3.4 mm. (*d* ) Disrupted microtubular shape. Note the decondensation of microtubules and the less condensed appearance of chromosomes. Scale bar = 2.9 mm. Red, tubulin (Alexa Fluor 594); blue, chromosomes (DAPI).



## 4.4.Results

### 4.4.1. Experiment 1

**Effects of transient exposure of in vitro-matured bovine oocytes to increasing NaCl concentrations on meiotic spindle status**

Table 4.1 details the effects of NaCl pre-treatment on meiotic spindle configurations. As the osmolality of the NaCl solution increased, percentages of oocytes showing an abnormal spindle configuration also significantly increased. Only half the oocytes exposed to 0.5% NaCl (433 mOsmol) (A3) showed a normal spindle configuration. However, no significant differences in terms of microtubule distributions or chromosome configurations were observed in oocytes treated with 0.25% NaCl (A2) compared to untreated control oocytes (A1).

**Effects of transient exposure of in vitro-matured bovine oocytes to increasing sucrose concentrations on meiotic spindle status**

Table 4.2 details the effects of sucrose pre-treatment on meiotic spindle configurations. No significant differences on microtubule configuration were observed when comparing oocytes in the control group (B1) and those exposed to 2.77% (B2) or 5.10% (B3) sucrose. However, only oocytes exposed to 2.77% of sucrose (B2) showed a similar normal spindle configuration rate as non-exposed oocytes (B1). As for the NaCl treatments, increasing sucrose concentrations led to increasing proportions of oocytes with decondensed or absent chromosomes. Oocytes exposed to 0.25% NaCl (B6) showed similar rates of normal spindles to non-treated oocytes (B1). No significant differences were observed between oocytes pre-treated with 0.25% NaCl (B6) or 2.77% sucrose (B2).

**Table 4.1.** Effects of previtrification treatment with increasing NaCl concentrations on the spindle configuration of *in vitro*-matured bovine oocytes

| | | | Microtubule morphology (% ± SEM)* | | | Chromosome morphology (% ± SEM)* | | |
|---|---|---|---|---|---|---|---|---|
| **Treatment** | **n** | **Total MII (% ± SEM)** | **Normal** | **Dispersed** | **Decondensed or absent** | **Normal** | **Dispersed** | **Decondensed or absent** |
| **A1** | 29 | 97.7 ± 2.3 | 85.0 ± 9.6[a] | 12.5 ± 9.5 | 2.5 ± 2.5[a] | 85.0 ± 9.6 [a] | 12.5 ± 9.5 | 2.5 ± 2.5[a] |
| **A2** | 40 | 100 ± 0.0 | 83.4 ± 0.0[ab] | 13.5 ± 5.6 | 3.1 ± 3.1[a] | 83.4 ± 8.1 [a] | 13.5 ± 5.6 | 3.1 ± 3.1[a] |
| **A3** | 33 | 100 ± 0.0 | 52.6 ± 0.1[bc] | 39.3 ± 6.0 | 9.1 ± 9.1[a] | 47.2 ± 7.0 [b] | 39.3 ± 6.0 | 9.1 ± 9.1[a] |
| **A4** | 41 | 90.9 ± 9.1 | 51.8 ± 8.1[bc] | 22.5 ± 5.3 | 25.7 ± 6.1[ab] | 46.0 ± 8.7 [b] | 22.5 ± 5.3 | 25.7 ± 6.1[ab] |
| **A5** | 43 | 95.5 ± 4.6 | 40.3 ± 4.2[cd] | 24.8 ± 7.0 | 34.9 ± 9.7[ab] | 37.5 ± 4.2 [bc] | 24.8 ± 7.0 | 34.9 ± 9.7[ab] |
| **A6** | 42 | 85.3 ± 6.9 | 16.5 ± 7.0[de] | 24.2 ± 2.8 | 59.4 ± 6.0[bc] | 16.5 ± 7.0 [bc] | 24.2 ± 2.8 | 59.4 ± 6.0[bc] |
| **A7** | 38 | 88.8 ± 6.6 | 15.5 ± 3.6[de] | 28.4 ± 5.8 | 56.2 ± 5.3[bc] | 9.6 ± 3.5 [c] | 28.4 ± 5.8 | 56.2 ± 5.3[bc] |
| **A8** | 41 | 88.1 ± 9.1 | 6.8 ± 6.8[e] | 18.7 ± 8.4 | 74.5 ± 12.5[c] | 6.8 ± 6.8 [c] | 18.7 ± 8.4 | 74.5 ± 12.5[c] |

[a-e] Values with different letters within each column differ significantly ($P<0.05$).* Percentages referred to the total number of oocytes reaching the MII stage. A1: Control oocytes. A2 – A8: Oocytes exposed to NaCl (0.25%, 0.5%, 1%, 1.5%, 2%, 3% and 4%, respectively).

**Table.4.2.** Effects of previtrification treatment with increasing sucrose concentrations on the spindle configuration of *in vitro*-matured bovine oocytes

| | | | Microtubule morphology (% ± SEM)* | | | Chromosome morphology (% ± SEM)* | | |
|---|---|---|---|---|---|---|---|---|
| **Treatment** | **n** | **Total MII (% ± SEM)** | **Normal** | **Dispersed** | **Decondensed or absent** | **Normal** | **Dispersed** | **Decondensed or absent** |
| **B1** | 70 | 83.2 ± 3.4 | 87.0 ± 4.7[a] | 8.3 ± 2.3[a] | 4.7 ± 2.9[a] | 87.0 ± 4.7[a] | 9.4 ± 2.8[a] | 3.6 ± 2.5[a] |
| **B2** | 61 | 87.7 ± 4.4 | 71.8 ± 5.0[ab] | 23.3 ± 4.2[ab] | 4.9 ± 2.2[a] | 69.1 ± 4.6[ab] | 22.7± 6.2[ab] | 8.2 ± 2.8[a] |
| **B3** | 72 | 88.1 ± 3.8 | 63.0 ± 4.6[ab] | 26.0 ± 5.6[ab] | 11.0 ± 2.8[ab] | 58.7± 3.4[b] | 30.3± 2.9[ab] | 11.0 ± 2.8[ab] |
| **B4** | 78 | 83.7 ± 6.4 | 57.2 ± 7.1[b] | 26.0 ± 6.6[ab] | 16.8 ± 6.9[ab] | 55.1 ± 7.0[b] | 31.5 ± 6.6[ab] | 13.5 ± 6.4[ab] |
| **B5** | 59 | 76.1 ± 6.5 | 31.5 ± 6.8[c] | 38.4 ± 4.0[b] | 30.2 ± 6.8[b] | 30.0 ± 6.3[c] | 50.1 ± 3.2[b] | 19.9 ± 4.6[b] |
| **B6** | 52 | 93.3 ± 4.1 | 75.6 ± 2.0[ab] | 20.9 ± 0.6[ab] | 3.5 ± 2.0[a] | 73.9 ± 3.5[ab] | 22.7 ± 2.0[ab] | 3.5 ± 2.0[a] |

[a-c] Values with different letters within each column differ significantly ($P<0.05$). * Percentages referred to the total number of oocytes reaching the MII stage. B1: Control oocytes. B2 – B5: Oocytes exposed to sucrose (2.77%, 5.10%, 10.54% and 15.57%, respectively). B6: oocytes exposed to 0.25% NaCl.

### 4.4.2. Experiment 2

**Cryotolerance of *in vitro*-matured bovine oocytes exposed to NaCl or sucrose prior to vitrification**

Table 3 compares the effects of vitrifying the oocytes with or without NaCl (0.25%) pre-treatment on meiotic spindle status. Significantly higher rates of oocytes exhibiting a metaphase II spindle were recorded in the control group (non-treated, non-vitrified oocytes) compared to the remaining vitrified oocyte groups. Pre-treatment with NaCl prior to vitrification induced a similar normal microtubule morphology rate to that observed after vitrification, though both rates were significantly lower than those observed for fresh control oocytes, mainly due greater proportions of oocytes with decondensed or absent microtubules in these two groups (C2 and C3). No significant differences were observed among treatment groups when percentages of dispersed microtubules were examined.

Significantly lower percentages of vitrified oocytes displaying a normal chromosomal morphology were observed compared to non-vitrified oocytes. However, oocytes exposed to 0.25% NaCl prior to vitrification/warming featured similar normal chromosome percentages to their non-vitrified counterparts.

**Cryotolerance of *in vitro*-matured bovine oocytes exposed to sucrose treatment prior vitrification**

As shown in Table 4, the vitrified oocyte groups showed significantly lower percentages of MII stage oocytes compared to control non-vitrified oocytes. Irrespective of the pre-treatment, significantly lower normal microtubule configuration rates were observed when vitrified oocytes were compared to control non-vitrified oocytes. Similarly, significantly lower normal chromosome configuration rates compared to control fresh oocytes were observed for vitrified oocytes, and higher percentages of oocytes with dispersed, decondensed or absent chromosomes in the cytoplasm were identified. However, when oocytes were vitrified after sucrose treatment, significantly higher percentages of oocytes with normal chromosome configurations were recorded when oocytes were exposed to 375 mOsm sucrose prior to vitrification compared to control vitrified oocytes, mainly due to a lower proportion of decondensed or absent

chromosomes. Similarly, the proportion of decondensed or absent microtubules significantly decreased after sucrose treatment.

### 4.4.3.Experiment 3

**Effects of sucrose pre-treatment on the developmental competence of vitrified/warmed bovine oocytes**

As shown in Table 5, significantly higher cleavage and blastocyst rates were recorded in the non-vitrified oocyte groups than vitrified oocyte groups, irrespective of whether oocytes had been subjected or not to sucrose treatment.

**Table 4.3.** Cryotolerance of *in vitro*-matured bovine oocytes subjected to NaCl treatment prior to vitrification.

| | | | Microtubule morphology (% ± SEM)* | | | Chromosome morphology (% ± SEM)* | | |
|---|---|---|---|---|---|---|---|---|
| **Treatment** | **n** | **Total MII (% ± SEM)** | **Normal** | **Dispersed** | **Decondensed or absent** | **Normal** | **Dispersed** | **Decondensed** |
| **C1** | 86 | 98.8 ± 0.9[a] | 79.1 ± 5.9[a] | 18.7 ± 4.4 | 2.2 ± 2.2[a] | 73.7 ± 9.7[a] | 25.2 ± 8.7 | 1.1 ± 1.1[a] |
| **C2** | 87 | 63.2 ± 9.6[b] | 40.2 ± 3.7[b] | 26.0 ± 4.6 | 33.8 ± 9.4[b] | 35.7 ± 13.2[b] | 42.6 ± 4.9 | 21.7 ± 8.7[b] |
| **C3** | 80 | 71.3 ± 3.6[b] | 41.9 ± 4.3[b] | 28.4 ± 8.5 | 29.7 ± 4.2[b] | 41.9 ± 4.3[ab] | 38.7± 3.8 | 19.4 ± 2.2[b] |

[a,b] Values with different letters within each column differ significantly (P<0.05). * Percentages referred to the total number of oocytes reaching the MII stage. C1: *In vitro*-matured; C2: *In vitro*-matured oocytes vitrified/ warmed by the cryotop method; C3: *In vitro*-matured oocytes exposed to 0.25% NaCl for 1 h and then vitrified and warmed by the cryotop method.

**Table 4.4.** Cryotolerance of *in vitro*-matured bovine oocytes subjected to sucrose treatment prior to vitrification.

| | | | Microtubule morphology (% ± SEM)* | | | Chromosome morphology (% ± SEM)* | | |
|---|---|---|---|---|---|---|---|---|
| **Treatment** | **n** | **Total MII (%± SEM)** | **Normal** | **Dispersed** | **Decondensed or absent** | **Normal** | **Dispersed** | **Decondensed or absent** |
| **D1** | 93 | 100 ± 0.0[a] | 81.7 ± 1.1[a] | 12.9 ± 1.1[a] | 5.4 ± 0.4[a] | 81.7 ± 1.1[a] | 12.9 ± 1.1[a] | 5.4 ± 0.4[a] |
| **D2** | 121 | 61.1 ± 1.3[b] | 24.4 ± 0.9[b] | 33.9 ± 1.1[b] | 41.8 ± 1.0[b] | 23.3 ± 0.9[b] | 40.5 ± 0.6[b] | 36.2 ± 0.7[b] |
| **D3** | 140 | 61.3 ± 1.3[b] | 32.9 ± 1.3[b] | 38.2 ± 1.0[c] | 28.9 ± 0.8[c] | 34.2 ± 1.2[c] | 42.7 ± 1.1[c] | 23.1 ± 0.7[c] |

[a-c] Values with different letters within each column differ significantly (P<0.05). * Percentages referred to the total number of oocytes reaching the MII stage. D1: *In vitro*-matured oocytes; D2: *In vitro*-matured oocytes vitrified/warmed by the cryotop method; D3: *In vitro*-matured oocytes exposed to 2.77% NaCl for 1 h and then vitrified and warmed by the cryotop method.

**Table 4.5.** Developmental competence of *in vitro*-matured bovine oocytes subjected to sucrose treatment prior to vitrification.

| Treatment | n | Cleavage (% ± SEM)* | Blastocysts on Day 7 (% ± SEM)* |
|---|---|---|---|
| **E1** | 193 | 63.7 ± 5.8[a] | 18.1 ± 2.4[a] |
| **E2** | 169 | 75.2 ± 3.4[a] | 19.5 ± 3.1[a] |
| **E3** | 184 | 27.7 ± 2.4[b] | 1.1 ± 0.9[b] |
| **E4** | 201 | 33.3 ± 3.8[b] | 1.5 ± 0.7[b] |

[a-c] Values with different letters within each column differ significantly ($P<0.05$). Control (non-vitrified): Oocytes matured *in vitro* for 24. E1: *In vitro*-matured oocytes; E2: *In vitro*-matured oocytes exposed to 2.77% NaCl for 1 h; E3: *In vitro*-matured oocytes vitrified/ warmed by the cryotop method; E4: *In vitro*-matured oocytes exposed to 2.77% NaCl for 1 h and then vitrified and warmed by the cryotop method.

## 4.5. Discussion

This study sought to examine whether pre-treating *in vitro*-matured cow oocytes with elevated NaCl or sucrose concentrations would improve their cryotolerance to vitrification, along with their *in vitro* developmental competence. To define a suitable sublethal stress treatment, in an initial experiment we examined the effects of different osmolality solutions on MII spindle structure.

Our cytotoxicity tests revealed significantly higher percentages of oocytes with abnormal spindle structures including disorganized or decondensed microtubules or chromosomes. These effects were dose-dependent, as normal spindle rates decreased as osmolality of the pretreatment solution increased. Consistent with this finding, Mullen *et al*. (2004) reported that osmolality was a significant predictor of the spindle morphology of human oocytes . Hyperosmotic effects of sucrose solutions at 600, 1200, and 2400 mOsm resulted in 44, 44, and 100% of the spindles with abnormal structure, respectively [12]. In effect, cow oocytes have been observed to behave as a perfect osmometer (i.e., they follow the Boyle–Van't Hoff relationship) over the range 265 to 800 mOsm [150].

Despite this background, Lin and collaborators. [47, 48] provided strong evidence that pig oocytes osmotically stressed by a transient increase in NaCl, sucrose or trehalose concentrations, offered higher developmental rates following cryopreservation, parthenogenic activation, or somatic cell nuclear transfer Osmotic stress has nevertheless been observed to be harmful to oocytes in several studies, even when similar osmotic pressures were applied for only 10 min [12, 318, 319]. In our study, 0.25% NaCl (375 mOsm) had no substantial effects on spindle morphology yet higher concentrations led to markedly reduced rates of oocytes showing normal spindle configurations. Similarly, we observed no adverse effects of 2.77% sucrose (375 mOsm) and selected this concentration for the pretreatment of IVM oocytes prior to vitrification. Since the type of initial stress and its intensity are determinants of the expression of stress-related proteins that will help combat subsequent stress, this initial stress can also compromise oocyte viability [320]. The difference in the percentages of oocytes showing normal microtubule distributions in the groups exposed to 375 and 433 mOsm sucrose solutions (71.78% vs 62.96%, respectively) was not as great as the difference detected between groups exposed to the same NaCl osmolality (83.35% vs

52.60%, respectively). This could indicate that elevated intracellular NaCl concentrations may have both osmotic effects and other consequences.

In our second experiment, the effects of exposure to NaCl or sucrose prior to vitrification on spindle status were tested. According to many other authors, vitrification/warming procedures lead to a reduction in rates of oocytes at metaphase II [7, 17, 37, 169, 170, 229]. In effect, we detected a lower proportion of oocytes at MII stage compared to controls (non-vitrified oocytes). Moreover, vitrified oocytes showed higher rates of anaphase II regardless of whether they had been subjected or not to osmotic pretreatment. Some permeable cryoprotectants used in vitrification solutions such as DMSO and EG could parthenogenetically activate *in vitro*-maturated sheep [321], pig [322] or buffalo [323] oocytes. Whether the osmotic stress caused by cryoprotectants contributes to this effect it is unknown [47]. According to Lin et al. [47], it is not known if the osmotic stress induced by NaCl causes the same effects and these authors speculated that this could be the reason for the improved cryotolerance and developmental competence of pig oocytes [47]. In our cytotoxicity tests, no significant differences were detected between rates of MII and AII in oocytes exposed to different osmolalities of NaCl or sucrose. However, differences in MII percentages emerged between control and vitrified oocytes in the second experiment, and vitrified oocytes showed greater percentage of anaphase II. This indicates that osmotic stress induced by NaCl or sucrose does not seem to cause parthenogenetic activation in *in vitro*-matured cow oocytes. Larman *et al*. [191] and Gook *et al*. [324] reported that besides the vitrification solution, cooling shock can induce spontaneous activation in matured oocytes. Succu *et al.* [170] showed that vitrified oocytes had a large proportion of spindle abnormalities when assessed immediately after warming. For this reason, we included a 60 min recovery period after warming prior to fixation to allow for spindle re-assembly. This post-warming interval should be sufficient for tubulin polymerization and to restore microtubule structure. The oocyte can then resume meiosis and signal the spindles to reorganize themselves into anaphase II [220]. Similarly, after high hydrostatic pressure treatment some authors have detected greater improvements in oocyte competence when left to recover for 1 h or 2 h before vitrification [45, 289]. Results have, however, been contradictory. According to Larman et al. [161] there is no reason to wait for microtubule recovery, especially if vitrification is carried out at 37°C. In the vitrified oocytes, we observed no significant effects of NaCl or sucrose

pretreatment on normal microtubule configuration rates. Neither were any effects of NaCl on rates of normal chromosome configurations detected. In contrast, oocytes treated with sucrose prior to vitrification showed higher rates of normally organized chromosomes compared to oocytes vitrified without sucrose pretreatment. Therefore, it seems that pretreatment with 375 mOsm NaCl or sucrose for 1 h prior to vitrification, offers some protection against chromosome decondensation as one of the negative effects of vitrification.

Exposure to 375 mOsm sucrose solution for 1 h had no deleterious effects on cleavage or development to the blastocyst stage of the *in vitro*-matured bovine oocytes. However, cryosurvival or developmental competence were not improved when used prior to vitrification in contrast with observations in pig oocytes. It has been described that the precisely controlled sublethal stress treatment of cells can improve embryo and gamete performance, and consequently enhances subsequent oocyte survival, fertilization, and *in vitro* development. The stress response triggers the induction of heat shock proteins (HSP), firstly detected after heat stress although later found to be involved in responses to other environmental stressors including cold temperature, osmotic effect of salts, acid change in pH, high hydrostatic pressure, and starvation. HSP participate in various cell functions including protein, DNA, and chromatin stabilization and repair, cell cycle control, redox regulation, energy metabolism, fatty acid/lipid metabolism, and the elimination of damaged proteins [279, 280]. The production of heat shock proteins might explain the improved survival after vitrification and developmental competence of pig oocytes observed after osmotic stress induced by NaCl or sucrose solutions [48]. In matured cow oocytes, HSP70 (a stress response HSP [325]) has been described to act as a molecular chaperone helping nascent polypeptides to form properly. HSP70 also acts as a microtube-associated protein interacting with polymerized tubulin through a region containing a sequence related to tubulin-binding motifs [326]. According to the structure and composition of cow oocytes, we used a lower osmolality solution as pre-treatment than that employed for pig oocytes, and this was perhaps insufficient to induce the production of adequate amounts of HSP to protect the oocyte from cryodamage. Thus, it could be speculated that to induce greater stress, bovine oocytes could be pretreated with higher concentrations of NaCl or sucrose but over a shorter period than 1 h. In line with previous observations, the greater the stress (below the lethal dose), the more HSP are produced and the higher the cryosurvival [45].

In our study, we did not observe the improved developmental competence achieved by Lin *et al*. [47, 48] in pig oocytes. A possible explanation for this could be the different amounts, composition and distribution of lipids [327]. Membrane structure also differs between the species. Thus, pig oocytes have double the total fatty acid reserves than cow oocytes, and phospholipids contain a higher proportion of polyunsaturated fatty acids than ruminant oocyte phospholipids [153]. These difference could have marked effects on the nature of membranes and may contribute to contrasting responses of oocytes to cryopreservation [140]. Cell membrane composition determines the properties of a cell. Cells with more flexible membranes are more permeable to water and intracellular cryoprotectants and will suffer less damage during cryopreservation [298]. Ultrastructural differences between *in vitro*-matured pig and cow oocytes may explain their different developmental competence after treatment with NaCl or sucrose prior to vitrification. Due to their large intracytoplasmic lipid droplets and other specific properties that hinder successful cryopreservation [20], pig oocytes might benefit from osmotic stress because it permits pre-adaptation of the cell to the subsequent stress of vitrification-warming [46].

In conclusion, treatment with 375 mOsm NaCl or sucrose for 1 h before vitrification had no detrimental effects on the meiotic spindle status of IVM bovine oocytes. In particular, sucrose pretreatment was unable to improve embryo development as observed in other species. There is a clear need for further work designed to improve the cryotolerance of bovine oocytes.

