UNIVERSITAT POLITÈCNICA DE VALÈNCIA

GENERATION OF OFFSPRING FROM CRYOPRESERVED RABBIT (*Oryctolagus cuniculus*) OOCYTES

Ph.D Thesis by

María Estrella Jiménez Trigos

Supervisors

Francisco Marco Jiménez

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A thesis submitted to the Polytechnic University of Valencia in partial fulfilement of the requirements for the degree of doctor of philosophy

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ABSTRACT/RESUMEN/RESUM

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ABSTRACT

The general aim of this thesis was to optimise the current methodologies of oocyte cryopreservation in order to obtain live offspring from cryopreserved rabbit oocytes.

In chapter 1, meiotic spindle configuration, cortical granules (CGs) distribution and oocyte developmental competence were evaluated after cryopreservation with the current slow-freezing and vitrification procedures. The meiotic spindle organisation was dramatically impaired regardless of the method used. Nevertheless, altered CG distribution is more evident in vitrified oocytes than in slow-frozen ones and the developmental rate to blastocyst stage after parthenogenetic activation was only obtained using slow-freezing method. From this chapter it may be concluded that both methodologies equally affect oocyte structure. However, slow-freezing method seems to be the recommended option for this species as a consequence of the sensitivity to high levels of cryoprotectants in this species.

The aim of the following two chapters was the optimisation of cryopreservation procedures using different strategies to modify the oocytes in order to make them more cryoresistant.

In chapter 2, Taxol and Cytochalasin B were employed to stabilise the cytoskeleton system during vitrification. The effect of these two molecules on the meiotic spindle and chromosome configuration and development to blastocyst stage after parthenogenesis activation were also evaluated. There were no significant differences in the structural configuration between vitrified groups. Regarding cleavage and blastocyst developmental rate, no statistical

differences were found between vitrified-non-treated and Taxol-treated oocytes, but no oocytes treated with Cytochalasin B reached this stage. Therefore, structural configuration and blastocyst development were not improved by this pre-treatment. Moreover, Cytochalasin B pre-treatment seems to cause a deleterious effect on developmental ability to blastocyst stage of these oocytes.

In chapter 3, oocytes were incubated with cholesterol-loaded methyl-β-cyclodextrin (CLC) to increase the membrane fluidity and stability and improve their developmental ability after parthenogenetic activation or intracytoplasmic sperm injection (ICSI). Cholesterol incorporation and its presence after cryopreservation were evaluated using confocal microscopy. Results showed that cholesterol was incorporated into the oocyte and remained, albeit in a lesser amount after cryopreservation procedures. However, no improvements on developmental competence were obtained after parthenogenetic activation or intracytoplasmic sperm injection.

In the last three chapters of this thesis, the main objective was to develop a reliable technique which would allow us to obtain live offspring from cryopreserved oocytes. For that purpose, *in vivo* fertilisation using intraoviductal oocyte transfer assisted by laparoscopy was considered a good alternative to bypass the inadequacy of conventional *in vitro* fertilisation in rabbit.

In chapter 4, two recipient models (ovariectomised or oviduct ligated immediately after transfer) were used to compare the ability of fresh oocytes to fertilise *in vivo*. This first work showed that embryo recovery rates in all transferred groups decreased significantly, but ligated oviduct recipients

provided significantly higher results compared to ovariectomised ones. For that reason, in the second experiment the ligated oviduct recipient model was used to generate live births. Results obtained in this chapter suggested that it was possible to obtain offspring from cryopreserved oocytes using this technique, but this kind of animal models compromised the use of the reproductive tract in a high percentage of females.

For that reason, chapter 5 was focused on the development of another type of animal model as an alternative. First, the ability of cyanoacrylate tissue adhesive to block the oviducts before the ovulation would take place was evaluated. Then, *in vivo* fertilisation ability of fresh transferred oocytes after blocking the oviduct with the adhesive was also assessed. Finally, slow frozen oocytes were transferred to generate live birth. Results showed that cyanoacrylate tissue adhesive was effective in blocking the oviduct, as no embryos were recovered in the blocked oviduct six days after artificial insemination (AI). Moreover, this method could fertilise fresh and also slow-frozen oocytes with a higher live birth rate than the previous recipient models. This study showed that successful production of live offspring using slow-frozen oocytes in combination with *in vivo* fertilisation was possible, which suggested that *in vivo* environment could help improve the results of oocyte cryopreservation.