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# CHAPTER V

## Cholesterol added prior to vitrification on the cryotolerance of immature and *in vitro* matured bovine oocytes

# Cholesterol added prior to vitrification on the cryotolerance of immature and *in vitro* matured bovine oocytes

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## 5.1. Abstract

This study examines whether incorporating cholesterol-loaded methyl-β-cyclodextrin (CLC) in the bovine oocyte plasma membrane improves oocyte tolerance to vitrification. *In vitro* matured oocytes were incubated with 2 mg/ml BODIPY-labeled CLC for different time intervals in FCS or PVA supplemented medium or exposed to different CLC concentrations to examine the subcellular localization of cholesterol by confocal microscopy live-cell imaging. Subsequently, the effects of optimized CLC concentrations and incubation times prior to vitrification on early embryo development were assessed. Then, we evaluated the effects of pretreatment with 2 mg/ml CLC for 30 min before the vitrification of immature (GV) and *in vitro* matured (MII) oocytes on developmental competence and gene expression. Our results indicate a high plasma membrane labeling intensity after 30 min of incubation with 2 mg/ml CLC for 30 min, regardless of the holding medium used. When oocytes were incubated with 1 mg/ml, 2 mg/ml and 3 mg/ml of CLC, intense labeling was observed at the plasma membrane after 40, 30 and 20 min, respectively. CLC pre-treatment before the vitrification of bovine oocytes did not affect subsequent cleavage and embryo development rates irrespective of CLC concentrations, incubation times or meiotic stage. However, pretreatment seems to improve the quality of embryos derived from vitrified oocytes, mainly when oocytes were vitrified at the GV stage.

## 5.2. Introduction

Widespread use of animal oocytes for procedures such as *in vitro* embryo production, nuclear transfer or gene banking has dramatically increased interest in oocyte cryopreservation in the agricultural and scientific communities [328]. The practical benefits of vitrification to preserve bovine oocytes are nevertheless limited since vitrified oocytes show impaired *in vitro* maturation and early embryo development. Moreover, blastocyst yields from vitrified-warmed bovine oocytes have not been markedly improved by many of the different cryodevices and vitrification protocols that have been attempted (reviewed in: [329]). Among the explanations provided for the inefficient cryopreservation of bovine oocytes are the large physical size, low volume-to-surface ratio of oocytes, their high lipid content, and their plasma membrane composition [330].

During oocyte cryopreservation, cooling and osmotic stress can cause irreversible damage to membrane integrity [13, 331]. Oocytes undergo substantial volume changes due to water and cryoprotectant movement during cryopreservation (reviewed in: [330]). This suggests that cells with more flexible membranes permeable to water and cryoprotectants are likely to suffer less damage that those with more rigid, less permeable membranes. The cholesterol/phospholipid ratio of the plasma membrane is a major determinant of membrane stability during cryopreservation [143, 290]. This is because membranes with high cholesterol content are more fluid at low temperatures and thus less susceptible to damage during cooling [141]. The incorporation of cholesterol in the plasma membrane should enhance membrane fluidity and permeability at low temperatures, and thereby increase oocyte tolerance to cryopreservation.

The addition of cholesterol to sperm plasma membrane in the bull [302, 303] or stallion [304] has been reported to improve sperm cryosurvival. In the latter studies, cholesterol was preloaded into different cyclodextrins. Cyclodextrins (CDs) are cyclic oligosaccharides consisting of five or more α-D-glucopyranose residues linked by α-1,4 glucosidic bonds that have a hydrophobic center capable of integrating lipids. Of the cyclodextrins, βCDs are the most efficient at extracting cholesterol from isolated or intact membranes in a variety of cells (reviewed in: [305]). This high affinity of βCDs for cholesterol, besides conferring the capacity to remove cholesterol from biological

membranes, also enables the formation of cholesterol inclusion complexes that donate cholesterol to the membrane increasing membrane cholesterol levels. βCD-cholesterol inclusion complexes are typically generated by mixing a cholesterol suspension with a cyclodextrin solution. The efficiency of cholesterol transfer from βCD inclusion complexes to biological membranes depends on the βCD:cholesterol molar ratio, βCD-cholesterol concentration, and treatment duration [306, 307].

In prior work in oocytes it was observed that the co-incubation of bovine immature [188] or *in vitro*-matured [32] oocytes with βCDs loaded with cholesterol (CLC) improved the nuclear maturation of oocytes after vitrification, but did not benefit embryo development to the blastocyst stage. These authors attributed the lack of effect of CLC treatment in improving cryotolerance that cholesterol was preferentially transferred to lipids or proteins present in the fetal calf serum (FCS) avoiding its incorporation into the oocyte. By replacing FCS with polyvinyl alcohol (PVA) during the transfer of cholesterol to bovine oocytes, Horvath and Seidel (2006) were able to improve cleavage rates and development to the 8-cell stage after vitrification. When examining the incorporation of cholesterol in the oocyte plasma membrane after incubation with CLC, Horvath and Seidel [32] and Jiménez-Trigos et al [308] observed that cholesterol was transported through the cumulus layers and the zona pellucida in oocytes incubated for 1 hour with cholesterol labeled with 22-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4yl) amino-23,24 bisnor-5-cholen-3β-ol (NBD). However, little information was provided regarding the exact localization of cholesterol within the oocyte or how much cholesterol entered the cell. One particular complication when examining the transport of cholesterol or other lipids by microscopy is the observation that coupling to a fluorophore can dramatically change the structural and biochemical properties of a molecule and its interactions with other membrane components [310]. Boron-dipyrromethene (BODIPY)-cholesterol closely mimics the membrane partitioning and trafficking actions of cholesterol and because of its excellent fluorescent properties, enables the direct monitoring of sterol movement by time-lapse imaging using trace amounts of the probe [311]. So far, BODIPY-cholesterol has been used as a cholesterol probe in model membranes and in trafficking studies in living cells [312, 313]. Through *in vivo* time-lapse analysis, the time-point at which this probe was located at the plasma membrane of mouse oocytes has been identified [332]. We therefore hypothesized that this new fluorescent cholesterol probe would be useful to determine the dose and incubation time at which cholesterol locates mainly at the plasma membrane of bovine

oocytes before vitrification. This will help establish whether an increase in the cholesterol/phospholipid ratio could benefit bovine oocyte cryotolerance.

Previous studies have shown that basic cryobiological differences exist between immature and mature oocytes. Mature metaphase II-stage (MII) oocytes can be difficult to cryopreserve, mainly because of the presence of the meiotic spindle and chromosome configuration. By contrast, in immature germinal vesicle-stage (GV) oocytes, spindle depolymerization during cryopreservation is avoided as chromatin is diffused in the diplotene state of prophase I and surrounded by a nuclear membrane [333-335]. Despite clear advantages of cryopreservation of GV oocytes, difficulties still exist with embryonic development after cryopreservation. Oocytes at GV stage are more sensitive to osmotic stress than MII oocytes [336]. Mature MII stage bovine oocytes show higher water and CPA permeability coefficients than GV stage oocytes [150, 151]. Consequently, GV oocytes are less able to tolerate potential water flux- or shear force related damage during volume changes [152]. Therefore, the improvement in membrane fluidity conferred by membrane cholesterol enrichment has strong potential to enhance tolerance of GV oocytes to vitrification.

The present study was designed to examine whether exposure of bovine oocytes to CLC before vitrification/warming could improve their cryotolerance and embryo development after *in vitro* fertilization. In a first set of experiments, we characterized intracellular trafficking and localization of fluorescently-labeled cholesterol in *in vitro* matured oocytes incubated with CLC either in FCS or PVA supplemented medium and assessed their effect on early embryo development after vitrification/warming. In a second set of experiments, different concentrations of CLC were compared in terms of the subcellular localization of fluorescently-labeled cholesterol. Subsequently, the effects of optimized CLC concentrations and incubation times prior to vitrification on early embryo development were assessed. Finally, immature or *in vitro* matured oocytes were vitrified after 30 min of incubation with 2 mg/ml CLC, and then fertilized and cultured to determine early embryo development and quality, and at the level of expression of specific genes that are potentially important in embryo survival. The expression patterns of genes involved in apoptosis (*BAX*), lipid metabolism (*CYP51*), imprinting (*DNMT3A, IGF2R, UBE2A*), heat (*HSPA1A*) and oxidative stress (*MnSOD*) were determined on Day 5 morulae by RT-qPCR.

## 5.3. Material and methods

### 5.3.1. Chemicals and supplies

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

### 5.3.2. Bovine oocyte collection and *in vitro* maturation

The methods used for the *in vitro* maturation of the bovine oocytes have been described elsewhere [316]. Briefly, bovine ovaries were collected at slaughter from a local abattoir (Escorxador Sabadell, S.A., Sabadell, Spain) and transported to the laboratory in phosphate buffered saline (PBS) at 35-37ºC. Cumulus oocyte complexes (COCs) were obtained by aspirating 2–10 mm follicles. Only COCs with three or more layers of cumulus cells and a homogeneous cytoplasm were selected to be matured *in vitro*. After three washes in modified PBS (PBS supplemented with 36 mg/ml pyruvate, 50 mg/ml gentamicin and 0.5 mg/ml bovine serum albumin (BSA)), groups of up to 50 COCs were placed in 500 µl of maturation medium in four-well plates and cultured for 24 h at 38.5ºC in a 5% $CO_2$ humidified air atmosphere. The maturation medium was comprised of TCM-199 supplemented with 10% (v/v) FCS, 10 µg/ml epidermal growth factor and 50 mg/ml gentamicin.

### 5.3.3. Cholesterol-loaded methyl-β-cyclodextrin (CLC)

The method described by Purdy and Graham [302] was used to prepare CLC. Briefly, 1 g of methyl-β-cyclodextrin was dissolved in 2 ml of methanol by vortexing. Separately, 200 mg cholesterol was dissolved in 1 ml of chloroform. A 0.45 ml aliquot of the cholesterol solution was then gently mixed with the 2 ml of cyclodextrin solution until combined to form a clear solution. The solvents were removed from the mixture with a stream of nitrogen gas and the crystals obtained were dried for 24 h, stored at room temperature, and designated cholesterol-loaded methyl-β-cyclodextrin (CLC).

### 5.3.4. Cholesterol imaging in living bovine oocytes treated with CLC

To determine cholesterol trafficking, oocytes were incubated together with different concentrations of CLC (see experimental design) complexed with 1 µM BODIPY-cholesterol (BPY-Chol. Avanti Polar Lipids, Alabama, USA) [311, 332] at 38.5ºC and 5%

$CO_2$ in air. BPY-Chol was visualized in living oocytes using a confocal microscope Leica TCS SP5 (Leica Microsystems GmbH, Mannheim, Germany). BPY-Chol was excited with a 488 nm argon laser with fluorescence emission detected in the 500-620 nm range using a HyD detector to identify the intracellular location of the fluorescent probe after incubation at different chase times (see experimental design).

The fluorescence intensity of plasma membrane and cytoplasm was quantified using ImageJ Software. After subtracting the background, cytoplasm mean intensity was measured by manually outlining the area corresponding to the oocyte cytoplasm, excluding the plasma membrane. A 55-pixel band was then created to surround the plasma membrane and mean fluorescence intensity for the plasma membrane was determined. The images were segmented into foreground and background by setting a threshold that was the same for all samples.

### 5.3.5. Oocyte vitrification and warming

**Vitrification protocol**

The vitrification/warming procedures employed were essentially as described by Morató *et al.* [37]. The holding medium (HM) used to formulate the vitrification-warming solutions consisted of HEPES-TCM-199 supplemented with 20% (v/v) FCS. All steps were performed under a laminar flow hood heated to 38.5ºC using a stereomicroscope to visualize each step. Partially denuded oocytes were transferred into equilibration solution (ES) consisting of 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethylsulfoxide (DMSO) in HM for 10 min. Subsequently, oocytes were moved to the vitrification solution (VS) containing 15% (v/v) DMSO, 15% (v/v) EG and 0.5 M sucrose dissolved in HM. After incubating for 30–40 s, oocytes were loaded onto a cryotop. Almost all the solution was removed to leave only a thin layer covering the oocytes and the cryotop plunged into liquid nitrogen. The entire process from exposure to VS to plunging in liquid nitrogen was completed in 90 seconds. When synthetic medium was required (see experimental design), 6% polyvinylpyrrolidone (PVP) (w/v) and 1 mg/ml fetuin were added to the equilibration and vitrification solutions instead of FCS.

**Warming protocol**

Vitrified oocytes were warmed by directly immersing the cryotop into the warming solution containing 1 M sucrose dissolved in HM for 1 min. Next, the recovered oocytes were transferred to the dilution solution containing 0.5 M sucrose dissolved in HM for 3

min. The oocytes were then incubated in HM for 5 min. After a final rinse in HM for 1 min, oocytes were transferred to the maturation medium and allowed to complete their *in vitro* maturation.

### 5.3.6 In vitro fertilization and embryo culture

*In vitro* matured oocytes (fresh and vitrified/warmed) were *in vitro* fertilized at 38.5ºC in a 5% $CO_2$ atmosphere. Frozen–thawed spermatozoa from Asturian bulls (ASEAVA, Llanera, Asturias, Spain) of proven fertility were used in all the experimental procedures. Motile spermatozoa were obtained by centrifuging frozen–thawed sperm on a discontinuous gradient (Bovipure, Nidacon International, Gothenburg, Sweden) for 10 min at 100×g at room temperature. Viable spermatozoa collected from the bottom were washed (Boviwash, Nidacon International, Gothenburg, Sweden) and pelleted by centrifugation at 100×g for 5 min. Spermatozoa were counted in a Neubauer hemocytometer and diluted in an appropriate volume of fertilization medium (Tyrode's medium supplemented with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/ml fatty acid-free BSA and 10 mg/ml heparin–sodium salt (Calbiochem, Darmstadt, Germany)) to a final concentration of $1 \times 10^6$ spermatozoa/ml. 100-µl droplets of diluted sperm were prepared under mineral oil and 20 to 25 oocytes/droplet co-incubated at 38.5°C, in a 5% $CO_2$ high humidity atmosphere.

After 18–20 h, presumptive zygotes were stripped of remaining cumulus cells by gentle vortexing after which they were cultured in 100-µl drops of synthetic oviductal fluid (SOF) [317] containing FCS (5%, v/v) under mineral oil at 38.5°C in 5% $CO_2$, 5% $O_2$, 90% $N_2$ for 8 days. Cleavage rates were recorded at 48 h post-insemination (hpi) and the number of blastocysts was determined on Days 7 and 8 post-insemination (pi). Day 8 embryos were classified according to the degree of blastocele expansion into three groups according to Morató *et al.* [337]: (1) non-expanded blastocysts, in which the blastocele volume was less than one-half of the total volume of the blastocyst; (2) expanded blastocysts, in which the blastocele volume was more than one-half of the total volume of the blastocyst; (3) hatched or hatching blastocysts, in which the expanded blastocyst was without a zona pellucida or had an opened zona pellucida.

**5.3.7. RNA extraction and real-time quantitative (rt)-PCR**

Total RNA was extracted from Day-5 morulae using the Picopure RNA extraction Kit (Thermo Fisher Scientific Inc, Waltham, MA, USA) following the manufacturer's instructions. Morulae harvested from each experimental group were pooled in groups (10 morulae) and washed three times in Dulbecco's-PBS at 38.5°C. Each pool was added to 100 µl of extraction buffer, snap frozen in liquid nitrogen, and stored at – 80°C until RNA isolation. A total of five pools for each experimental group were analyzed..

For RNA extraction, samples were vortexed for 15 s and incubated with lysis solution at 42ºC for 30 min. The samples were then centrifuged at 3,000×g for 3 min. The upper aqueous phase containing RNA was carefully transferred to a new tube without disturbing the interface. RNA was precipitated by the addition of an equal volume of 70% ethanol and loaded onto a spin column from a PicoPure™ RNA Isolation kit (Arcturus, Mountain View, CA, USA) according to the manufacturer's instructions. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA). Total RNA (40 ng) was reverse-transcribed to produce cDNA using Multiscribe™ Reverse Transcriptase (Applied biosystems, Foster City, CA, USA) primed with random primers. In all cases, a reverse transcriptase negative control was used to evaluate genomic DNA contamination.

The relative abundance of mRNA transcripts was calculated using the ΔΔcq method with the conserved helix-loop-helix ubiquitous kinase (*CHUK*) (Falco *et al.*, 2006) as a reference gene on a Bio-Rad CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc, Mississaugua, Canada) and QuantiFast SYBR Green PCR Master Mix (Qiagen, Toronto, Canada). The genes analyzed included *BAX, CYP51, DNMT3A, IGF2R, UBE2A, HSPA1A* and *MnSOD*, along with the reference gene. In each sample, cDNA was analyzed in quadruplicate to determine relative levels of each transcript of interest. In all cases, a reverse transcriptase negative control was included. The qRT-PCR reaction mix contained 12.5 µl 2× QuantiFast SYBR Green PCR Master Mix, 2µl (1875 nM) forward and reverse primers (Integrated DNA Technologies, Inc., IA, USA) specific for the genes of interest and 2 µl of cDNA template. The final volume was made up to 25 µl using nuclease-free water. Reactions were run at 95°C for 5 min followed by 40 cycles of 95ºC for 10 s and 60ºC for 1 min and a standard dissociation curve.

Primer sequences and approximate sizes of the amplified fragments of all transcripts are provided in Table 1. The efficiency of primer amplification was 90 to 100%. Non-template controls were not amplified or returned a Cq value 10 points higher than the average Cq for the genes. Expression levels of the target genes were normalized to average expression levels of the *CHUK* gene, which was expressed at constant levels (Cq values) in all samples and was stable under the conditions used.

**Table 5.1: Primers used for reverse transcription–quantitative polymerase chain reaction**

| Gene | Primer sequence | Fragment size | GenBank accession no. | Sequence references |
|---|---|---|---|---|
| *DNMT3A* | F: CCGTAGTGTCCAAGACCAATC<br>R: GCTGAGGCAAATCCTCGTAAC | 186 | BC114063 | [338] |
| *HSPA1A* | F: AAGGTG CTGGACAAGTGCCAGGAGGTGA<br>R: ACTTGGAAGTAAACAGAAACG GGTGAAAA | 503 | U09861 | [339] |
| *UBE2A* | F: GGGCTCCGTCTGAGAACAACATC<br>R: CATACTCCCGCTTGTTCTCCTGG | 336 | XM_864331 | [340] |
| *CYP51* | F: GCTCATTAGTTTGGGGGTGA<br>R: TCCCCACCCATCCTTACATA | 218 | NM_001025319.2 | [340] |
| *IGF2R* | F: CAGGTCTTGCAACTGGTGTATGA<br>R: TTGTCCAGGGAGATCAGCATG | 137 | J03527 | [341] |
| *BAX* | F: TCTGACGGCAACTTCAACTG<br>R: TGGGTGTCCCAAAGTAGGAG | 214 | NM_173894.1 | [342] |
| *MnSOD* | F: CCC ATGAAGCCCTTTCTAATCCTG<br>R: TTCAGAGGCGCTACTATTTCCTTC | 307 | L22092.1 | [339] |
| *CHUK* | F: TGATGGAATCTCTGGAACAGCG<br>R: TGCTTACAGCCCAACAACTTGC | 180 | NM_174021.2 | [343] |

Abbreviations: F, forward; R, reverse

### 5.3.8. Experimental design

#### 5.3.8.1. Experiment 1: Characterize the effects of CLC added to a FCS-supplemented or chemically-defined medium before vitrification on *in vitro* matured bovine oocytes.

**Experiment 1a. Live-cell imaging of cholesterol transport within *in vitro* matured bovine oocytes incubated with CLC in a FCS-supplemented or chemically-defined medium**.

After 22 h of IVM, oocytes were denuded by gentle pipetting and incubated in 2 mg/ml BODIPY-labeled CLC at 38.5ºC in HEPES-TCM-199 medium containing either 10% (v/v) FCS or 0.05% (w/v) PVA. BPY-Chol fluorescence was visualized in living oocytes at 30, 45, 60 and 75 min using a confocal microscope. A total of 20 oocytes were examined in each group in 3 different replicates.

**Experiment 1b. Effects of CLC added to FCS-supplemented or chemically-defined medium before vitrification on the developmental competence of *in vitro* matured oocytes.**

After 22 h of IVM and according to the results obtained in the previous experiment, partially denuded oocytes were randomly distributed into five groups: A and C) oocytes maintained in medium containing FCS; B) oocytes incubated in medium containing FCS with 2 mg/ml CLC; D) oocytes incubated in medium containing PVA with 2 mg/ml CLC; and E) oocytes incubated in medium containing PVA without CLC. Incubation with or without CLC was carried out at 38.5ºC in 5% $CO_2$ for 30 min. Oocytes were then washed 3 times in the corresponding handling medium without CLC. Oocytes were then vitrified/warmed as previously described using a FCS-supplemented medium (group A) or a synthetic medium containing 6% PVP and 1 mg/ml fetuin (groups B to E). After warming, oocytes were allowed to recover for 2 additional hours in IVM medium. Oocytes *in vitro* matured for 24 h were used as non-vitrified controls.

After 24 h of IVM, oocytes from each treatment group were *in vitro* fertilized and cultured. Cleavage rates were determined 48 hpi and blastocyst rates on Days 7 and 8 pi (8 replicates per group).

#### 5.3.8.2. Experiment 2: Cholesterol tracking in living oocytes treated with different CLC concentrations and effects on embryo developmental competence after vitrification/warming

**Experiment 2a. Live-cell imaging of cholesterol transport into *in vitro* matured bovine oocytes incubated with different CLC concentrations.**

After 22 h in IVM, oocytes were denuded by gentle pipetting and incubated with 1 mg/ml, 2 mg/ml or 3 mg/ml BODIPY-labeled CLC in HEPES-buffered TCM-199 containing 10% (v/v) FCS at 38.5ºC. BPY-Chol fluorescence was visualized in living oocytes using a confocal microscope at 10, 20, 30, 40 and 60 min. A total of 30 oocytes were examined in each group in 3 different replicates.

**Experiment 2.b. Effects of different CLC concentrations prior to vitrification on the developmental competence of *in vitro* matured bovine oocytes**

After 22 h of *in vitro* maturation and according to the results obtained in Experiment 2a, oocytes were randomly distributed into 4 groups: Control Vit group = no CLC treatment; CLC 1 mg/ml 40' group = oocytes incubated with 1 mg/ml CLC for 40 min; CLC 2 mg/ml 30' group = oocytes incubated with 2 mg/ml CLC for 30 min; CLC 3 mg/ml 20' group = oocytes incubated with 3 mg/ml CLC for 20 min. Oocytes were then washed 3 times in a medium without CLC and vitrified/warmed as previously described but using media supplemented with FCS. After warming, oocytes were allowed to recover for 2 additional hours in IVM medium. Oocytes *in vitro* matured for 24 h were used as non-vitrified controls.

After 24 h of IVM, oocytes from each treatment group were *in vitro* fertilized and cultured. Cleavage rates were determined 48 hpi and blastocyst rates on Days 7 and 8 pi (4 replicates per group).

#### 5.3.8.3. Experiment 3: Effects of CLC treatment before vitrification of immature and *in vitro* matured oocytes on developmental competence and specific gene expression.

After follicle aspiration, a proportion of the COCs was randomly divided into the following groups: GV-VIT = vitrified GV oocytes; and GV-CLC-VIT = GV oocytes incubated with 2 mg/ml CLC for 30 min and vitrified. After vitrification, VIT group oocytes were warmed and transferred back to the maturation medium.

The rest of the COCs were matured *in vitro* for 22 h and randomly divided into three groups: Control = fresh non-vitrified MII oocytes; MII-VIT = vitrified MII oocytes; and MII-CLC-VIT = MII oocytes incubated with 2 mg/ml CLC for 30 min and vitrified. After vitrification, VIT group oocytes were warmed and transferred back to the maturation medium. After 24 h of IVM, oocytes from each treatment group were *in vitro* fertilized and cultured as previously described. In a first set of experiments (5 replicates), embryo development was allowed to continue until Day 5 when morulae from the different treatment groups were harvested for RNA extraction and RT-qPCR. In a second set of experiments, cleavage rates were determined 48 hpi and blastocyst rates on Days 7 and 8 pi (6 replicates per group)

**5.3.9. Statistical analysis**

All statistical tests were performed using the software package SPSS version 21.0 for Windows (IBM Corp.; Chicago, Illinois, USA). Data are provided as the mean ± standard error of the mean (SEM) and significance was set at $P \leq 0.05$. The Shapiro-Wilk and Levene tests were used to check the normality of data and homogeneity of variances, respectively.

On the one hand, the effects of CLC, handling medium and vitrification medium on the development competence of *in vitro* matured and fertilized bovine oocytes were tested through a three-way analysis of variance followed by post-hoc Sidak's test. The effects of CLC treatment of *in vitro* matured bovine oocytes in an FCS-supplemented or chemically-defined medium before vitrification on the developmental competence of embryos derived from these oocytes were evaluated through one-way ANOVA, followed by post-hoc Sidak's test. Embryo developmental competence of immature and *in vitro*-matured bovine oocytes pretreated with CLC before vitrification was evaluated through a two-way ANOVA (factors: stage and treatment) followed by post-hoc Sidak's test.

With regard to analysis of gene expression, relative expression of six out of seven genes (all except *CYP51*) did not match with parametric assumptions. For this reason, we attempted to transform our data through square root ($\sqrt{x}$) or arcsin square root (arcsin $\sqrt{x}$). Transformation was only successful in the case of arcsin √(*IGF2R*). Therefore, the effects of treatment (i.e. fresh, vitrified-warmed control, vitrified-warmed + CLC) and stage (GV, MII) upon the expression of *CYP51* and *IGF2R* were tested through a two-way analysis of

variance (ANOVA) followed by Sidak's test for pair-wise comparisons. For the relative transcript abundance of the other genes (*DNMT3, HSPA1A, MnSOD, BAX* and *UBE2A*), we performed a non-parametric Scheirer-Ray-Hare ANOVA for ranked data (again, factors were treatment and stage). Following calculation of the 'H' statistic, the Mann-Whitney test was run for pair-wise comparisons.

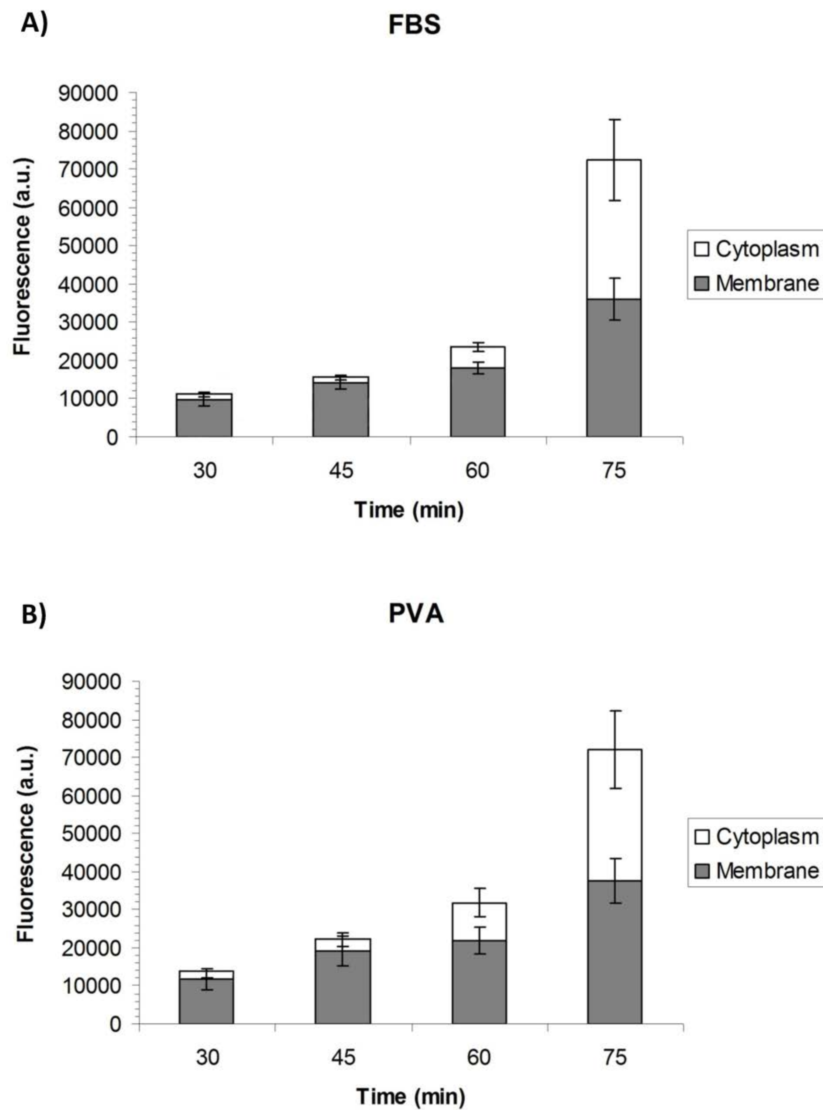
## 5.4. Results

### 5.4.1. Experiment 1: Characterize the effects of CLC added to a FCS-supplemented or chemically-defined medium before vitrification on *in vitro* matured bovine oocytes.

#### Experiment 1a. Live-cell imaging of cholesterol transport within *in vitro* matured bovine oocytes incubated with CLC in a FCS-supplemented or chemically-defined medium.

To examine whether cholesterol in a medium supplemented with FCS was transported into the oocyte, cholesterol internalization at different chase times was imaged in living bovine oocytes incubated with 2 mg/ml CLC in a medium containing FCS (Fig 1A) or PVA (Fig 1B). After 30 min of incubation, the plasma membrane showed a high labeling intensity relative to the cytoplasm. As chase time lengthened, cholesterol inclusion in the cytoplasm also increased considerably. After 75 min, cholesterol trafficking through the plasma membrane into the cytoplasm showed the same pattern when the medium was supplemented with FCS or with PVA.

**Figure 5.1. Subcellular localization of BPY-Chol in *in vitro* matured bovine oocytes.** Fluorescence intensity observed at the membrane and in the cytoplasm of bovine oocytes treated with 2 mg/ml CLC in a medium containing FCS or PVA at different chase times: 30, 45, 60 and 75 minutes. Mean ± SEM values for each subcellular compartment over time.



**Experiment 1b. Effects of CLC added to FCS-supplemented or chemically-defined medium before vitrification on the developmental competence of IVM oocytes**

Given that cholesterol labeling could be observed at the plasma membrane after 30 min of incubation in the previous experiments, *in vitro* matured oocytes were vitrified/warmed following pre-treatment with 2 mg/ml CLC for 30 min in a FCS- or PVA-supplemented medium. The vitrification medium used for this experiment was supplemented with PVP and fetuin to avoid the use of FCS during the vitrification process. Table 2 shows the results of oocyte survival and embryo development after oocytes were subjected to each treatment. Significantly higher survival, cleavage and blastocyst rates were observed for non-vitrified oocytes than vitrified oocytes regardless of CLC treatment or the holding medium used. When comparing the vitrification groups, oocytes from group C, held in FCS-supplemented medium before vitrification in synthetic medium showed a significantly lower survival rate compared to the other vitrification groups. Oocytes from group C also showed significantly lower cleavage rates when compared to the group of oocytes held and vitrified in media containing FCS (group A) while no significant differences were observed for oocytes in groups B, D and E when compared to groups A and C. No significant differences in Day 7-blastocyst rates were observed among the vitrification groups. However, oocytes from group D showed significantly lower Day 8-blastocysts rate than oocytes from group A. When evaluating embryo expansion as an indicator of embryo quality, CLC pretreatment of oocytes in the presence of PVA (group D) resulted in significantly reduced expansion rates on Day 8 pi when compared to those recorded in fresh non-vitrified oocytes, while the remaining vitrification groups showed similar rates of expansion to the control and D groups. Only embryos derived from oocytes from group A (held and vitrified in media supplemented with FCS) were able to hatch, although no significant differences in rates were observed between this group and the remaining vitrification groups.

**Table 5.2. Effect of CLC treatment of *in vitro* matured bovine oocytes in an FCS-supplemented or chemically-defined medium before vitrification on the developmental competence of embryos derived from these oocytes.**

| | CLC | Handling medium | Vit medium | n | Mean % ± SEM | | | | Day 8 embryo Mean % ± SEM | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Survival | Cleavage | Blastocyst Day 7 | Blastocyst Day 8 | Non-expanded | Expanded | Hatched |
| **Control** | - | FCS | - | 552 | 95.7 ± 1.6 [a] | 75.1 ± 2.6 [a] | 19.8 ± 1.9 [a] | 22.0 ± 1.8 [a] | 21.3 ± 4.2 [a] | 58.8 ± 5.0 [a] | 19.9 ± 4.8[a] |
| **A** | - | FCS | FCS | 383 | 71.6 ± 3.2 [b] | 44.4 ± 2.7 [b] | 4.3 ± 1.0 [b] | 7.2 ±1.0 [b] | 64.9 ± 10.0 [b] | 31.5 ± 9.5 [ab] | 3.6 ± 3.6 [b] |
| **B** | + | FCS | PVP+ F | 121 | 60.6 ± 12.3 [b] | 31.5 ± 8.5 [bc] | 3.1 ± 1.3 [b] | 5.5 ± 2.5 [bc] | 33.3 ± 19.2 [ab] | 66.7 ± 19.2 [ab] | 0 [b] |
| **C** | - | FCS | PVP+ F | 109 | 49.7 ± 2.8 [c] | 24.8 ± 3.7 [c] | 2.6 ± 1.7[b] | 4.6 ± 2.2 [bc] | 55.6 ± 29.4 [ab] | 44.4 ± 29.4 [ab] | 0 [b] |
| **D** | + | PVA | PVP+ F | 186 | 66.0 ± 5.5 [b] | 37.4 ± 4.0 [bc] | 2.6 ± 1.0 [b] | 3.5 ± 1.0 [c] | 90.0± 10.0[b] | 10.0±10.0 [b] | 0 [b] |
| **E** | - | PVA | PVP+ F | 293 | 64.0± 5.3 [b] | 34.6 ± 3.1 [bc] | 2.7 ± 0.7 [b] | 5.9 ± 0.9 [bc] | 68.5± 10.2[b] | 31.5±10.2 [ab] | 0 [b] |

CLC treatment: oocytes were incubated with 2 mg/ml CLC for 30 min. Handling medium: the handling medium used during CLC incubation contained PVA or FCS. Vit medium: vitrification/warming medium contained FCS or PVP+Fetuin. Data are provided as the mean ± SEM (standard error of the mean). Values with different subscripts within columns differ significantly ($P<0.05$).

### 5.4.2. Experiment 2: Cholesterol tracking in living bovine oocytes treated with different CLC concentrations and effect on embryo development competence after vitrification/warming

**Experiment 2a. Live-cell imaging of cholesterol transport into *in vitro* matured bovine oocytes incubated with different CLC concentrations**.

Internalization of fluorescence-labeled cholesterol was followed by confocal microscopy imaging of living bovine oocytes incubated with 1 mg/ml (Fig 2A), 2 mg/ml (Fig 2B) or 3 mg/ml CLC (Fig 2C) at different chase times. As in the previous experiment, total cell fluorescence increased with incubation time. When oocytes incubated with 2 mg/ml or 3 mg/ml CLC were imaged after 10 min, intense labeling was observed at the plasma membrane while there was essentially no labeling in the cytoplasm. This pattern was repeated when the incubation time was extended to 20 min in the presence of 1 mg/ml CLC. For the highest CLC concentration (3 mg/ml), fluorescence could be observed diffusing into the cytoplasm as early as 20 min, while for 1 mg/ml CLC and 2 mg/ml CLC, 40 and 30 min respectively were required before fluorescence spread into the cytoplasm. Fluorescence intensity in the cytoplasm increased with incubation time and higher levels of fluorescence were observed for oocytes incubated with the highest CLC concentrations (2 and 3 mg/ml). Images of cholesterol internalization into the oocytes are provided in Fig 3.

**Figure 5.2. Subcellular localization of BPY-Chol in *in vitro* matured bovine oocytes incubated with CLC.** Fluorescence intensity observed at the plasma membrane and in the cytoplasm of bovine oocytes treated with 1, 2 or 3 mg/ml CLC at different chase times: 10, 20, 30, 40 and 60 minutes. Mean ± SEM values for each subcellular compartment over time.

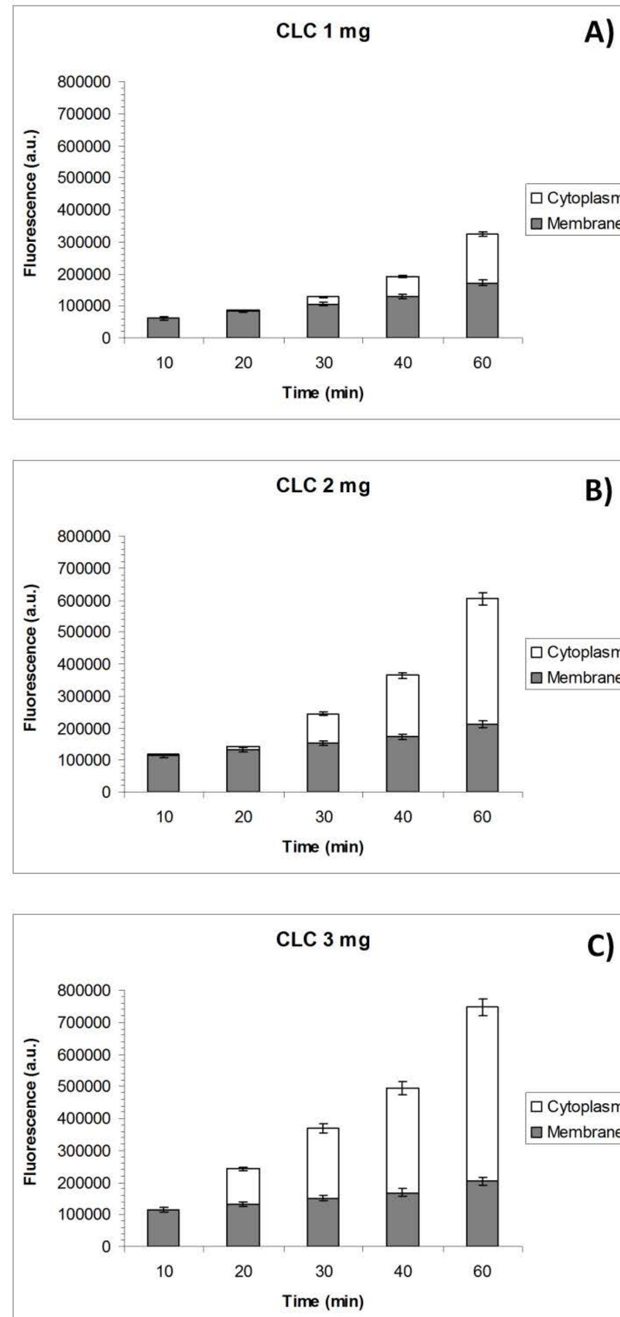
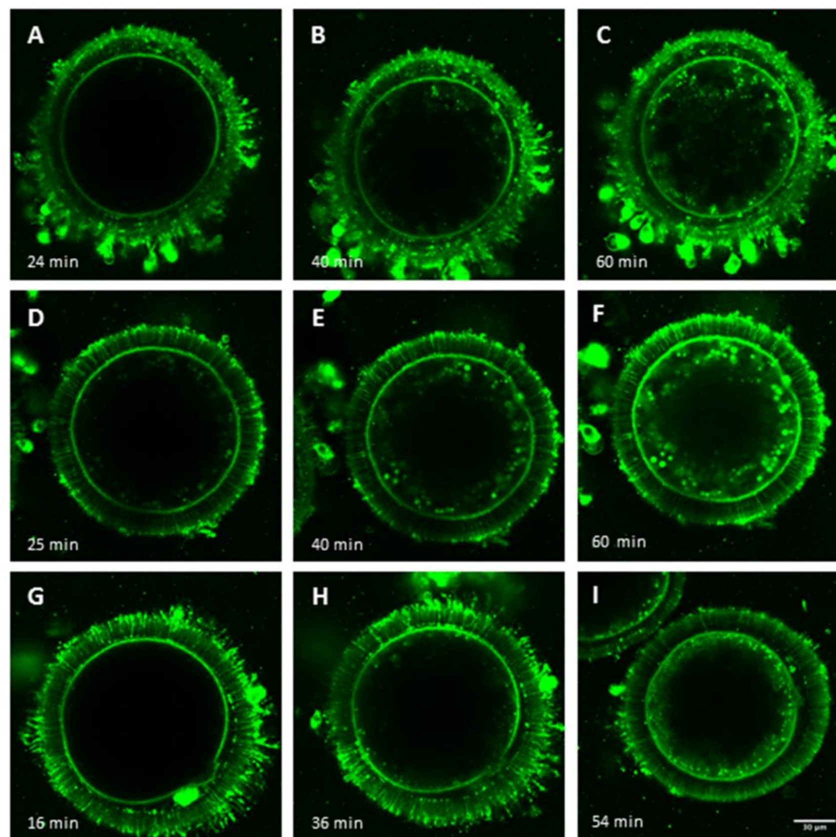


**Figure 5.3. Immunofluorescence localization of BODIPY-Cholesterol in *in vitro* matured oocytes.** The plasma membrane and cytoplasm localization of cholesterol was detected using the BODIPY fluorescent probe in *in vitro* matured bovine oocytes incubated with different concentrations of CLC. (A-C) oocytes incubated with 1 mg/ml of CLC imaged at 20, 40 and 60 minutes; (D-F) oocytes incubated with 2 mg/ml of CLC imaged at 30, 40 and 60 minutes; (G-I) oocytes incubated with 3 mg/ml of CLC imaged at 10, 20 and 40 minutes. BPY-Chol could be detected only at the plasma membrane (A, G), at the plasma membrane and diffusing into the cytoplasm (B,D,H) or at the plasma membrane and mostly distributed in the cytoplasm (E,C,F,I). Fluorescence intensity was quantified using ImageJ software. Scale bar = 30 µm.



**Experiment 2.b: Effects of different CLC concentrations prior to vitrification on the developmental competence of *in vitro* matured bovine oocytes.**

To select the incubation time and CLC concentration for this experiment we looked for high fluorescence intensity at the plasma membrane level along with some scattered fluorescence in the cytoplasm (<25%). This level of fluorescence indicated that the plasma membrane contained relatively high amount of CLC and that the low diffusion of CLC into the cytoplasm would not be likely to impair oocyte survival after vitrification. Thus, based on the results obtained in Experiment 2a and these fluorescence criteria, we tested different incubation times and concentrations of CLC prior to vitrification of *in vitro* matured oocytes to assess their effects on early *in vitro* embryo development (Table 5.3). While differences among treatments were not observed in terms of survival rates, cleavage rates were significantly reduced in the groups of oocytes treated with 1 mg/m CLC for 20 min or 2 mg/ml for 30 min when compared with control fresh oocytes, while this was not observed in the remaining vitrification groups. Day 7 and Day 8 blastocyst yields were significantly higher for fresh non-vitrified oocytes when compared to the vitrified oocytes. Among the vitrification groups, no significant differences in Day 7 and Day 8 blastocyst yields were observed, regardless of the concentration or incubation time.

**Table 5.3. Comparison of different incubation times and concentrations of CLC prior to vitrification on the developmental competence of *in vitro*-matured bovine oocytes**.

| Treatment | n | Mean % ± SEM | | | | Day 8 embryos Mean % ± SEM | | |
|---|---|---|---|---|---|---|---|---|
| | | Survival | Cleavage | Blastocysts Day 7 | Blastocysts Day 8 | Non-expanded | Expanded | Hatched |
| **Control** | 191 | 91.9 ± 5.7 | 62.4 ± 4.7 [a] | 15.9 ± 1.6 [a] | 18.7 ± 0.1[a] | 36.0 ± 22.4[a] | 50.4 ± 16.8 | 13.6 ± 8.2 |
| **Control Vit** | 101 | 80.2 ± 0.2 | 48.4 ± 6.7 [ab] | 7.1 ± 1.4[b] | 8.8 ± 1.1[b] | 33.3 ± 19.2[a] | 66.7 ±19.2 | 0 |
| **CLC 1 mg/ml 40’** | 107 | 80.5 ± 5.3 | 33.6 ± 1.9 [b] | 2.0 ± 1.2[b] | 5.0 ± 1.4[b] | 75.0 ± 25.0[b] | 25.0 ± 25.0 | 0 |
| **CLC 2 mg/ml 30’** | 114 | 80.5 ± 4.9 | 37.1 ± 6.8 [b] | 5.7 ± 3.2[b] | 8.4 ± 1.8[b] | 63.9 ± 21.7[a, c] | 36.1 ± 21.7 | 0 |
| **CLC 3 mg/ml 20’** | 101 | 80.8 ± 13.7 | 51.0 ± 13.0 [ab] | 6.0 ± 2.2[b] | 7.4 ± 2.0[b] | 72.2 ± 14.7[b, c] | 27.8 ±14.7 | 0 |

Treatments: Control = oocytes *in vitro* matured for 24 h; Control Vit: no CLC treatment before vitrification; CLC 1 mg/ml 40’ = oocytes incubated with 1mg/ml CLC for 40 min before vitrification; CLC 2 mg/ml 30’ = oocytes incubated with 2 mg/ml CLC for 30 min before vitrification; CLC 3 mg/ml 20’= oocytes incubated with 3 mg/ml CLC for 20 min before vitrification . Data are provided as the mean ± SEM. Values with different subscripts within columns differ significantly ($P$<0.05).

### 5.4.3. Experiment 3: Effects of CLC treatment before vitrification of immature and *in vitro* matured oocytes on developmental competence and specific gene expression.

Table 4 shows the *in vitro* embryo developmental competence of oocytes –immature or *in vitro*-matured – pretreated with 2 mg/ml CLC for 30 min prior to vitrification. All vitrification treatments led to significantly lower percentages of embryo cleavage and Day 7 or Day 8 blastocyst yields ($P<0.05$) compared to fresh non-vitrified oocytes. Cleavage and blastocyst rates were similar for all vitrified/warmed oocytes, regardless of CLC treatment or nuclear stage. Furthermore, all vitrified oocyte groups returned similar rates of blastocyst expansion to the control fresh oocyte group except for a significantly higher percentage observed for the vitrified MII oocytes. Only matured MII oocytes treated with CLC before vitrification also demonstrated similar percentages of blastocyst hatching to control fresh oocytes, although rates did not differ statistically from those observed in the other vitrification groups.

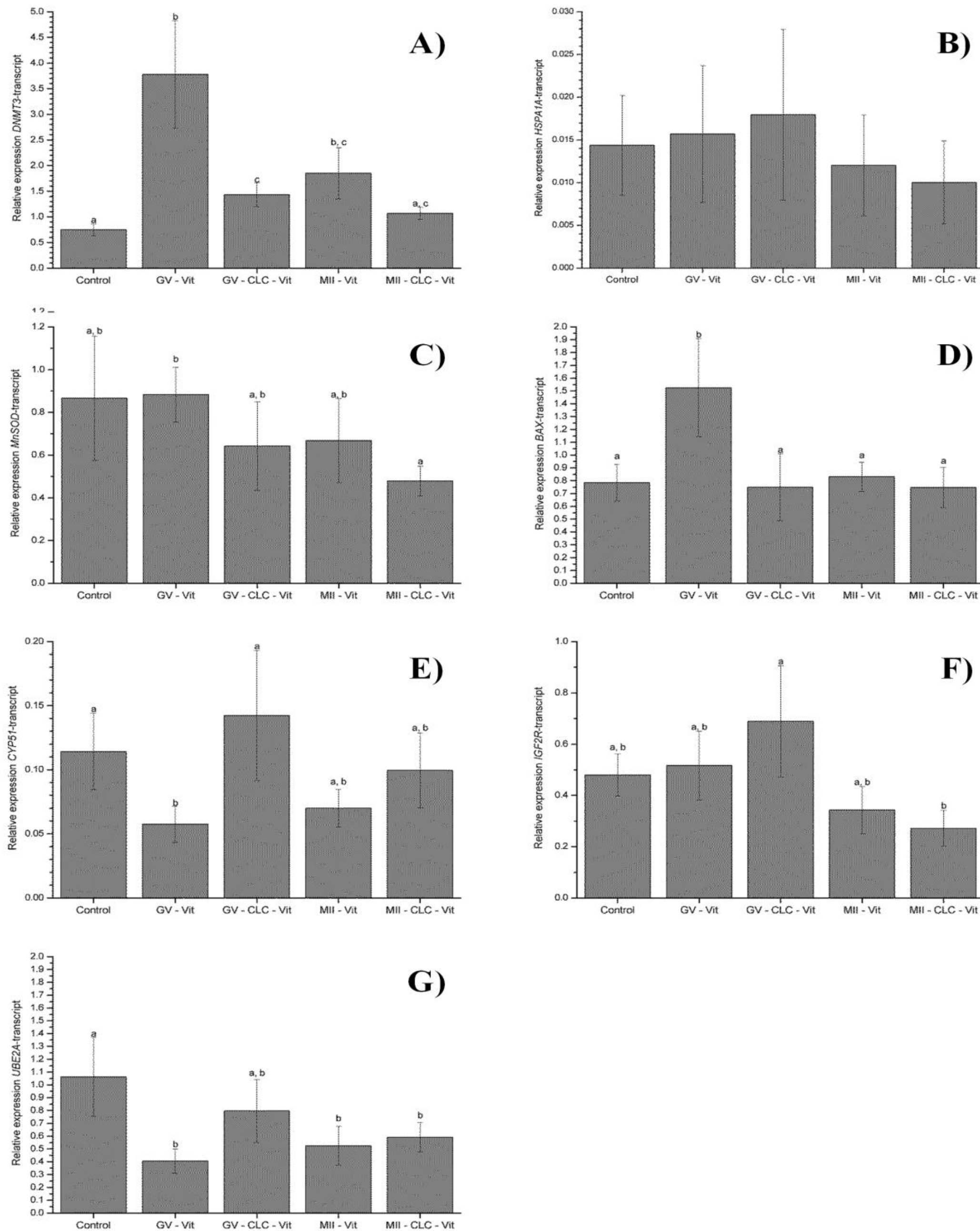
**Table 5.4. Embryo developmental competence of immature and *in vitro*-matured bovine oocytes pretreated with CLC before vitrification.**

| Treatment | n | Mean % ± SEM | | | | Day 8 embryos Mean % ± SEM | | |
|---|---|---|---|---|---|---|---|---|
| | | Survival | Cleavage | Blastocyst Day 7 | Blastocyst Day 8 | Non-expanded | Expanded | Hatched |
| **Control** | 185 | 93.4 ± 4.3[a] | 66.7 ± 3.6[a] | 18.4 ± 1.6[a] | 19.4 ± 0.8[a] | 42.6 ± 10.9[a] | 35.6 ±10.2[a] | 21.8 ± 10.0[a] |
| **GV-VIT** | 238 | 72.0 ± 7.2[ab] | 41.4 ±5.5[b] | 5.6 ± 1.1[b] | 7.3 ± 0.8[b] | 66.7 ±16.7[ab] | 27.8 ± 12.7[a] | 5.6 ± 5.6[b] |
| **GV- CLC-VIT** | 236 | 64.0 ± 4.9[b] | 40.9 ± 4.8[b] | 5.8 ± 0.7[b] | 5.7 ± 1.5[b] | 75.00 ±11.2[b] | 25.0 ±11.2[a] | 0.0 ± 0.0[b] |
| **MII-VIT** | 180 | 60.8 ± 10.0[b] | 39.9 ± 5.5[b] | 7.3 ±2.1[b] | 7.0 ± 1.3[b] | 38.9 ± 15.3[a] | 61.1 ± 15.3[b] | 0.0 ± 0.0[b] |
| **MII-CLC-VIT** | 206 | 69.0 ± 5.1[b] | 41.7 ±2.2[b] | 8.9 ± 1.3[b] | 9.9 ± 1.7[b] | 36.1 ± 15.8[a] | 55.6 ±13.4[ab] | 8.3 ± 5.7[ab] |

Treatments: Control = fresh non-vitrified MII oocytes; GV VIT = vitrified GV oocytes; GV-CLC-VIT = GV oocytes vitrified after 30 min incubation with 2 mg/ml CLC; MII VIT = vitrified MII oocytes; MII-CLC-VIT = MII oocytes vitrified after 30 min incubation with 2 mg/ml CLC. Data are provided as the mean ± SEM. Values with different subscripts within columns differ significantly ($P<0.05$).

In order to determine whether changes in the expression of developmentally-important genes were associated with the different vitrification protocols, we next evaluated the levels of *DNMT3A, HSPA1A, MnSOD, BAX, CYP51, IGF2R, UBE2A* mRNAs *in vitro* produced morulae (Fig 4). No significant differences were detected in relative mRNA abundances for *HSPA1A*, *MnSOD* and *IGF2R* in morulae derived from GV vitrified oocytes or MII vitrified oocytes compared to non-vitrified oocytes, regardless of CLC treatment. *DNMT3A* and *BAX* gene expression was significantly higher in morulae from vitrified GV oocytes. The expression of both genes in CLC-treated vitrified GV oocytes remained similar when compared to the levels observed in non-vitrified fresh oocytes. In contrast, *CYP51* expression was significantly lower in morulae from vitrified GV oocytes compared to morulae from CLC-treated vitrified or non-vitrified oocytes. While the expression of the *BAX* gene in vitrified MII oocytes remained similar to that in the fresh oocyte control group regardless of CLC treatment, *DNMT3A* expression was significantly higher in morulae from vitrified MII oocytes, and no differences were observed in morulae from oocytes vitrified after CLC treatment. *UBE2A* gene expression was significantly lower in morulae from GV- or MII vitrified or MII-CLC-vitrified oocytes, while expression levels of this gene in morulae derived from GV-CLC-vitrified oocytes remained similar to those recorded in control fresh oocytes.

**Figure 5.4. Relative expression levels of the genes *DNMT3* (A), *HSPA1A* (B), *MnSOD* (C), *BAX* (D), *CYP51* (E), *IGF2R (F)*, *UBE2A* (G) recorded in *in vitro* produced morulae.** GV = oocytes at the germinal vesicle stage; MII = oocytes at the metaphase II stage; Control = fresh, non-vitrified oocytes; Vit = vitrified oocytes; CLC +Vit = Oocytes pre-treated with 2 mg/ml CLC for 30 min and vitrified. Data are provided as the mean ± SEM. Different subscripts indicate significant differences ($P$<0.05) in gene expression.



## 5.5. Discussion

Cryopreserved oocytes show highly compromised survival and developmental capacities due to the morphological and cytological damage induced by the cryopreservation process. Cells with more flexible membranes that are more permeable to water and intracellular cryoprotectants are known to incur less damage during cryopreservation [290]. Adding cholesterol to the incubation medium increases membrane fluidity at lower temperatures by modifying how membrane phospholipids interact with each other, making membranes less contracted at lower temperatures [344].

Horvath and Seidel [32] detected no effects of pre-treatment of *in vitro* mature bovine oocytes with CLC in the presence of FCS before vitrification on subsequent embryo developmental competence. In contrast, when a synthetic medium was used during CLC treatment (2 mg/ml CLC for 1 h), cleavage and development up to eight-cell embryo rates were significantly higher for oocytes pretreated with CLC prior to vitrification. These authors attributed the lack of effect of CLC treatment in improving cryotolerance that cholesterol was preferentially transferred to lipids or proteins present in the fetal calf serum (FCS) avoiding its incorporation into the oocyte [32]. In the present study, through in vivo confocal microscopy and BPY-Chol labeling, we observed cholesterol trafficking into oocytes at a specific CLC concentration (2 mg/ml) using a handling medium supplemented either with FCS or PVA. Moreover, in both the FCS and PVA supplemented media, CLC was observed at the plasma membrane 30 min after incubation. Based on these results, we compared the effect on embryo development of CLC treatment (2 mg/ml for 30 min) prior to vitrification using the two supplements. Our results showed that neither prior CLC treatment, nor the use of FCS or PVA, conferred any benefits in terms of embryo development up to the blastocyst stage. It has been shown that both cholesterol depletion and cholesterol enrichment of the plasma membrane may affect cellular functions [305]. In our study, we postulate that any improvements attributable to plasma membrane cholesterol enrichment may have been offset by cell alterations resulting from changes in phospholipid distributions and modified cholesterol distribution among the cell compartments due to the presence of

CLC [305, 345, 346]. This may have had an effect on the oocyte plasma membrane and on the subsequent developmental competence of the oocyte after vitrification.

The results obtained in this study for the use of FCS or synthetic macromolecular components in the vitrification solution are comparable to those of other studies examining small volume supports for vitrification [20, 37, 96]. The solutions used for oocyte *in vitro* maturation, handling, vitrification or warming usually contain FCS [20, 21, 37, 347, 348]. Since we observed no improvement in embryo developmental competence after the vitrification of oocytes handled or vitrified in synthetic media, FCS was used as a supplement for the rest of our experiments. It has been already reported that the efficiency of cholesterol transfer from βCD inclusion complexes to biological membranes depends on βCD-cholesterol concentration and exposure duration [306, 349, 350]. Because no differences in embryo development were observed here after incubation with 2mg/ml CLC for 30 min we used confocal microscopy to identify the time-point at which fluorescent-tagged cholesterol was primarily located at the plasma membrane using three different concentrations of CLC: 40 min for 1 mg/ml , 30 min for 2 mg/ml and 20 min for 3 mg/ml. Cholesterol manipulation as carried out in this study may have modified the plasma membrane properties of matured oocytes pre-treated with different CLC concentrations for varying time intervals. However, we observed no correlation between oocyte cryotolerance and embryo cleavage or embryo development up to the blastocyst stage. The effects of cholesterol on membranes depend on lipid composition and fatty acid saturation. Cholesterol and sphingomyelin are thought to form ordered lipid domains (rafts) in mammalian cell membranes [305]. Cholesterol disrupts the highly ordered gel phase of brain sphingomyelin, leading to a more fluid membrane, while reducing the fluidity of brain phosphatidylcholine bilayers [351]. We propose that the irregular incorporation of labeled lipids into the different cholesterol domains of the oocyte plasma membrane may preclude increases in membrane fluidity in response to the addition of cholesterol.

Cryobiological differences between immature and *in vitro* matured oocytes prompted our subsequent studies in which oocytes at the GV or MII stage were pretreated with 2 mg/ml CLC for 30 min before vitrification, after which the impact on embryo development and gene expression was determined. Although no differences in cleavage and blastocyst yields were observed after CLC treatment, differences were detected in

the relative abundance of some gene transcripts during early embryo development. For example, the relative abundance of *DNMT3A* transcripts in morulae derived from immature and *in vitro* matured vitrified oocytes was significantly higher compared to abundances observed in morulae derived from fresh oocytes. Similarly, Shirazi et al. [352] found that the overall expression of *DNMT3B in morulae derived from* vitrified immature ovine oocytes was greater than in morulae derived from non-vitrified fresh oocytes. *DNMT3A* and *DNMT3B* drive most *de novo* methylation during embryonic reprogramming whereby methyl groups are added to previously unmodified DNA [353]. DNA methylation exerts essential functions in many genetic processes such as X chromosome inactivation, genome imprinting, and transcriptional silencing of specific genes and repetitive elements [354, 355]. Given its role in de novo methylation, abnormal levels of *DNMT3A* expression could suggest that embryos derived from vitrified oocytes are at higher risk for problems in these processes, which could be ameliorated by CLC treatment prior to vitrification. Importantly, CLC treatment prior to vitrification of both GV and MII bovine oocytes gave rise to morulae showing levels of *DNMT3A* transcripts approaching those of morulae derived from non-vitrified oocytes. Similarly, when the expression of *UBE2A*, another gene related to developmental epigenetics, was analyzed, morulae derived from GV vitrified after CLC treatment showed similar expression levels to those derived from non-vitrified oocytes. Further work is needed to assess global levels of DNA methylation and histone acetylation in oocytes pretreated with CLC prior to vitrification.

The expression of *BAX* was also significantly higher in morulae derived from vitrified GV oocytes than morulae derived from fresh or CLC-treated GV oocytes. When *BAX* is overexpressed in cells, apoptotic death is accelerated and previous research has shown that morphologically poor quality or fragmented embryos show higher expression levels of this gene [356, 357]. In response to pre-treatment with CLC before vitrification of GV oocytes, *BAX* gene expression levels returned to similar levels to those observed in fresh control oocytes. This suggests that CLC may have helped improve the quality of embryos obtained after oocyte vitrification. *CYP51* (cytochrome P450 family 51 or lanosterol 14a-demethylase *CYP51*) is required for de novo cholesterol synthesis during embryogenesis, which is essential for the regulation of membrane fluidity and thereby related to survival after embryo vitrification. Because blastocyst quality correlates positively with embryo survival after vitrification [358, 359], CLC treatment before

vitrification of GV oocytes may also have served to improve embryo quality after vitrification of GV oocytes. While *CYP51* gene expression was significantly lower in morulae derived from vitrified GV oocytes, morulae derived from GV-CLC vitrified oocytes showed similar relative abundances of mRNA transcripts to morulae derived from fresh non-vitrified oocytes.

Embryos also show changes in expression of genes reflecting the stress response to suboptimal conditions [339]. Differences in gene transcript levels between morulae derived from vitrified oocytes treated with CLC compared to those without were higher for immature oocytes. This suggests that vitrification at the GV stage may be particularly harmful for the oocyte and that treatment with 2 mg/ml CLC for 30 min may protect the oocyte from specific stresses, giving rise to pre-embryos of higher developmental potential. Even though differences in developmental competence in terms of blastocyst rates were not observed after CLC treatment, differences in the relative abundances of some gene transcripts important for early embryo development were detected. Differences in the effects of CLC treatment between GV and MII oocytes might be related to their different cryotolerances,. For example, hydraulic conductivity is 2-fold lower in GV oocytes compared to MII oocytes [150, 151]. Thus, MII stage bovine oocytes have higher water and CPA permeability coefficients than GV stage oocytes [150, 151]. This implies that GV oocytes are less able to tolerate water flux or shear force related damage during volume changes [152]. Accordingly, GV oocytes may benifit more from membrane cholesterol enrichment through CLC treatment. Since membranes are stabilized by cholesterol and remain more fluid at lower temperatures, stresses associated with membrane distortion due to dehydration and shrinkage during vitrification, especially damaging for GV stage oocytes, are likely to be be reduced [360].

In conclusion, CLC pre-treatment before the vitrification of bovine *in vitro* matured oocytes does not appear to affect subsequent cleavage and embryo development rates, irrespective of the presence of FCS in the handling medium, the CLC concentration, incubation time or meiotic oocyte stage (GV or MII). However, relative differences in the expression of genes related to embryo development in oocytes pretreated with 2 mg/ml suggests that CLC does improve some aspects of embryo quality after vitrification, particularly when derived from oocytes vitrified at the GV stage. Further

studies will obviously be necessary to determine whether the changes in gene expression that we have identified, and the processes in which they participate, can be further enhanced to optimize oocyte quality after vitrification.

## 5.6. Acknowledgments

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# CHAPTER VI

## Vitrification of in vitro matured bovine oocytes in media supplemented with a biopolymer produced by an Antarctic bacterium

Chapter VI

# Vitrification of in vitro matured bovine oocytes in media supplemented with a biopolymer produced by an Antartic bacterium

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## 6.1. Abstract

Previous reports [267] have already demonstrated that the exopolysaccharide produced by *Pseudomonas* sp. ID1 (M1 EPS) can be applied as an agent for cell cryopreservation, alone or in combination with other cryoprotectants. After 21h of *in vitro* maturation, oocytes form prepubertal and adult cows were vitrified/warmed in media supplemented with various concentrations of M1 EPS (0 mg/mL, 0.001mg/mL, 0.01 mg/mL, 0.1 mg/mL and 1 mg/mL). After warming, oocytes were allowed to recover for 1 additional hour in IVM medium. Fresh, non-vitrified oocytes were used as a control. In the first experiment, at 24 h of IVM, oocytes from all treatments were completely denuded, fixed and microtubule and chromosome distribution was analysed by immunocytochemistry under a fluorescent microscope. In the second experiment, oocytes were in vitro fertilized at 24 h of IVM and cleavage and blastocysts rates were recorded at days 2, 7 and 8, respectively. In the third experiment, blastocyst gene expression were analysed by RT-PCR. Vitrification of prepubertal oocytes in an M1 EPS supplemented medium triggered significantly lower percentages of decondensation or absence of chromosomes and microtubules than when oocytes were vitrified in media without M1 EPS. When oocytes were vitrified and warmed in media supplemented with 0 and 0.01 mg/ml M1 EPS, the percentage of oocytes with normal spindle configuration was significantly lower for prepubertal than for cow oocytes. In conclusion, supplementation with M1 EPS concentrations during vitrification and warming did not induce adverse changes in the spindle of bovine oocytes, regardless of the concentration used. Although a more severe damage on spindle configuration could be observed after vitrification of prepubertal oocytes, EPS supplementation during vitrification and warming seem to have a greater benefit during vitrification of prepubertal than adult bovine oocytes. Cleavage rates were similar among vitrification treatments, regardless of the M1 EPS supplementation. Oocytes vitrified in media supplemented with 0.001, 0.01 or 0.1 mg/mL did not differ from oocytes vitrified without M1 EPS in terms of blastocyst yield. However, the hatching ability of blastocysts derived from cow oocytes vitrified with 0.01 or 0.1 mg/mL of M1 EPS was similar to blastocysts from fresh control oocytes, while none of the embryos from other treatments reached the hatched stage. The developmental competence from vitrified oocytes was likely impaired by changes in gene expression of some gens related with epigenetics (DNA methylation and chromatine acetylation) and blastocyst quality (BAX, BCL2).

## 6.2. Introduction

Apart of many advantages for the application of assisted reproductive techniques in bovine and the role in biodiversity preservation, bovine oocyte cryopreservation represents the most suitable animal model to study the effects of oocyte vitrification in human. Human oocyte cryopreservation is becoming one of the most demanding options for female fertility preservation. Most of live births from cryopreserved oocytes are from in vivo matured oocytes obtained from standard ovarian stimulation cycles and only few of them are from in vitro matured oocytes. However, recovery of immature oocytes without ovarian stimulation followed by in vitro maturation and cryopreservation is an important alternative for woman who hormonal stimulation is contraindicated or cancer patients who cannot delay their gondotoxic cancer treatment [361].

Nevertheless of the importance and wide application as female fertility preservation option, concerns about the possible effects of oocyte cryopreservation on clinical safety have to be considered. The consequences of oocyte cryopreservation on further early embryo development, as well as on obstetric and perinatal outcomes and long term development should be examined. Oocyte cryopreservation, as well as other assisted reproductive techniques such as in vitro maturation, in vitro fertilization and in vitro culture can perturb epigenetic gene regulation, leading to abnormal phenotypes [362-368]. In this respect, bovine oocyte vitrification is generally considered a suitable animal model for studying the effects of oocyte vitrification in human, because of close similarity between the oocyte structure and embryo development in human and bovine species [369].

In recent decades, numerous attempts have been made to improve cryopreservation outcomes by limiting the oocyte damage. Biological molecules isolated from organisms that live under subfreezing conditions could be used to protect oocytes from cryoinjuries suffered during cryopreservation process [24-27]. In order to survive extremes of temperature some organisms (such as fishes, insects, plants, bacteria and yeasts) have developed unique defenses against their environment, leading to the biosynthesis of large diversity of molecular compounds to sustain life at extreme

conditions [256, 259-263, 265, 370, 371]. Interestingly, biological molecules such as proteins, glycoproteins and carbohydrates can interact with ice in different ways. While some of these molecules act as ice nucleators, others act by preventing ice crystal formation or by inhibiting the growth of existing ice crystals [243, 255, 256, 261, 372, 373]. Rubinsky et al. [241] noticed that antifreeze glycoproteins (AFGPs) from Antarctic fishes seriously improved the viability of immature pig oocytes and 2-cell stage mouse embryos after vitrification, and suggest that antifreeze proteins own the capacity to inhibit ice crystal formation and stabilize the plasma membrane.

Since then, different studies have been performed to assess the effect of AFP on oocyte vitrification [24-27, 242].

Aside from AFP, other molecular compounds such as polysaccharides may exert a cryoprotectant role. The extracellular exudates, composed primarily of exopolysaccharides, that are produced by psychrophilic and ice-dwelling microorganisms have reached a prominent position among the cryoprotectants [260-263, 265, 266, 371, 374, 375].

Exopolysaccharides (EPS) produced by Pseudomonas sp. ID1, a cold-adapted bacterium isolated from marine sediment collected from South Shetland Islands (Antarctica), is a high molecular weight heteropolysaccharide (>2 × $10^6$Da) composed mainly of glucose, galactose and fucose. This biopolymer also contains small amounts of uronic acid, and aminoacids (glutamic acid, aspartic acid, glycine and alanine) [267]. EPS from Pseudomonas sp. ID1 (M1 EPS) is able to preserve its cell structure and maintain its viability after being submitted to freezing temperatures of −20◦C and −80◦C, demonstrating that EPS production is an adaptation mechanism for survival in cold marine environments such as the Antarctica [267]. Pseudomonas sp. ID1 EPS, not only conferred cryoprotection for the strain itself, but also for E. coli cells, suggesting it can thus be applied as an agent for cell cryopreservation, alone or in combination with other cryoprotectants. Several studies have demonstrated that EPS production in bacteria at low temperatures is a cold temperature adaptation mechanism [269, 270, 376, 377]. Other authors have reported that the EPS cryoprotective activity not only benefits the cold-adapted bacterial producer, but also non-producing cells, such as E. coli, suggesting a universal cryoprotectant role for these biopolymers ([271, 272]. There

were no studies that have investigated the effect of EPS on the outcome of oocyte vitrification.

On the basis of the above mentioned, we hypothesized that EPS synthetized by Pseudomonas sp ID1 could protect in vitro matured bovine oocytes from most current damage suffered during cryopreservation and enhance embryo development after warming. Thus, the aim of this study was to examine whether the addition of M1 EPS, produced by Pseudomonas sp. ID1, to vitrification and warming solutions for in vitro matured bovine oocytes would improve their cryotolerance. In the first experiment, microtubule and chromosome configurations were analyzed by immunocytochemistry after exposure of oocytes to various concentrations of M1 EPS in vitrification and warming solutions. In the second experiment, oocytes were treated with different concentrations of M1 EPS in vitrification and warming solutions fertilized and cultured up to the blastocyst stage. Finally, differences in terms of gene expression on blastocysts obtained from oocytes exposed to different concentrations of M1 EPS during vitrification-warming process were analyzed by RT-PCR.

## 6.3. Materials and methods

### 6.3.1. Chemicals and supplies

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) unless otherwise stated.

### 6.3.2. Bovine oocyte collection and in vitro maturation

The methods used for the in vitro maturation of the oocytes have been described elsewhere [316], with some modifications. Briefly, ovaries from slaughtered cows were transported from a local abattoir to the laboratory in phosphate buffered saline (PBS) at 35-37ºC. Cumulus oocyte complexes (COCs) were obtained by aspirating 2–10 mm follicles. Only COCs with three or more layers of cumulus cells and a homogeneous cytoplasm were selected to be matured in vitro. After three washes in modified PBS (PBS supplemented with 36 mg/mL pyruvate, 50 mg/mL gentamicin and 0.5 mg/mL bovine serum albumin (BSA)), groups of up to 20 COCs were placed in 100 µL-drops of maturation medium and cultured for 24 h at 38.5 ºC in a 5% $CO_2$ humidified air atmosphere. The maturation medium was comprised of TCM-199 supplemented with 10% (v/v) FCS, 10 ng/mL epidermal growth factor and 50 mg/mL gentamicin.

### 6.3.3 Polysaccharide M1 supplementation

Pseudomonas sp. ID1 cells were removed from culture by centrifugation (6000 rpm, 25 min, 4◦C) in order to recover the exopolysaccharide. The supernatant free of cells was reserved and pellets were washed with a Ringer solution (Scharlau, Scarlab S.L, Barcelona, Spain) for three times and centrifuged at 40,000 × g for 20 min at 4◦C, to remove the EPS adhered to the cell surface. Washing supernatants were pooled with the first culture supernatant previously reserved, and then were submitted to a tangential flow filtration process through 0.22 µm membranes (Millipore) (Carrión O., et al 2015). The filtrate was subjected to dialysis with sterile distilled water 1:10 (v/v) through 10,000 Da membranes (Millipore) to remove salts, pigments and other components of the culture medium and to obtain the EPS in a concentrated and purified form. Finally, the purified EPS was freeze-dried [267]. It was stored in hermetic sealed flasks in a fresh and dry place.  The working solution of M1 EPS was prepared by adding 100 mg of CLC to 10 mL of HEPES-buffered TCM-199 (10mg/mL) at 37ºC and mixing vigorously. 1mL aliquots of working solution were stored at -20ºC until use. Different

concentrations of M1 EPS (0.001mg/mL, 0.01mg/mL, 0.1 mg/mL and 1mg/mL) solution were added individually to equilibrium, vitrification, warming and dilution solutions after slowly rewarming the frozen M1 EPS and thoroughly mixing to ensure homogenous dilution.

### 6.3.4. Oocyte vitrification and warming

The vitrification-warming procedure was essentially as described by [37]. MII oocytes were vitrified in equilibrium solution and vitrification solution, supplemented with or without M1 polysaccharide during the whole vitrification and warming procedure.

#### Vitrification protocol

The holding medium (HM) used to formulate the vitrification-warming solutions consisted of H-TCM-199 supplemented with 20% (v/v) FCS. Partially denuded oocytes were transferred into equilibration solution (ES) consisting of 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethylsulfoxide (DMSO) in HM for 8 min. Subsequently, oocytes were moved to the vitrification solution (VS) containing 15% (v/v) DMSO, 15% (v/v) EG and 0.5 M sucrose dissolved in HM. After incubating for 30–40 s, oocytes (up to 6) were loaded onto the cryotop, almost all the solution was removed to leave only a thin layer covering the oocytes and plunged into liquid nitrogen. The entire process from exposure in VS to plunging was completed with less than 90 seconds.

#### Warming protocol

Vitrified oocytes were warmed by directly immersing the cryotop into the warming solution containing 1 M sucrose dissolved in HM for 1 min. Afterwards, the recovered oocytes were transferred to the dilution solution containing 0.5 M sucrose dissolved in HM for 3 min. Next, the oocytes were incubated in HM for 5 min. After a final rinse in HM for 1 min, oocytes were transferred to the maturation medium at 38.5ºC in humidified air with 5% $CO^2$ for approximately 1hour – 1hour and a half, before being used for further experiments.

### 6.3.5. Oocyte immunohistochemistry and laser-scanning confocal microscopy

After in vitro maturation, oocytes were denuded of cumulus cells by gentle pipetting in PBS before immunostaining for tubulin and chromatin. Fresh in vitro-matured oocytes were used as controls to determine normal patterns of microtubule and chromatin

organization. Samples of oocytes from the experimental groups were fixed in 4% (w/v) formaldehyde PBS (30 min, 38.5ºC), permeabilized using Triton X-100 (2.5% (v/v) in PBS) for 15 min, and then immunostained for tubulin and chromatin as described elsewhere [208]. The fixed oocytes were incubated with the anti-α-tubulin monoclonal antibody (Molecular Probes, Paisley, UK) (1:250) overnight, followed by incubation with the anti-mouse IgG antibody Alexa Fluor 488 (Molecular Probes, Paisley, UK) (1:5000) for 1 h. Between incubations, the oocytes were washed three times in pre-warmed PBS for 5 min. Fixed and stained oocytes were mounted on poly-L-lysine treated coverslips fitted with a self-adhesive reinforcement ring in a 3-µl drop of Vectashield containing 125 $ng.ml^{-1}$ of 4',6-diamidino-2-phenylindole (DAPI) (Vectorlabs, Burlingame, CA), and flattened with a coverslip. The preparation was sealed with nail varnish and stored at 4ºC protected from light until observation within the following 2 days. Meiotic spindle configuration was assessed under an epifluorescence microscope (Axioscop 40FL, Carl Zeiss, Germany). The criteria used to classify chromosome and microtubule distributions have been described elsewhere (Albarracin et al. 2005). Briefly, the meiotic spindle was defined as normal when the classic symmetrical barrel shape was observed, with chromosomes aligned regularly in a compact group along the equatorial plane. In contrast, abnormal spindles showed disorganized, clumped, dispersed, or unidentifiable spindle elements and chromatin was abnormally organized (showing clustering or dispersal from the spindle centre).

### 6.3.6. In vitro fertilization and embryo culture

In vitro matured oocytes (fresh and vitrified/warmed) were in vitro fertilized at 38.5ºC in a 5% $CO^2$ atmosphere. Briefly, frozen–thawed spermatozoa from Asturian bulls (ASEAVA, Llanera, Asturias, Spain) of proven fertility were used in all the experimental procedures. High motility and good morphology spermatozoa were obtained by centrifuging frozen–thawed sperm for 10 min at 300 x g at room temperature on a discontinuous gradient composed by 1 ml of 40% and 1 ml of 80% Bovipure (Nidacon Laboratories AB, Göthenborg, Swede) according to the manufacturer´s specification. Viable spermatozoa collected from the bottom were washed with 3ml of Boviwash (Nidacon International, Gothenburg, Sweden) and pelleted by centrifugation at 300 x g for 5 min. Spermatozoa were counted in a Neubauer chamber and diluted in an appropriate volume of fertilization medium (Tyrode's medium supplemented with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM

Na-pyruvate, 6 mg/mL fatty acid-free BSA and 10 mg/mL heparin–sodium salt) to a final concentration of 1 x $10^6$ spermatozoa/mL. 100-µL droplets of diluted sperm were made under mineral oil and 20 oocytes/droplet were co-incubated at 38.5 °C, 5% $CO_2$ and high humidity.

After 18–20 h, the presumptive zygotes were stripped of remaining cumulus cells by vortexing and cultured in 100-µl drops of synthetic oviductal fluid (SOF) (Caisson Labs, Smithfield, UT) (Holm, et al. 1999) supplemented with BSA (0.85%, w/v), 88.6 µg/ml sodium pyruvate, non-essential amino acids (2%, v/v), essential amino acids (1%, v/v), 0.1% gentamicin and 2% serum under mineral oil. BSA (15%, w/v), FCS (2%, v/v) under mineral oil 38.5°C in 5% $CO_2$, 5% $O_2$, 90% $N_2$ for 8 days.

Cleavage rates were recorded at 48 hpi and the number of blastocysts was determined on day 7 and 8 post-insemination. Day 8 embryos were classified according to the degree of blastocoel expansion into three groups according to [337]: (1) non-expanded blastocysts: blastocoel volume less than one-half of the total volume of the blastocyst; (2) expanded blastocysts: blastocoel volume more than one-half of the total volume of the blastocyst; (3) hatching or hatched blastocysts: expanded blastocyst with an opened zona pellucida or lacking the zona pellucida.

### 6.3.7. RNA extraction and quantitative real-time PCR analysis (qPCR)

The analysis of gene expression was performed in day 8 blastocysts collected from the 6 different treatment groups. Blastocysts were washed three times with PBS Dulbecco's supplemented with PVA at 38.5 °C and then pipetted into 0.5 ml micro tubes. Immediately, tubes were plunged into liquid nitrogen and stored at −80 °C until processed further.

First, poly-(A)-RNA was extracted from blastocysts in pools of five using Dynabeads mRNA Direct Extraction Kit (Dynal Biotech, Oslo, Norway), following the manufacturer's instructions with minor modifications [378].

For poly-(A)-RNA extraction, each pool of blastocysts were lysed in 50 µl of Lysis buffer for 5 minutes with gently pipetting, and the fluid lysate was then hybridized with 10 µl pre-washed beads for 5 minutes with gently shaking. After hybridization, poly-(A)-RNA-bead complexes were washed twice in 50 µl of Washing buffer A and two

more times in 50 µl of Washing buffer B. Next, the samples were eluted in 16 µl of Elution buffer (Tris HCl) and heated to 70 °C for 5 minutes. Immediately after extraction, 4 µl of qScript cDNAsupermix (Quanta Biosciences; Gaithersburg, MD, USA) were added and the Reverse Transcription (RT-PCR) reaction was carried out using oligo-dT primers, random primers, dNTPs and qScript reverse transcriptase. The RT-PCR reaction was performed for 5 minutes at 25 °C, followed by 1 h at 42 °C to allow the RT-PCR of mRNA and 10 minutes at 70 °C to denature the RT enzyme. After RT-PCR, cDNA was diluted with 25 µl of Tris HCl (elution solution).

Quantification of relative abundance of mRNA transcripts was performed by the qPCR method using a 7500 Real Time PCR System (Applied Biosystems, Foster City, California, USA). The qPCR reaction mix contained 10 µl of Fast SYBR Green Master Mix (Applied Biosystems, Foster City, California, USA), 2 µl of forward and 2 µl of reverse primers (Life Technologies, Madrid, Spain) and 2 µl of cDNA template. Nuclease-free water was added up to a final volume of 20 µl. The PCR amplification was carried out for one cycle of denaturation at 95 °C for 10 minutes, 45 cycles of amplification with denaturation step at 95 °C for 15 seconds, annealing step for 1 minute at 60ºC, (the appropriate annealing temperature of primers), and extension step at 72 °C for 40 seconds. Fluorescence data were acquired during the 72 °C extension steps. To verify the identity of the amplified PCR product, melting curve analysis and gel electrophoresis (in a 2% agarose gel containing 0.6 µg/mL ethidium bromide) were performed. The melting protocol consisted of heating samples from 50 to 95 °C, holding at each temperature for 5 seconds, while monitoring fluorescence. In each run there were three technical replicates from each of the three biological replicates per individual gene. Furthermore, for each probe set a negative control for the template and a negative control for the reverse transcription were also amplified by PCR in order to ensure that no cross-contamination occurred.

Quantitative PCR analysis of day 8 blastocysts with seven candidate genes (BAX, BCL2-like 1; DNMT3A; UBE2A; SCLC2A3; KAT2A; HDAC1) was performed in comparison with the glyceraldehide-3-phosphate dehydrogenase (GAPDH) gene and H2A histone family member Z (H2AFZ) (endogenous control genes) (Table 6.1). The comparative threshold cycle (Ct) method was used to quantify relative gene expression levels and quantification was normalized to the endogenous control (housekeeping

(HK) genes: GADPH and H2AFZ. Fluorescence data were acquired after each elongation step to determine the threshold cycle for each sample. The threshold cycle, which is set in the log-linear phase, reflects the PCR cycle number at which the fluorescence generated within a given reaction is just above background fluorescence. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative Ct method, the ΔCt value was determined by subtracting the mean between GAPDH and H2AFZ Ct value for each sample from the Ct value of each target gene of the sample for each replicate separately. Calculation of ΔΔCt involved using the ΔCt value from blastocysts derived from non-vitrified oocytes (i.e fresh control) as an arbitrary constant to subtract from all other ΔCt sample values. Fold differences in relative transcript abundance were calculated for target genes assuming an amplification efficiency of 100% and using the formula $2^{-(\Delta\Delta Ct)}$. Primer sequences, amplicon size and GenBank accession numbers for each gene are provided in Table 6.1. The efficiency of primer amplification was 100%. Non-template controls were not amplified or returned a Ct value 10 points higher than the average Ct value for the genes.

**6.3.8. Experimental design**

Three experiments were conducted as three (Experiments 1 and 3) or at least nine (Experiment 2) replicates.

**6.3.8.1. Experiment 1. Effects of M1 EPS supplementation during vitrification and warming on the meiotic spindle status of in vitro-matured bovine oocytes.**

After 21 h of in vitro maturation, sets of 546 heifer oocytes and 405 cow oocytes were randomly allocated to six groups (A1–A6, B1-B6). Oocytes from groups A2–A6 and B2-B6 were vitrified and warmed in vitrification and warming solutions containing 0 mg/mL, 0.001mg/mL, 0.01mg/mL, 0.1 mg/mL and 1mg/mL M1 EPS. Oocytes in group A1 and B1 non-vitrified served as controls. After vitrification-warming, oocytes were left to recover for 1 h in maturation medium. Subsequently, all oocytes were fixed in 4% (v/v) PFA and immunostained to examine meiotic spindle status.

#### 6.3.8.2. Experiment 2. Effects of M1 EPS supplementation during vitrification and warming on the developmental competence of in vitro-matured bovine oocytes.

835 heifer oocytes and 1062 cow oocytes were randomly allocated to six groups: C1 and D1, comprised of heifer and cow oocytes matured in vitro for 24 h; C2 and D2, of heifer and cow oocytes vitrified and warmed by the cryotop method without M1 EPS supplementation; C3-C6 and D3-D6, of heifer and cow oocytes vitrified/warmed by the cryotop method supplemented with 0.001mg/mL, 0.01mg/mL, 0.1 mg/mL and 1mg/mL M1 EPS, respectively. Oocytes in groups C2-C6 and D2-D6 were allowed to mature for an additional 1 h – 1 h 30 in maturation media after warming. After maturation, the oocytes were fertilized and at 18-20 hpi, presumptive zygotes survival were analyzed and survived zygotes were placed in culture drops. Cleavage rates were determined at 48 hpi and blastocyst rates recorded on Day 7 and 8.

#### 6.3.8.3. Experiment 3. Effects of M1 EPS supplementation during vitrification and warming of in vitro-matured bovine oocytes on the gene expression of the resulting blastocysts.

Day 8 blastocysts from experiment 2 were harvested for RNA extraction and qPCR analysis (three replicates).

### 6.3.9. Statistical analysis

A statistical package (IBM SPSS Version 21.0 for Windows; IBM Corp.; Chicago, Illinois, USA) was used to evaluate all data. First, data were checked through Shapiro-Wilk and Levene tests for normality and homogeneity of variances, respectively. The significance of differences in chromosome and microtubule configurations, cleavage rates and blastocyst yield were analysed by a two-way analysis of variance ANOVA followed by Sidak's test for pair-wise comparisons. Data that were not normally distributed were compared using the Kruskal–Wallis test. The significance of differences on the relative abundance of the transcrips between blastocysts from vitrified oocytes were analyzed by Kruskall-Wallis test followed by Mann-Whitney.

Data are expressed as means ± standard error of the mean (SEM). The level of significance was set at $P \leq 0.05$ in all cases.

**Table 6.1. Primers used for reverse transcription–quantitative polymerase chain reaction**

| NCBI official name (gene symbol) | Sequence (5' to 3') | Amplicon size (pb) | GenBank accession no. |
|---|---|---|---|
| Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | F: AGTCCACTGGGGTCTTCACTAC | 243 | NM_001034034.2 |
| | R: CAGTGGTCATAAGTCCCTCCAC | | |
| H2A histone family, member Z (H2AFZ) | F: GCGTATTACCCCTCGTCACTTG | 227 | NM_174809.2 |
| | R: GTCCACTGGAATCACCAACACTG | | |
| BCL2 associated X apoptosis regulator (BAX) | F: GAGAGGTCTTTTTCCGAGTGGC | 237 | NM_173894.1 |
| | R: TGTCCCAAAGTAGGAGAGGAG | | |
| BCL2-like 1 (BCL2-L1) | F: CCACTTAGGACCCACTTCTGAC | 188 | BC147863.1 |
| | R: GGGTGCTTCCTACAGCTACAGT | | |
| DNA methyltransferase 3 alpha (DNMT3A) | F: CCTCAGCTCCCCCTACTTATTC | 199 | NM_001206502.1 |
| | R: AGCTGTGAGCTTACTCCTGAGC | | |
| Solute carrier family 2 member 3 (SLC2A3) | F: TTCAGCTCAGGAGAGACCAAG | 237 | NM_174603.3 |
| | R: TGTACGGAAGTGAAGGAGGAG | | |
| K(lysine) acetyltransferase 2A (KAT2A) | F: AGGATGTGGCTACCTACAAGG | 190 | XM_015468132.1 |
| | R: GCACCAGCTTGTCCTTCTCTAC | | |
| Histone deacetylase 1 (HDAC1) | F: CTGAGGAGATGACCAAGTACC | 167 | NM_001037444.2 |
| | R: CCACCAGTAGACAGCTGACAGA | | |
| Ubiquitin-conjugating enzyme E2A (UBE2A) | F: GTCTACTCTGGGGTGGTGCTAA | 155 | XM_005887215.2 |
| | R: CCTCCTGCAACCTCTTGAAGTC | | |

## 6.4. Results

### 6.4.1. Experiment 1. Effects of M1 EPS supplementation during vitrification and warming on the meiotic spindle status of in vitro-matured bovine oocytes

**Spindle configuration of in vitro matured prepubertal heifer oocytes vitrified and warmed in media supplemented with M1 EPS**

Table 6.2 details the effects of M1 EPS supplementation during vitrification and warming on meiotic spindle configurations. Significantly higher rates of oocytes exhibiting an MII spindle were recorded in the control group (untreated, non-vitrified oocytes) compared with the heifer oocytes vitrified with 0.001 mg/mL

Significantly higher rates of heifer oocytes exhibiting a normal configuration spindle were recorded in the control group (non-vitrified oocytes) compared with the remaining vitrified oocyte groups. However only heifer oocytes vitrified and warmed with non-supplemented M1 EPS media showed higher percentages of decondensed or absent chromosomes and microtubules compared with control group.

**Spindle configuration of in vitro matured adult cow oocytes vitrified and warmed in media supplemented with M1 EPS**

Table 6.3 details the effects of M1 EPS supplementation during vitrification and warming on meiotic spindle configurations. Significantly higher rates of oocytes exhibiting an MII spindle were recorded in the control group (untreated, non-vitrified oocytes) compared with cow oocytes vitrified with 1 mg/mL

However, only cow oocytes vitrified and warmed with 0.1mg/mL M1 EPS showed significantly lower rates of normal spindle configuration compared to the control group.

Heifer oocytes presented lower normal spindle morphology rates than cow oocytes when their vitrification and warming media were supplemented with 0 or 0.01mg/mL M1 EPS. When oocytes were vitrified and warmed in media supplemented with 0 and 1 mg/mL M1 EPS, the percentage of oocytes with decondensed or absent chromosome and microtubules was significantly higher for heifer oocytes than for cow oocytes

**Table 6.2.** Effects of treatment with increasing M1 polysaccharide concentrations in vitrification and warming solutions on the spindle configuration of *in vitro*-matured prepubertal heifer oocytes

| | | | Microtubule morphology (% ± SEM)* | | | Chromosome morphology (% ± SEM)* | | |
|---|---|---|---|---|---|---|---|---|
| **Treatment** | **n** | **Total MII (% ± SEM)** | **Normal** | **Dispersed** | **Decondensed or absent** | **Normal** | **Dispersed** | **Decondensed or absent** |
| **Control (H)** | 88 | 92.7 ± 1.9 [1] | 71.7 ± 5.2 [1] | 12.3 ± 3.2 | 16.1 ± 2.1 [1] | 70.2 ±4.3 [1] | 16.8 ± 4.2 | 13.0 ± 2.4 [1] |
| **0 mg/mL** | 81 | 76.2 ± 4.0 [1,2] | 21.3 ± 3.0 [2a] | 39.8 ± 6.6 | 38.9 ± 3.8 [2a] | 19.5 ± 1.9 [2a] | 41.6 ± 8.2 | 38.9 ± 6.3 [2a] |
| **0.001 mg/mL** | 108 | 68.9 ±5.4 [2] | 31.6 ± 3.8 [2] | 36.6 ± 4.0 | 31.8 ± 6.3 [1,2] | 30.3 ± 4.1 [2] | 37.9 ± 5.2 | 31.8 ± 6.3 [1,2] |
| **0.01 mg/mL** | 86 | 71.1 ± 4.9 [1,2] | 31.8 ± 8.6 [2a] | 32.9 ± 11.2 | 35.4 ± 5.5 [1,2] | 28.0 ± 5.9 [2a] | 40.1 ± 6.5 | 31.9 ± 6.6 [1,2] |
| **0.1 mg/mL** | 87 | 73.4 ± 9.1 [1,2] | 44.7 ±1.7 [2] | 35.4 ± 6.5 | 20.0 ± 6.9 [1,2] | 41.8 ± 1.1 [2] | 40.8 ± 6.8 | 17.4 ± 6.0 [1,2] |
| **1 mg/mL** | 96 | 79.3 ±5.8 [1,2] | 37.7 ± 7.4 [2] | 27.4 ± 6.8 | 34.9 ± 3.7 [1,2a] | 37.7 ± 7.4 [2] | 30.0 ± 6.3 | 32.3 ± 5.1 [1,2a] |

[1,2] Values with different numbers within each column differ significantly (P<0.05) between treatments. [a,b] Values with different letters within each column differ significantly (P<0.05) between age. * Values show the number of oocytes with given morphology as a percentage of the total number of oocytes reaching the MII stage.

**Table 6.3.** Effects of treatment with increasing M1 polysaccharide concentrations in vitrification and warming solutions on the spindle configuration of *in vitro*-matured adult cow oocytes

| | | | Microtubule morphology (% ± SEM)* | | | Chromosome morphology (% ± SEM)* | | |
|---|---|---|---|---|---|---|---|---|
| **Treatment** | **n** | **Total MII (% ± SEM)** | **Normal** | **Dispersed** | **Decondensed or absent** | **Normal** | **Dispersed** | **Decondensed or absent** |
| **Control (C)** | 98 | 95.1 ± 2.1 [1] | 67.7 ± 6.7 [1] | 23.9 ± 4.9 | 8.4 ± 4.8 [1] | 68.1 ± 5.2 [1] | 24.2 ± 2.7 | 7.7 ± 4.1 [1] |
| **0 mg/mL** | 72 | 71.6 ± 1.8 [1,2] | 54.0 ± 6.5 [1,2b] | 29.2 ± 9.1 | 16.8 ± 3.6 [1b] | 55.7 ± 6.1 [1,2b] | 29.0 ± 9.1 | 15.3 ± 3.6 [1b] |
| **0.001 mg/mL** | 47 | 69.1 ±2.4 [1,2] | 50.0 ± 0 [1,2] | 28.6 ± 1.4 | 21.4 ± 1.4 [1] | 50.0 ± 0 [1,2] | 28.6 ± 1.4 | 21.4 ± 1.4 [1] |
| **0.01 mg/mL** | 41 | 86.4 ± 8.1 [1,2] | 57.0 ± 4.1 [1,2 b] | 17.2 ± 0.5 | 25.8 ± 3.6 [1] | 60.0 ± 1.1 [1,2b] | 19.9 ± 2.3 | 20.1 ± 3.4 [1] |
| **0.1 mg/mL** | 65 | 78.3 ± 4.2 [1,2] | 37.6 ± 6.9 [2] | 32.7 ± 1.9 | 29.8 ± 6.5 [1] | 35.8 ± 8.3 [2] | 44.5 ± 8.1 | 19.7 ± 0.8 [1] |
| **1 mg/mL** | 82 | 69.2 ± 5.0 [2] | 50.4 ± 11.6 [1,2] | 30.9 ± 5.1 | 18.7 ± 6.7 [1b] | 50.5 ± 13.4 [1,2] | 35.8 ± 6.5 | 13.7 ± 8.1 [1b] |

[1,2] Values with different numbers within each column differ significantly (P<0.05) between treatments. [a,b]Values with different letters within each column differ significantly (P<0.05) between age. * Values show the number of oocytes with given morphology as a percentage of the total number of oocytes reaching the MII stage.

### 6.4.2. Experiment 2. Effects of M1 EPS supplementation during vitrification and warming on the developmental competence of in vitro-matured bovine oocytes.

#### Effects of M1 EPS supplementation during vitrification and warming on the developmental competence of vitrified–warmed prepubertal heifer oocytes

Table 6.4 compared the effects of vitrifying prepubertal heifer oocytes with different concentrations of M1 EPS on vitrification and warming media on the subsequent developmental competence. As indicated in Table 6.4, significantly higher cleavage and blastocyst rates were recorded in the non-vitrified oocyte than in vitrified oocyte groups. Moreover, oocytes vitrified and warmed with 0.01 mg/mL M1 EPS supplementation or without supplementation showed no significant differences in day 8 blastocyst rates when compared with non-vitrified group. No significant differences were observed among vitrified groups.

#### Effects of M1 EPS supplementation during vitrification and warming on the developmental competence of vitrified–warmed adult cow oocytes

Table 6.5 compared the effects of vitrifying cow oocytes with different concentrations of M1 EPS on vitrification and warming media on the subsequent developmental competence. As indicated in Table 6.5, significantly higher cleavage and blastocyst rates were recorded in the non-vitrified oocyte than vitrified oocyte groups. No significant differences were observed among vitrified groups, except for cow oocytes supplemented with 1mg/mLshowed significantly lower day 8 blastocyst rates (2.9%) compared to those supplemented with 0.01mg/mL(9.5%). Blastocyst rates were significantly lower in heifer (12.9%) than in cow control groups (24.3%). Among vitrified groups, 0.001mg/mL group day 7 blastocyst rates were significantly higher in cow (5.3%) than in heifer (1.4%).

**Table 6.4.** Effects of M1 EPS supplementation during vitrification and warming on the developmental competence of vitrified–warmed oocytes from prepubertal heifers.

| Treatment | n | Mean % ± SEM | | | D8 embryo Mean % ± SEM | | |
|---|---|---|---|---|---|---|---|
| | | Cleavage | Blastocyst Day 7 | Blastocyst Day 8 | Non-expanded | Expanded | Hatched |
| **Control** | 278 | 72.4 ± 3.2 | 12.9 ± 2.2 [a] | 13.8 ± 1.6 [a] | 30.1 ± 4.5 | 42.4±7.3 | 27.5±9.6 |
| **0 mg/mL** | 156 | 57.1 ± 3.7 | 5.5 ± 1.1 | 6.2 ± 1.4 [1,2] | 75.0 ± 17.1 | 25.0 ± 17.1 | 0 |
| **0.001 mg/mL** | 184 | 44.1 ± 6.0 | 1.4 ± 0.9 [a] | 3.1 ± 1.0 [1,2] | 100 ± 0 | 0 | 0 |
| **0.01 mg/mL** | 158 | 52.0 ± 4.4 | 3.2 ± 0.9 | 5.9 ± 1.9 [1,2] | 66.7 ± 33.3 | 13.3 ± 13.3 | 20.0 ± 20.0 |
| **0.1 mg/mL** | 160 | 51.4 ± 6.5 | 2.2 ± 0.9 | 2.8 ± 0.9 [1,2] | 40.0 ± 24.5 | 60.0 ± 24.5 | 0 |
| **1 mg/mL** | 177 | 52.7 ± 4.2 | 2.2 ± 0.7 | 1.9 ± 0.8 [1,2] | 60.0 ± 24.5 | 40.0 ± 24.5 | 0 |

[1,2] Values with different numbers within each column differ significantly (P<0.05) between treatments. [a,b]Values with different letters within each column differ significantly (P<0.05) between age.

**Table 6.5.** Effects of M1 EPS supplementation during vitrification and warming on the developmental competence of vitrified–warmed adult cow oocytes

| Treatment | | Mean % ± SEM | | | D8 embryo Mean % ± SEM | | |
|---|---|---|---|---|---|---|---|
| | n | Cleavage | Blastocyst Day 7 | Blastocyst Day 8 | Non-expanded | Expanded | Hatched |
| **Control** | 407 | 74.9 ± 4.3 | 23.9 ± 2.0 [b] | 24.3 ± 2.0 [b] | 16.1 ± 6.3 | 44.0±6.1 | 38.8 ±6.6 |
| **0 mg/mL** | 230 | 62 6 ± 3.6 | 3.3 ± 1.0 | 5.6 ± 1.5 [1,2] | 41.7 ± 20.1 | 58.3 ± 20.1 | 0 |
| **0.001 mg/mL** | 179 | 66.2 ± 4.1 | 5.3 ± 1.3 [b] | 5.7 ±1.3 [1,2] | 42.9 ± 17.0 | 35.7 ± 14.3 | 21.4 ± 14.9 |
| **0.01 mg/mL** | 209 | 53.5 ± 5.3 | 5.6 ± 1.2 | 9.5 ± 2.0 [1] | 38.3 ± 14.5 | 40.0 ± 12.7 | 21.7 ± 10.6 |
| **0.1 mg/mL** | 217 | 60.1 ± 6.4 | 3.2 ± 1.6 | 5.7 ± 1.5 [1,2] | 18.8 ± 13.2 | 77.1 ± 13.0 | 4.2 ± 4.2 |
| **1 mg/mL** | 227 | 56.7 ± 5.4 | 1.9 ± 0.7 | 2.9 ± 0.9 [2] | 66.7 ± 21.1 | 33.3 ± 21.1 | 0 |

[1,2] Values with different numbers within each column differ significantly ($P<0.05$) between treatments. [a,b]Values with different letters within each column differ significantly ($P<0.05$) between age.

### 6.4.3. Experiment 3. Effects of M1 EPS supplementation during in vitro matured bovine oocyte vitrification and warming on the gene expression of the resulting day 8 embryos.

When the relative expression levels of the genes BAX, BCL2-like 1, DNMT3A, SLC2A3, UBE2A, KAT2A and HDAC-1 were assessed from *in vitro* produced day 8 blastocysts (Figure x), no significant changes in relative mRNA abundance for SLC2A3, UBE2A and HDAC-1 genes were found between blastocyst from vitrified oocytes regardless of M1 EPS treatment. As can be observed in figure 6.2 the supplementation of oocyte vitrification and warming media with M1 EPS affected the expression levels of some gene transcripts related to apoptotis (BAX, BCL2-like 1 and ratio BAX/BCL2-like 1), the novo methylation (DNMT3A) and histone acetylation KAT2A), depending on the concentration employed.

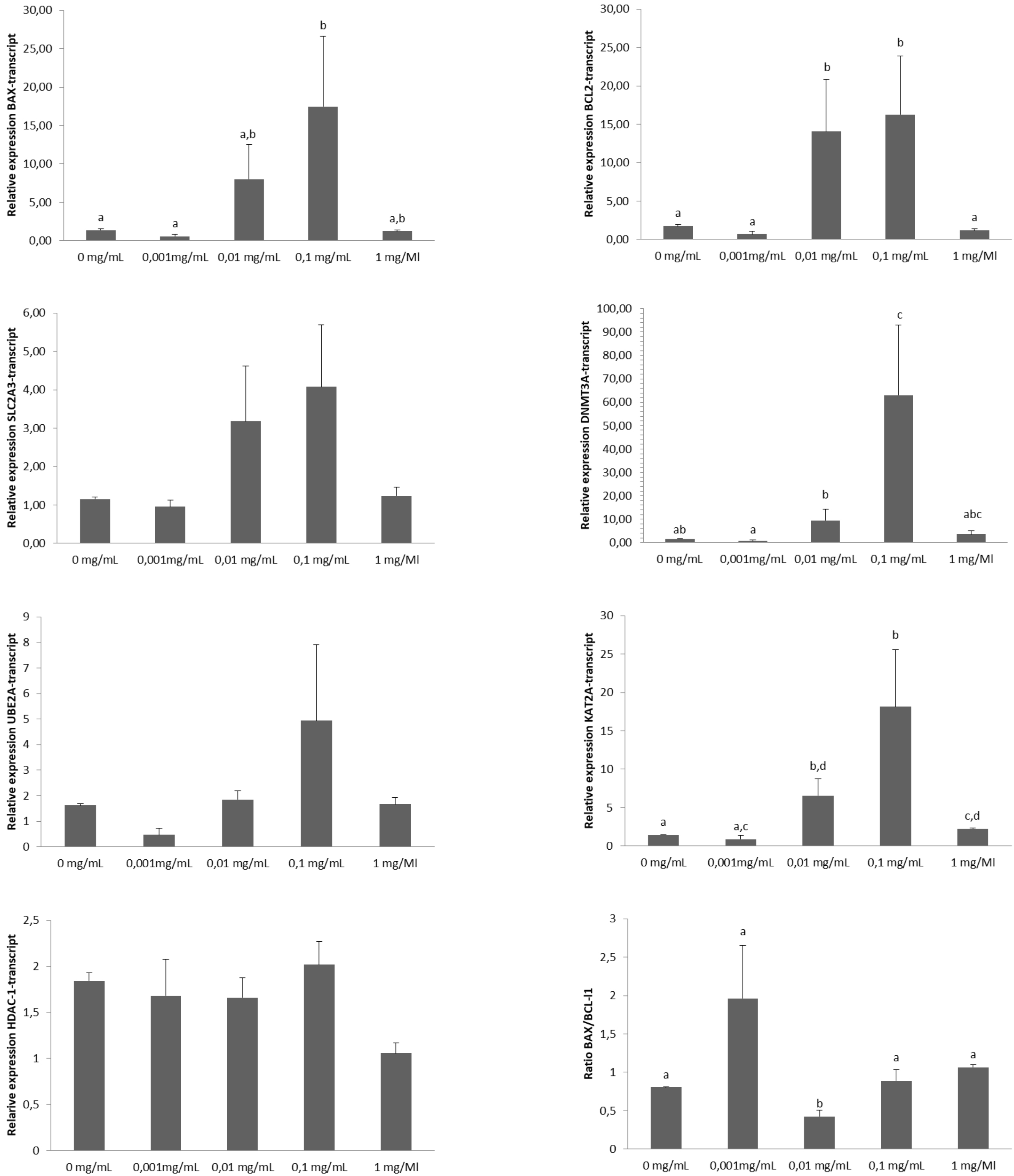
The relative abundance of the proapoptotic BAX transcript in embryos obtained from oocytes vitrified and warmed in media supplemented with 0.1mg/ml M1 EPS was higher ($p<0.05$) than that from 0mg/ml (vitrification control without M1 EPS) and 0.001mg/ml groups. Embryos from 0.01mg/ml and 0.1mg/ml groups showed higher BCL2-like 1 mRNA abundance compared to those from 0mg/ml, 0.001mg/ml and 1mg/ml groups. The lowest BAX/BCL2-like 1 ratio was observed in 0.01mg/ml group. No significant differences in BAX/BCL2-like 1 ratio were observed between all the other groups.

Embryos obtained from oocytes vitrified and warmed with 0.1mg/ml M1 EPS supplementation showed the highest DNMT3A expression levels compared to all other groups. However, DNMT3A expression levels were higher for 0.01mg/ml than for 0.001mg/ml. The relative KAT2A transcript abundance was higher in 0.1 mg/ml and 0.01mg/ml than it was for 0mg/ml and 0.001mg/ml.
Similar expression patterns among gene transcripts seem to be repeated for each vitrification group. The relative expression of all transcripts was upregulated in blastocysts from 0.1mg/ml and 0.01mg/ml groups compared to blastocysts from fresh control (embryos obtained from non- vitrified oocytes), whereas it was downregulated in 0.001mg/ml group compared to fresh control for all transcripts except for HDAC1 and SLC2A3.

Furthermore, gene expression in 1mg/ml and 0.001mg/ml groups remained similar to embryos from oocytes vitrified and warmed without M1 EPS supplementation. Except for the relative abundance of KAT2A which was significantly higher in 1mg/ml group compared to non- supplemented vitrification group (0mg/ml).

Highest gene expression levels were observed in 0.1mg/ml group. However, no significant differences were observed between 0.1mg/ml and 0.01mg/ml groups for the relative expression of any transcript, except for DNMT3A and BAX/BCL2-like 1 ratio transcripts.

**Figure 6.2.** Relative abundance (as mean ± SEM) of BAX, BCL2-like 1, DNMT3A, SLC2A3, UBE2A, KAT2A, HDAC-1 and ratio BAX/BCL2-like 1 transcripts recorded in day 8 blastocysts produced from IVM bovine oocytes vitrified and warmed in media supplemented with different concentrations of M1 EPS



Different letters (a, b,c,d ) mean significant differences ($P < 0.05$) between treatments (IVP blastocysts from IVF oocytes vitrified and warmed in media supplemented with 0mg/mL, 0.001mg/mL, 0.01mg/mL, 0.1mg/mL or 1mg/mL) within a given gene (i.e. BAX, BCL2-like 1, DNMT3A, SLC2A3, UBE2A, KAT2A, HDAC-1 or ratio BAX/BCL2-like 1).

## 6.5. Discussion

The aim of the present study was to examine whether the addition of M1 EPS, produced by Pseudomonas sp. ID1, to vitrification and warming solutions for in vitro matured bovine oocytes would improve their cryotolerance to vitrification, in addition to their in vitro developmental competence.

In an initial experiment we examined the effects of different M1 EPS concentrations on MII spindle structure. Differences on the cryotolerance related to the age of the animal were also analysed, comparing the effects observed in prepubertal heifer and adult cow oocytes. When heifer oocytes were vitrified, lower rates of normal spindle configuration were recorded compared to the control group (non-vitrified oocytes). However, in adult cow oocytes that effect was only observed in oocytes vitrified and warmed with 0.1mg/mL M1 EPS, but not in the other vitrification groups; suggesting that cow oocytes could have higher tolerance to cryopreservation compared to heifer oocytes. In fact, direct comparison showed that when oocytes were vitrified and warmed in media supplemented without M1 EPS and with 1 mg/mLM1 EPS, the percentage of oocytes with decondensed or absent chromosome and microtubules was significantly higher for heifer oocytes than for cow oocytes. Moreover, heifer oocytes presented lower normal spindle morphology rates than cow oocytes when their vitrification and warming media were supplemented with 0.01mg/mLM1 EPS or non-supplemented.

M1 EPS supplementation during oocyte vitrification and warming could have a great benefit for heifer oocytes, based on that only those heifer oocytes non-supplemented with M1 EPS showed higher percentages of decondensed or absent chromosomes and microtubules compared with control group. Instead no differences in percentages of decondensed or absent were observed in all other vitrified groups EPS supplemented compared to control group, non-vitrified oocytes.  .

Higher M1 EPS concentrations seemed to impair spindle configuration in cow oocytes, as 1mg/ml group presented lower percentages of oocytes reaching metaphase II stage and 0.1mg/ml is the only group that showed lower normal spindle configuration

compared to fresh control group. Contrarily, lower M1 EPS had a detrimental effect for heifer spindle organization, as lower percentages of oocytes reaching metaphase II were observed in 0.001mg/ml group and group non supplemented oocytes presented higher decondensed or absent chromosomes and microtubules.

According to our results, the supplementation of vitrification media with 500 ng/mL and 1000 ng/mL antifreeze protein III (AFPIII) during the vitrification of in vitro matured bovine cumulus oocyte complexes showed no differences in the rates of normal spindle organization compared to vitrified oocytes without AFPIII supplementation [252]. Contrarily, vitrification solution supplementation with 1mM antifreeze glycoprotein 8 (AFGP8) seems to reduce significantly the percentages of bovine oocytes with abnormal meiotic spindle organization and misaligned chromosomes compared to non-supplemented group [25].

Previous studies also reported the sensitivity of the oocyte meiotic spindle to cryopreservation was also different depending on the sexual maturity of the donor animal [6, 170]. Succu et al observed increased CPA toxicity in prepuberal oocytes compared to adult oocyte in ovine. They attributed this increased CPA toxicity observed in prepuberal oocytes to a deficiency of molecular and/or structural factors involved in spindle configuration restoring in prepubertal oocyte [170]. In bovine oocytes Albarracin et al [6] also reported different meiotic spindle sensitivity to cryoprotectants between prepubertal heifers and adult cows. But they found that oocytes retrieved from adult cows were more sensitive to exposure to CPAs, while vitrification seemed to have worse effects on heifer oocytes [6].

In the second experiment, the effects of M1 EPS supplementation during vitrification and warming on the developmental competence of in vitro matured oocytes were analyzed.
In recent decades, several studies have been used ice binding proteins or synthetic ice blockers to increase the survival and development after oocyte cryopreservation ([20, 24-27, 241, 242]. Other biomolecules such as lipoproteins or polysaccharides that interact with ice in nature either by triggering ice nucleation or by inhibition of ice nucleation and growth, may exert a cryoprotectant role [256, 261, 266, 371, 379]. Exopolysaccharides (EPS) produced  to sustain live in extreme conditions have reached

a prominent position among the cryoprotectants [[263-265]. However, as far as we are concerned none of them are tested on improving oocyte cryotolerance.

Fish AFP III added as a non-permeable cryoprotectant during oocyte vitrification demonstrated to protect mature [24, 26, 242] and immature mouse oocytes [27] from most current damage suffered during cryopreservation and enhance embryo development after warming. On bovine specie, AFGP8 supplementation during vitrification effectively protects MII-stage oocytes against chilling injury, as well as improves their cryosurvival and subsequent embryonic development [25]. Similarly to our results, recently [252] reported different concentrations (500 and 1000 ng/mL) AFP III into vitrification media did not preserve in vitro matured bovine oocyte meiotic spindle organization against the cryoinjuries and did not improve blastocyst formation. Zhou XL et al [20] reported that addition of ice blockers X-1000 and/or Z-1000, copolymers of polyvinyl alcohol and polyglycerol, in vitrification solution did not affect the survival rate and developmental competence of immature bovine oocytes vitrified and warmed by cryotop method.

Lee HH, et al. [24] compared the effect of adding three AFP from different source on murine oocyte vitrification-warming solutions and concluded that AFPs from bacteria (Flavobacterium frigoris ice-binding protein (FfIBP)), yeast (Glaciozyma sp. ice-binding protein (LeIBP)) and fish (Type III AFP) added to the vitrification and warming solutions individually, improve murine oocyte quality and embryo development.

We examined whether the addition of M1 EPS, produced by Pseudomonas sp. ID1, to vitrification and warming solutions for in vitro matured bovine oocytes would improve their developmental comptence. We did not observed any beneficial effect of M1 EPS supplementation on developmental competence in terms of cleavage and blastocyst rates. Although different EPS concentrations added to vitrification and warming solutions could improve the developmental competence of the resulting embryos, as for example in cow oocytes 0.01mg/ml group presented significantly higher blastocyst rates compared to 1mg/ml (9.5% vs 2.9% day 8 blastocyst rates), this improvement is not significantly ($p>0.05$) compared to non-supplemented vitrified oocytes (5.6%). Nevertheless, the hatchability of those embryos have been improved by the

supplementation of vitrification and warming media with some M1 EPS concentrations, 0.001mg/ml and 0.01mg/ml.

The impairment on in vitro developmental competence on cow oocytes caused by the highest M1 EPS concentration (1mg/ml) coincides with the effects observed on meiotic spindle organization. However, that coincidence was not observed in heifer oocytes. According to previous reports [380-383], blastocyst rates in this study were significantly lower in heifer (12.9%) than in cow control groups (24.3%). However, among vitrified groups, this difference was only observed on 0.001mg/mL group day 7 blastocyst rates that were significantly higher in cow (5.3%) than in heifer (1.4%). The possible mechanisms proposed in different studies for the reduced developmental potential in prepubertal oocytes include, incomplete cytoplasmatic maturation, impaired protein synthesis and altered metabolism compared to those from adult animals [380, 381, 384-386]. Some reports attributed that deficient developmental capacity of prepubertal oocytes to the abnormal cytoplasmic maturation of these oocytes (Damiani P et al. 1996). Nevertheless, in the present study, similar percentages of metaphase II and normal spindle configuration were observed between heifer and cow untreated oocytes.

According to other reports, bovine oocyte vitrification impair further embryo development, reflected in the lower blastocyst rates observed in vitrified oocytes compared to control group [20, 22, 37, 230].

Further research would be needed to clarify the exact mechanism of action of the M1 EPS to cryoprotect bacteria cells, in order to optimize their application for oocyte cryopreservation. EPS produced by Pseudomonas sp. ID is a high molecular weight heteropolysaccharide (>2 × $10^6$Da) composed mainly of glucose, galactose and fucose. Polysaccharides in the nature interact with ice either by triggering ice nucleation or by inhibition of ice nucleation and growth [262]. For example, a high molecular weight polysaccharide from *Bacillus thuringiensis* was found to inhibit ice nucleation [260]. Another polysaccharide with a fatty acid component from the Alaskan beetle *Upis ceramboides* exhibited a pronounced thermal hysteresis due to ice binding [261]. Boreal birch pollen polysaccharides showed ice-nucleating as well as ice-binding ‘antifreeze’ activities [262]. This biopolymer also contains small amounts of uronic acid, and

aminoacids (glutamic acid, aspartic acid, glycine and alanine) [267]. Similar to extracellular polysaccharides from Colwellia psychrerythraea 34H, endowed with ice recrystallization inhibition activity [266], the results of chemical and spectroscopic analyses of the purified capsular material from Pseudomonas M1 revealed the presence of amino acids in the composition. In fact, the presence of aminoacids is crucial for the interaction with ice, in several different kinds of AFP [265, 268] and are quite uncommon among the composition of several EPS produced by many marine bacteria [387].

Recently, the improvement in the efficacy of oocyte cryopreservation allows the application of this technique for human fertility preservation. However, apart from well-known structural damage suffered at different cellular levels during cryopreservation, recently some concerns have emerged about the safety of oocyte cryopreservation for the progeny. Although with low incidence [388], some studies reported the association of oocyte cryopreservation with some altered gene expression and some of these changes may originate from epigenetic disorders [95, 235-240, 352, 389-394].

Bovine oocyte vitrification represents a more useful animal model for studying the potential effects of oocyte vitrification on health and development of human embryo than mice [369, 395].

Stress suffered during oocyte vitrification and warming can decrease de reserves and synthesis of maternal factors encoded by maternal-effect genes in the oocyte, which have been proven to play crucial roles in regulating epigenetic marks and maintain DNA imprints during preimplantation development [233].

Bovine embryos produced from vitrified oocytes may have a different gene expression profile compared with those produced from non-vitrified oocytes; this difference could be due to the modifications in epigenetic marks that control gene expression during embryo development. Considering these alterations produced by oocyte vitrification, in the present study we compared the effects of different concentrations of M1 EPS supplementation during in vitro matured bovine oocyte vitrification and warming on the gene expression of the resulting day 8 embryos. Although in general no marked differences on cleavage and blastocyst yield were observed between oocytes vitrified and warmed in media supplemented with different concentrations of M1 EPS or non-

supplemented, differences in relative abundance of some gene transcripts during early embryo development were found.

In this report, the relative abundance of DNMT3A transcripts in blastocysts derived from vitrified oocytes supplemented with 0.1mg/ml M1 EPS showed the highest DNMT3A expression levels compared to all other groups. DNMT3A expression was upregulated in all vitrification groups compared to blastocysts derived from non-vitrified oocytes, except for 0.001mg/ml where it was downregulated. Similarly in a previous study we reported the relative abundance of DNMT3A transcript in morulae derived from immature and in vitro matured vitrified oocytes was significantly higher compared to those morulae derived from fresh oocytes. Signifying that there existed relatively high levels of *DNMT3s* activities during development in vitro for morula and blastocysts derived from vitrified oocytes.  During bovine embryo development, DNMT3A mRNA expression begins at the 2-cell stage and continues to the blastocyst stage [396]. In bovine, it was demonstrated that methylation decreased from the two-cell stage to the 6- to 8-cell stage and increased thereafter up to the blastocyst stage [397].

DNMT3A are expressed from maternal mRNAs stored during oogenesis, and conduct most de novo methylation during oocyte maturation and pre- and post-implantation embryo development periods [353, 398]. In fact, DNMT3A exerts essential functions in the control of development-related gene expression during oogenesis and early embryogenesis, such as regulation of transcription, epigenetic reprogramming, the establishment of both maternal and paternal imprints, X chromosome inactivation, and other cellular functions [[353, 399-402] and it usually causes gene silencing [402].

Higher levels of embryonic methylation that might be caused for the increased expression of DNMT3A could negatively influence the development competence of bovine embryos derived from vitrified oocytes [403]. But the downregulation observed in 0.001mg/ml group could also cause the development of different mutant phenotypes, malformations and syndromes related to impairing gene expression [404]. So, given the role in de novo methylation, any altered level of DNMT3A expression might entail an imprinting problem in embryos from vitrified oocytes. However, further studies need to be carried out on the global levels of DNA methylation and histone acetylation in

blastocysts after oocyte vitrification with M1 EPS addition or not, before meaningful conclusions can be drawn.

However, quantitatively most of our results for DNMT3A are contradictory to Chen et al [240] who described DNMT3A downregulation in bovine blastocysts derived from vitrified oocytes. On the other hand, Cheng et al [239] reported no statistically significant difference in DNMT3A relative abundance in mouse blastocysts derived from vitrified oocytes. Those divergences among experiments into the same species may be due to the different protocols used for the embryo production, that influence on the transcriptional levels of the resulting embryos [273, 405, 406] and to the different methods to assess gene expression profile. The only clear conclusion extracted from those findings is that oocyte cryopreservation has the potential to alter DNMTs expression. This issue should be further analysed to clearly address what is the exact result of the altered DNMTs expression on the oocytes and early embryos because other studies suggested the abnormal expression of DNMTs results in methylation defects and aberrant embryo development [239, 240, 352].

Although the mechanism behind M1 EPS supplementation during oocyte vitrification and warming can change the gene expression in the derived blastocyst is unclear, the loss of mRNAs stored in mature oocytes during vitrification and warming cause epigenetic modification that would inevitably have implications for epigenetic inheritance during early embryonic development [235-240]. Thus, mRNA abundance decreased in oocytes after thawing, causing potential disturbance of the imprinting in the preimplantation embryo. The inheritance of genomic imprints was strictly due to the embryonic maintenance of DNA methylation [407]. However, Cheng et al [239] demonstrated that during preimplantational embryo development expression of some DNMTs altered during oocyte vitrification could generally return to normal levels in blastocysts. In the same way a change in DNMT3b relative expression was also observed in ovine blastocysts produced from vitrified oocytes, that were increased at morula stage compared to fresh control and then decreased at blastocyst stage [352].
This study in bovine oocyte vitrification could serve as a good model to study the effects of oocyte vitrification on DNMT3A expression in humans, because DNMT gene expression is largely conserved among nonrodent species [408].

Besides from DNA methylation, histone modification by acetylation and deacetylation is another mechanism of epigenetic regulation [409] and is involved in genome reprograming. These functions are controlled by the actions of two families of enzymes, histone acetyltransferases and histone deacetylases. Posttranslational modifications by acetylation of specific lysine residues on N-terminal portion of histones composing the nucleosome, neutralizes the positive charges of the histone, diminishing their affinity for DNA and, thereby, increasing the accessibility of transcriptional factors to the DNA. This effect is reversed by histone deacetylation, which implies gene repression.

In this study the gene expression of the acetyltransferase KAT2A was significantly upregulated in blastocysts derived from oocytes vitrified in media supplemented with 0.01mg/ml, 0.1mg/ml and 1mg/ml M1 EPS. No significant differences for blastocysts from 0.001mg/ml supplemented and non-supplemented groups were observed compared to fresh control. Contrarily Chen et al 2016 found KAT2A was significantly reduced in blastocysts from vitrified group compared to fresh control.

KAT2A are one of the acetyltransferases responsible to increase the acetylation levels of histone H3 at lysine 9 (acH3K9). Mc graw et al 2003 suggested that maternal mRNA KAT2A could be more important during the early stages of development prior to blastocyst stage to produce the nuclear KAT2A (GCN5) protein which will be necessary to decondensate the chromatin and required further in development, after the genome activation [410].

As demonstrated, the pattern of KAT2A expression is different from vitrified groups. The excessive upregulation of the acetylase KAT2A gene expression in 0.1mg/ml and 0.01mg/ml groups could lead to some epigenetic defects not reflected in the blastocyst rates. Aberrations in transcript levels of histone-modifying genes during preimplantation development might have effects on the embryonic development ([411].

Oocyte vitrification altered histone acetylation levels in mouse [235, 412] and pig [413] oocytes. However, further studies to determine the histone acetylation levels in blastocysts obtained from oocytes vitrified with M1 EPS supplemented media or not, before meaningful conclusions can be drawn.

No significant differences were found in the relative expression of the deacetylase HDAC1-transcript between vitrified groups and with blastocyst from non-vitrified

oocytes in the current study. Concordantly with our results, Shirazi et al [352] reported no differences in the expression level of HDAC-1 in ovine blastocysts produced from vitrified GV oocytes. Contrarily, in Chen et al [240] study, HDAC-1 relative abundance was downregulated in bovine blastocysts from vitrified MII oocytes. In bovine, the transition from oocyte to embryonic genome is highly dependent on HADC1expression [410].

Chen et al [240] found DNMT3A, HDAC1, KAT2A downregulation in blastocysts derived from vitrified oocytes, this differences could be due to differences in oocyte origin, in vitro culture conditions, they used ICSI for fertilization whereas we used IVF, and they used early embryos while we used expanded and hatched blastocysts (or day 8 blastocysts). Moreover, some differences in the PCR analysis may also lead to some differences.

Concerning about genes related with apoptosis analyzed in blastocyst in this study, BCL2 is considered an anti-apoptotic gene and *BAX* is pro- apoptotic and its overexpression accelerates cellular apoptotic pathways [414, 415]. In several studies, the mRNA expression levels of *BAX* are used to evaluate embryos quality developed in vitro [416-419]. Considered individually, in this study blastocysts derived from vitrified oocytes followed the same expression pattern for BAX and BCL2 that was observed for all the other above mentioned transcripts. Higher expression levels were observed in blastocysts derived from vitrified oocytes supplemented with 0.1mg/ml and 0.01mg/ml M1 EPS and no significant differences were observed between remainder groups. Nevertheless, this situation changes if the relation between these two transcripts is considered, the ratio bax/bcl2. Bax/Bcl2 ratio indicates the tendency of a cell to undergo apoptosis [420]. According to Yang and Rajamahendran [414] good quality embryos have a greater concentration of *BCL2* protein than that of *BAX* protein, whereas in lower quality embryos there is more *BAX* than *BCL2*. In our study data from ratio BAX/BCL2-like 1 showed that blastocysts obtained from oocytes vitrified with 0.01mg/ml M1 EPS supplementation had higher expression levels of BCL-2 compared to BAX ($P<0.05$), then this concentration could be considered the treatment that obtain blastocyst with the highest quality based on this assumption. Remaining groups did not presented differences compared to blastocysts obtained from oocytes non-vitrified. In relation to this, we could infer that although oocyte vitrifcation cause a marked decrease on blastocyst rates, in general oocyte vitrification did not impair the blastocyst quality.

Embryos developed intro blastocyst might have adjusted some of their epigenetic alterations and been finally allowed to pass through the early cleavage stages.

It is important take into account, that the genetic aberrations that block development in early stages prior to blastocyst stage will not be observed in those blastocysts analysed by rtPCR. Embryos with aberrant gene expression incompatible with embryo development was already sift out, they dead in previous sages before to reach the blastocyst stage. Then, these alterations will not be observed at blastocyst stage. The analysis of the gene expression in those blastocysts derived from vitrified oocytes could be useful to understand the epigenetic effects of these changes for the progeny. It is important for calf industry to avoid economic loses derived from transferring inviable embryos, but is especially important in improving the methods of oocyte cryopreservation for preserving fertility in human.

The lower developmental competence (lower blastocyst rates) observed in the embryos produced from vitrified oocytes could be, at least partially explained by the disruption of the physiological epigenetic profile of the oocyte due to the stress to which the oocytes suffered during vitrification and warming.

Although cleavage rate on Day 2 after IVF did not differ significantly between vitrified groups and fresh control group, few of those cleaved embryos from vitrified group reached blastocyst stage compared to those from control group. It could reflect that although the efficiency of the vitrification method allowed development until the cleavage of oocytes, those cleaved embryos had reduced potential to develop into blastocysts. It was probably associated with aberrant alterations in expression of some genes with key roles for important developmental events during early embryonic development, such as epigenetic reprograming.

In conclusion, M1 EPS supplementation did not have a clear effect on increasing the cryotolerance of bovine oocytes. Supplementation with various M1 EPS concentrations during vitrification and warming did not induce adverse changes in the spindle of bovine oocytes. Spindle configuration from heifer oocytes was more severely impaired by vitrification compared with cow oocytes. Besides, heifer oocytes seem to receive a greater benefit from EPS supplementation during vitrification and warming than cow oocytes. The data from present study indicated that developmental competence from vitrified oocytes seem to be impaired by changes in gene expression of some gens

related with epigenetics (DNA methylation and chromatin acetylation) and blastocyst quality (BAX, BCL2). Further experiments are needed to clarify whether M1 EPS supplementation during bovine oocyte vitrification could have some protective effect for damage suffered during cryopreservation.

# CHAPTER VII

## General discussion

Vitrification is the most sought cryopreservation method for animal oocytes of medical, genetic, and agricultural importance. The Cryotop method has been demonstrated to be a more efficient approach for bovine oocyte vitrification [89]. However, the practical use of cow oocyte vitrification is limited because of the specially high sensitivity of these oocytes to cooling and cryopreservation procedures and the low rates of blastocyst production that have been observed after warming compared with non-vitrified controls [96]. The high concentrations of cryoprotective agents used for vitrification as well as chilling injury and osmotic stress may damage the oocyte at structural and molecular levels [113, 127, 161, 394, 421] and consequently impair the subsequent embryo development. In the last few decades, several approaches have been used to improve the efficiency of cryopreservation protocols, through varying concentration and types of cryoprotectants, studying different times and temperature, examining different samples containers, or adding additives, however blastocyst yield has not been significantly improved.

For this purpose, in this thesis we have investigated different strategies to ameliorate the developmental competence of bovine oocytes after vitrification and warming processes. In general we have observed that induced osmotic stress tolerance prior to vitrification, the increase of cholesterol content into ooplasm prior to vitrification and the addition of ice-binding polysaccharides to vitrification and warming solutions, have no effect on oocyte cryotolerance. Thus the aim of this general discussion is to integrate all the results reported in this thesis to highlight the most interesting findings.

The results of earlier investigations suggest that sublethal stress, properly defined and applied appropriately before manipulation, could improve the cryotolerance of oocytes through adaptation mechanisms enhancing their subsequent stress tolerance [46]. Thus, pretreatment with high hydrostatic pressure or osmotic, heat or oxidative stress seems to improve morphological intactness, fertilizing ability and developmental competence after various in vitro and in vivo procedures, such as cryopreservation, parthenogenic activation or somatic cell nuclear transfer [46, 278]. In the pig, osmotic stress produced by the exposure of oocytes to increased concentrations of sodium chloride, sucrose or trehalose before manipulation has been reported to improve both cryotolerance to vitrification and developmental competence [47, 48]. However, to the best of our knowledge, how the effect of temporarily increased NaCl or sucrose concentrations may

influence cryotolerance and developmental competence of bovine oocytes had not yet been examined. For this reason, we designed an experiment to examine whether pretreatment of IVM cow oocytes with elevated NaCl or sucrose concentrations would improve their cryotolerance to vitrification, in addition to their in vitro developmental competence. To define a suitable sublethal stress treatment, in an initial experiment we examined the effects of solutions with different osmolality on MII spindle structure. The cytotoxicity tests revealed a significantly higher percentage of oocytes with abnormal spindle structures, including disorganized or decondensed microtubules or chromosomes, after exposure of oocytes to the NaCl or sucrose solutions. These effects were dose dependent, because normal spindle rates decreased as the osmolality of the pretreatment solution increased. Consistent with this finding, Mullen et al. [12] reported that osmolality was a significant predictor of the spindle morphology of human oocytes. Hyperosmotic effects of sucrose solutions at 600, 1200, and 2400 mOsmol resulted in 44%, 44%, and 100% of spindles with an abnormal structure, respectively [12]. In effect, cow oocytes have been observed to behave as a perfect osmometer (i.e. they follow the Boyle–Van't Hoff relationship) over the range 265–800 mOsmol [150].

Despite this, Lin et al. [47, 48] provided strong evidence that pig oocytes osmotically stressed by a transient increase in NaCl, sucrose or trehalose concentrations, had higher developmental rates following cryopreservation, parthenogenic activation or somatic cell nuclear transfer. Nevertheless, osmotic stress has been observed to be harmful to oocytes in several studies, even when similar osmotic pressures were applied for only 10 min [12, 319, 336]. In the present study, 0.25% NaCl (375 mOsmol) had no substantial effects on spindle morphology, yet higher concentrations led to a marked reduction in the number of oocytes showing normal spindle configurations. Similarly, we selected the concentration 2.77% sucrose (375 mOsmol) for the pretreatment of IVM oocytes before vitrification because we observed no adverse effects. This is why the type of initial stress and its intensity are determinants of the expression of stress-related proteins that will help to combat subsequent stress, but this initial stress can also compromise oocyte viability [320]. The difference in the percentage of oocytes with normal microtubule distributions in groups exposed to 375 and 433 mOsmol sucrose solutions (71.78% vs 62.96%, respectively) was not as great as the difference between groups exposed to 375 and 433 mOsmol NaCl solutions (83.35% vs 52.60%, respectively). This could indicate that elevated intracellular NaCl concentrations may

have both osmotic effects and other consequences, like possibly an ionic effect [422-424].

NaCl or sucrose treatment prior to vitrification did not avoid the reduction in the number of oocytes at MII caused by vitrification-warming. Moreover, there were a greater number of oocytes at the anaphase II stage after vitrification, regardless of whether they had been subjected to osmotic pretreatment. Some permeable cryoprotectants used in vitrification solutions, such as DMSO and EG, could parthenogenetically activate IVM oocytes from the sheep [321], pig [322] or buffalo [323]. According to Lin et al. [47], is not known whether the osmotic stress induced by NaCl causes the same effects as CPAs. In the cytotoxicity tests performed in this thesis without vitrification, no significant differences were detected in the number of oocytes at the MII and anaphase II stages between groups exposed to NaCl or sucrose solutions with different osmolalities. However, differences in the percentage of oocytes at the MII stage emerged between control and vitrified oocytes, and the vitrified group had a greater percentage of oocytes at anaphase II. This indicates that **osmotic stress induced by NaCl or sucrose does not seem to cause parthenogenetic activation in IVM cow oocytes,** at least at concentrations used in our study.

We observed no significant effects of NaCl or sucrose pretreatment on normal microtubule configuration rates in vitrified oocytes. Nor were there any effects of NaCl on the rate of detection of normal chromosome configurations. In contrast, the rate of normally organized chromosomes was higher in oocytes treated with sucrose before than in oocytes vitrified without sucrose pretreatment. Therefore, it seems that **pretreatment with 375 mOsmol NaCl or sucrose for 1 hour before vitrification offers some protection against chromosome decondensation,** one of the negative effects of vitrification. Exposure to 375 mOsmol sucrose solution for 1 h had no deleterious effects on cleavage or development to the blastocyst stage of IVM bovine oocytes. However, **cryosurvival or developmental competence were not improved when 375 mOsmol sucrose solution was used before vitrification**, in contrast with observations in pig oocytes [48]. It has been reported that precisely controlled sublethal stress treatment of cells can improve embryo and gamete performance, and consequently enhance subsequent oocyte survival, fertilization and in vitro development [278]. The stress response triggers the induction of heat shock proteins (HSPs), first

detected after heat stress, although later found to be involved in responses to other environmental stressors, including cold temperature, osmotic effect of salts, acid change in pH, high hydrostatic pressure and starvation [46]. HSPs participate in various cell functions, including protein, DNA, and chromatin stabilisation and repair, cell cycle control, redox regulation, energy metabolism, fatty acid/lipid metabolism and the elimination of damaged proteins [281]. The production of HSPs may explain the improved survival after vitrification and developmental competence of pig oocytes after osmotic stress induced by NaCl or sucrose solutions [48]. According to the structure and composition of cow oocytes, the solution we used to pretreat bovine oocytes had a lower osmolality than that used to pretreat pig oocytes, and the **osmolality of this solution was perhaps insufficient to induce the production of adequate amounts of HSP to protect the oocyte from cryodamage**. Thus, it could be speculated that to induce greater stress, bovine oocytes could be pretreated with higher concentrations of NaCl or sucrose but over a shorter period than 1 h. In line with previous observations, the greater the stress (below the lethal dose), the more HSPs are produced and the higher the cryosurvival (Du et al. 2008a). We did not observe the improved developmental competence reported by Lin et al. [47, 48] in pig oocytes. A possible explanation for this could be the different amounts, composition and distribution of lipids [327]. Membrane structure also differs between the two species. Thus, pig oocytes have double the total fatty acid reserve than cow oocytes, and phospholipids contain a higher proportion of polyunsaturated fatty acids than ruminant oocyte phospholipids [153]. These differences could have marked effects on the nature of membranes and may contribute to the different responses of oocytes from the two species to cryopreservation [140]. Cell membrane composition determines the properties of a cell. Cells with more flexible membranes are more permeable to water and intracellular cryoprotectants, and will incur less damage during cryopreservation [298]. Ultrastructural differences between IVM pig and cow oocytes may explain their different developmental competence after treatment with NaCl or sucrose before vitrification. Because of their large intracytoplasmic lipid droplets and other specific properties that hinder successful cryopreservation [20], pig oocytes may benefit from osmotic stress because it permits pre-adaptation of the cell to the subsequent stress of vitrification–warming [46].

In order to increase membrane fluidity and permeability at low temperatures, cholesterol can be added to the plasma membrane, thereby providing an alternative method for increasing oocyte tolerance to cryopreservation. In oocytes, co-incubation of bovine immature [188] or *in vitro* matured oocytes [32] with methyl-β-cyclodextrins loaded with cholesterol (CLC) improved nuclear maturation of oocytes after vitrification but failed to improve their embryo development up to the blastocyst stage. These authors attributed the lack of effect of CLC treatment on improving cryotolerance to the possible abduction of cholesterol contained into cyclodextrins by fatty acid-free albumin and lipoproteins from FCS, avoiding its incorporation into the oocyte. Thus, in order to determine the incubation time at a given concentration at which cholesterol locates mainly at the plasma membrane and whether FCS supplementation interferes in the cholesterol trafficking, we designed an experiment using a new cholesterol fluorescent prove, BODIPY-cholesterol, to **characterize the intracellular trafficking and localization of fluorescently-labeled cholesterol in in vitro matured oocytes incubated with CLC either in a FCS or PVA** supplemented medium. This would allow assessing whether an increase of the cholesterol/phospholipids ratio prior to in vitro matured bovine oocyte vitrification has any benefit upon bovine oocytes cryotolerance. In this thesis, we proved that, although some molecules of cholesterol could be requested for some lipids or certain proteins present in the FCS, as observed as the lower fluorescence intensity in the group handled in FCS supplemented medium compared to those from synthetic medium, **CLC is still able to diffuse trough the zona pellucida and plasma membrane and to be incorporated into the oocyte cytoplasm when oocytes are handled in a medium supplemented with FCS**. Moreover, as far as we are concerned, this is the first study that demonstrates the incorporation of CLC into bovine oocytes in vitro matured by confocal microscopy using bodipy-cholesterol. However, neither a previous CLC treatment nor the use of FCS supplemented or synthetic media conferred any special improvement in terms of embryo development up to blastocyst stage. It has been shown that small changes in cholesterol both cholesterol depletion and cholesterol enrichment of plasma membrane may affect cellular functions [305]. In this study, the possible improving in membrane fluidity conferred by a greater and faster membrane cholesterol enrichment, observed as an greater fluorescence intensity when handling media was supplemented with PVA, might have been counteracted by the cellular alterations caused by the changes in the phospholipids distributions and the alteration of the cholesterol distribution among

cellular compartments due to CLC presence [305, 345, 346], which may had have effect on the oocyte plasma membrane and on its subsequent developmental competence after vitrification.

Because no differences in embryo development were observed after incubation with 2mg/ml CLC for 30 min and taking into account that the efficiency of cholesterol transfer from the βCDs inclusion complexes to biological membranes depends on βCD-–cholesterol concentration and duration of the exposure [306, 349, 350], we used live-confocal to identify the exact time-point at which the fluorescent tagged cholesterol was mainly located at the plasma membrane using three different concentrations of CLC. The results showed that the best time for CLC incubation was 40 min for 1mg/ml, 30 min for 2mg/ml and 20 for 3mg/ml CLC, to guarantee that plasma membrane contained the maximum amount of CLC but a low diffusion of CLC into the cytoplasm. Because is well known that a higher lipid content into cytoplasm can impair the cryopreservation success [150, 425-430]. However, co-incubation with the resulting CLC concentrations and incubation times prior to vitrification did not exert any effect on early embryo development. Despite the fact that cholesterol manipulation could have modified biophysical plasma membrane properties of bovine mature oocytes pretreated with different CLC concentrations and incubation times, it was noticeable that no systematic relation with oocyte cryotolerance was observed in terms of embryo cleavage or embryo development up to the blastocyst stage. It is known that cholesterol exerts different effects on membrane domains depending on their lipid composition or the degree of saturation of the fatty acids. So, it can be hypothesized that the CLC did not incorporate equally into the different cholesterol domains of the oocyte plasma membrane and, therefore, did not necessarily turned into a rise in membrane fluidity after the addition of cholesterol.

Considering the cryobiological differences between immature and in vitro matured oocytes, explained in the literature review of this thesis (Chapter II), bovine immature or in vitro matured oocytes were vitrified after a 30 min incubation with 2 mg/ml CLC, fertilized and cultured to evaluate early embryo development and quality in terms of gene expression. Even though differences in developmental competence in terms of blastocyst rates were not observed after CLC treatment, differences in relative abundance of some gene transcripts important for early embryo development were

found. Embryos show changes in the gene expression pattern that reflect stress response to a suboptimal condition [339]. **Differences in levels of gene transcripts in morulae derived from vitrified oocytes non-treated with CLC were higher for the immature oocytes. It suggest that vitrification at GV stage might be more harmful for the oocyte and then the treatment with 2 mg/ml CLC for 30min could protect the oocyte from some of these stressful processes, at least for these which are involved the transcripts studied**, as methylation, apoptosis and lipid metabolism thus giving rise to pre-embryos of higher developmental potential. This difference in the effect of CLC treatment between GV oocytes and MII oocytes might be related to the different cryotolerance between them, explained for different factors [333-335]. For instance, the hydraulic conductivity is 2-fold lower for GV oocytes compared to matured MII oocytes [150, 151]. Thus, MII stage bovine oocytes have higher water and CPA permeability coefficients than GV stage oocytes [150, 151] and GV oocytes would be less able to tolerate potential water flux or shear force related damage during volume changes [152]. For this reason GV oocytes could receive more profit from the membrane cholesterol enrichment for the CLC treatment. Because considering that membranes are stabilized by cholesterol and remain more fluid at lower temperatures, then stresses associated with membrane distortion due to dehydration and shrinkage during vitrification processes, especially important for GV stage oocytes, could be reduced [360].

In recent decades, numerous attempts have been made to improve cryopreservation outcomes by limiting the oocyte damage. Biological molecules isolated from organisms that live under subfreezing conditions could be used to protect oocytes from cryoinjuries suffered during cryopreservation process (Lee HH et al 2015, Jo JW et al 2011, 2012; Liang S et al 2016). These molecules could act by blocking ice formation and recrystallization during warming, protecting the cell membranes during cryopreservation. Pseudomonas sp. ID1, a cold-adapted bacterium isolated from marine sediment from Antartica, produces an exopolysaccharide (M1 EPS) with cell cryoprotective activity as a cold-adaptation mechanism. We hypothesized in **Chapter VI**, that EPS synthetized by Pseudomonas sp ID1 could protect in vitro matured bovine oocytes from most current damage suffered during cryopreservation and enhance embryo development after warming. Thus, the aim of this study was to examine whether the addition of M1 EPS, produced by Pseudomonas sp. ID1, to vitrification and

warming solutions for in vitro matured bovine oocytes would improve their cryotolerance.

First we analyzed microtubule and chromosome configurations by immunocytochemistry after exposure of oocytes to various concentrations of M1 EPS in vitrification and warming solutions We observed no significant effects of M1 EPS treatment on decondensed or absent microtubule and chromosome configuration rates in vitrified cow oocytes. In contrast, those heifer oocytes vitrified without M1 EPS treatment showed higher percentages of decondensed or absent chromosomes and microtubules compared with control group. No differences in percentages of decondensed or absent chromosomes and microtubules were observed in all other heifer oocytes vitrified with EPS supplementation compared to control group. Therefore, it seems that **M1 EPS supplementation during vitrification and warming of prepubertal heifer oocytes confers some protection against chromosome decondensation,** one of the negative effects of vitrification.

Different EPS concentrations seem to exert different effect on meiotic spindle organization after vitrification and warming depending on the sexual maturity of the animal. Higher M1 EPS concentrations seemed to impair spindle configuration in cow oocytes, as 1mg/ml group presented lower percentages of oocytes reaching metaphase II stage and 0.1mg/ml is the only group that showed lower normal spindle configuration compared to fresh control group. Contrarily, lower M1 EPS concentrations had a detrimental effect for heifer spindle organization, as lower percentages of oocytes reaching metaphase II were observed in 0.001mg/ml group and group non supplemented oocytes presented higher decondensed or absent chromosomes and microtubules.

Previous studies also reported the sensitivity of the oocyte meiotic spindle to cryopreservation was also different depending on the sexual maturity of the donor animal [169, 205]. In bovine oocytes Albarracin et al [169] reported that vitrification seemed to have worse effects on heifer oocytes. Succu et al [205] observed increased CPA toxicity in prepuberal oocytes compared to adult oocyte in ovine. They attributed this increased CPA toxicity observed in prepuberal oocytes to a deficiency of molecular and/or structural factors involved in spindle configuration restoring in prepubertal oocyte [170].

In the second experiment, we examined whether the addition of different concentrations of M1 EPS to vitrification and warming solutions for in vitro matured bovine oocytes would improve their developmental competence. Although some particular EPS concentrations added to vitrification and warming solutions could improve blastocyst rates, as for example in cow oocytes 0.01mg/ml group presented significantly higher blastocyst rates compared to 1mg/ml, this improvement is not significantly ($p>0.05$) compared to non-supplemented vitrified oocytes (5.6%). The impairment on in vitro developmental competence on cow oocytes caused by the highest M1 EPS concentration (1mg/ml) coincides with the effects observed on meiotic spindle organization. However, that coincidence was not observed in heifer oocytes.

Exposure to M1 EPS during vitrification and warming had no deleterious effects on cleavage or development to the blastocyst stage of IVM bovine oocytes. Moreover, the hatchability of those embyos have been improved by the supplementation of vitrification and warming media with some M1 EPS concentrations, 0.001mg/ml and 0.01mg/ml.

According to previous reports [380-382] significantly lower blastocyst rates were recorded in heifer than in cow for non-vitrified oocytes. That difference was also observed in 0.001mg.mL-1 group.

Finally, differences in terms of gene expression on blastocysts obtained from oocytes exposed to different concentrations of M1 EPS during vitrification-warming process were analyzed by RT-PCR. Although in general no marked differences on cleavage and blastocyst yield were observed between oocytes vitrified and warmed in media supplemented with different concentrations of M1 EPS or non-supplemented, differences in relative abundance of some gene transcripts during early embryo development were found. The lower developmental competence (lower blastocyst rates) observed in the embryos produced from vitrified oocytes could be, at least partially explained by the disruption of the physiological epigenetic profile of the oocyte due to the stress to which the oocytes suffered during vitrification and warming. Data from present study indicated that developmental competence from vitrified oocytes seem to be impaired by changes in gene expression of some gens related with epigenetics (DNA methylation and chromatin acetylation) and blastocyst quality (BAX, BCL2).

Further research would be needed to clarify the exact mechanism of action of the M1 EPS to cryoprotect bacteria cells, in order to optimize their application for oocyte cryopreservation.

# CHAPTER VIII

## Conclusions

From the results presented in this dissertation three blocks with its conclusions can be drawn:

Exposition to increased concentrations of NaCl or sucrose prior to in vitro-matured bovine oocytes vitrification

1. Treatment with 375 mOsmol NaCl or sucrose solution for 1 h before vitrification had no detrimental effects on the meiotic spindle status of IVM bovine oocytes. Treatment with NaCl or sucrose concentrations higher than 375mOsm gave rise to higher abnormal spindle rates.
2. In particular, sucrose pretreatment was unable to improve embryo development as observed in other species.
3. There is a clear need for further work designed to improve the cryotolerance of bovine oocytes.

Impacts of cholesterol added before vitrification on the cryotolerance of immature and in vitro-matured bovine oocytes

4. In vivo-imaging of intracellular trafficking of BODIPY-labeled cholesterol-loaded cyclodextrins CLC revealed high fluorescence intensity of the plasma membrane after incubation of *in vitro* matured oocytes with 2 mg/ml CLC for 30 minutes compared to the low labelling of the cytoplasm, either in a FCS or PVA supplemented medium.
5. However higher amounts of cholesterol were incorporated into oocyte membranes when the media was supplemented with polyvinyl alcohol compared fetal calf serum. Although some molecules of cholesterol could be requested for some lipids or certain proteins present in the FCS, cholesterol is still able to diffuse trough the zona pellucida and plasma membrane and to be incorporated into the oocyte cytoplasm when oocytes are handled in a medium supplemented with FCS
6. Cholesterol-loaded cyclodextrins pretreatment prior to bovine oocyte vitrification did not improved survival, and day 7 or day 8 blastocyst rates regardless of the application of the determined cholesterol loaded cyclodextrins treatment or the holding medium used.
7. Live-confocal microscopy allowed to identify the exact time-point at which the fluorescent tagged cholesterol was mainly located at the plasma membrane using

three different concentrations of cholesterol-loaded cyclodextrins: 40 min for 1mg/mL, 30 min for 2mg/ml and 20 for 3mg/ml. Higher incubation times caused internalization of cholesterol loaded cyclodextrins into cytoplasm.

8. Cholesterol-loaded cyclodextrins addition prior to in vitro matured bovine oocyte vitrification at 1mg/ml, 2mg/ml and 3 mg/ml for 40 minutes, 30 minutes and 20 minutes, respectively, did not improve their developmental competence after warming.
9. Pretreatment with 2mg/ml for 30 minutes prior immature and in vitro matured bovine oocyte vitrification did not improved cleavage or blastocyst rates.
10. However, the treatment of oocytes with 2mg/ml cholesterol loaded cyclodextrins before vitrification, altered the expression of genes related to lipid metabolism (CYP51), apoptosis (BAX) and DNA methylation (DNMT3A) in bovine morulae, mainly when oocytes were vitrified at germinal vesicle stage.
11. Based on the changes on the relative expression of genes which related to the embryo development, the use of a 2 mg/ml of cholesterol-loaded cyclodextrins for 30min may have contributed to a improve quality of those embryos obtained after vitrification, mainly on those oocytes vitrified at the GV stage.

Effects of exopolysaccharide supplementation during vitrification and warming in the spindle organization, developmental competence and gene expression of in vitro matured bovine oocytes.

12. M1 EPS supplementation during vitrification and warming of in vitro matured prepubertal heifer oocytes protected the meiotic spindle against chromosome and microtubule decondensation caused by vitrification.
13. Based on the lower percentage of cow oocytes reaching metaphase-II stage after 1mg/ml EPS addition during vitrification and warming and the lower normal spindle configuration observed in cow oocytes after 0.1mg/ml supplementation, we can conclude that the highest EPS concentrations impaired cryotolerance on co oocytes.
14. EPS supplementation during vitrification and warming failed to improve the developmental competence in terms of cleavage and blastocyst rates of in vitro matured bovine oocytes, regardless of the sexual maturity of the animal.

15. Developmental competence from vitrified oocytes seem to be impaired by changes in gene expression of some gens related with epigenetics (DNA methylation and chromatine acetylation) and blastocyst quality (BAX, BCL2).
16. Based on significantly higher expression levels of BCL-2 compared to BAX in those blastocysts obtained from oocytes vitrified with 0.01mg/ml M1 EPS supplementation, we can conclude this concentration could be considered the treatment that obtain the highest blastocyst quality.
17. Further research would be needed to clarify the exact mechanism of action of the M1 EPS to cryoprotect bacteria cells, in order to optimize their application for oocyte cryopreservation.

# CHAPTER IX

## References

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