Thus, this method was employed in the last chapter of this thesis to generate live offspring from vitrified rabbit oocytes for the first time. Results obtained revealed that there were no differences in the rate of birth between vitrified and slow-frozen transferred oocytes. Nevertheless, based on the results with

fresh oocytes, further experiments are still needed if the efficiency of cryopreservation procedures are to be improved.

RESUMEN

El objetivo general de esta tesis fue la optimización de las actuales metodologías de crioconservación ovocitaria para obtener descendencia viva a partir de óvulos crioconservados de coneja.

En el capítulo 1 se evaluó la configuración del huso meiótico, la distribución de los gránulos corticales (GCs) y la capacidad de desarrollo de los ovocitos tras su crioconservación mediante los procedimientos actuales de congelación y vitrificación. La organización del huso meiótico fue alterada drásticamente independientemente del método utilizado. Sin embargo, la alteración de la distribución de los GCs fue más evidente en el caso de los ovocitos vitrificados y la tasa de desarrollo hasta el estadío de blastocito tras la activación partenogenota solamente se obtuvo utilizando el método de congelación. De este capítulo se puede concluir que ambas metodologías afectan por igual la estructura del ovocito. Sin embargo, el método de congelación parece ser la opción más recomendable en el conejo como consecuencia de la sensibilidad que presenta esta especie a las altas concentraciones de crioprotectores.

El objetivo de los dos siguientes capítulos fue la optimización de los procedimientos de crioconservación mediante el uso de diferentes estrategias para modificar a los ovocitos y hacerlos así más crioresistentes.

En el capítulo 2, se emplearon el Taxol y el Cytochalasin B para estabilizar el sistema del citoesqueleto durante la vitrificación. El efecto de estas dos moléculas sobre la configuración del huso meiótico y los cromosomas y el

desarrollo hasta blastocisto tras la activación partenogenota fueron también evaluados. No se observaron diferencias en la estructura entre los diferentes grupos de ovocitos vitrificados. Tampoco se encontraron diferencias significativas en la división y la tasa de desarrollo hasta blastocisto entre el grupo vitrificado-no tratado y el grupo de ovocitos tratados con Taxol, pero ninguno de los ovocitos tratados con Cytochalasin B alcanzó dicho estadío. Por tanto, la configuración estructural y la capacidad de desarrollo no mejoraron tras este tratamiento. Incluso el tratamiento con Cytochlasin B pareció generar un efecto perjudicial sobre el desarrollo de estos ovocitos.

En el capítulo 3, los ovocitos fueron incubados con ciclodextrinas cargadas con colesterol (CLC) para incrementar la fluidez y la estabilidad de la membrana y mejorar así su capacidad de desarrollo tras el procedimiento de crioconservación y la activación partenogenota o la inyección intracitoplasmática de espermatozoide (ICSI). La incorporación de colesterol en el ovocito y su permanencia tras la crioconservación fue evaluada utilizando microscopía confocal. Los resultados mostraron que el colesterol era incorporado dentro del ovocito y que permanecía, aunque en menor cantidad, tras los procedimientos de crioconservación. Sin embargo, no se obtuvieron mejoras en la capacidad de desarrollo de los mismos tras la activación partenogenota o la ICSI.

En los últimos tres capítulos de esta tesis, el principal objetivo fue el desarrollo de una técnica fiable que permitiera obtener descendencia viva a partir de estos ovocitos crioconservados. Para este objetivo, se empleó la fecundación *in vivo* utilizando la transferencia intraoviductal de ovocitos asistida mediante

laparoscopía como una buena alternativa para sobrepasar la deficiencia de las técnicas de fertilización *in vitro* en la especie cunícola.

En el capítulo 4, se emplearon dos modelos de hembras receptoras (ovariectomizadas o cuyo oviducto fue ligado inmediatamente tras la transferencia) para comparar la habilidad de los ovocitos frescos transferidos de ser fertilizados *in vivo*. Este primer trabajo mostró que en todos los grupos transferidos, la tasa de recuperación embrionaria disminuyó significativamente. Sin embargo, en el grupo de hembras receptoras cuyo oviducto fue ligado, se obtuvieron mejores resultados que en las hembras ovariectomizadas. Por esta razón, el modelo de hembras receptoras cuyos oviductos fueron ligados tras la transferencia fue el de elección para obtener descendencia viva a partir de óvulos congelados. Los resultados obtenidos en este capítulo sugirieron que era posible obtener descendencia a partir de óvulos crioconservados, pero estos dos tipos de modelo animal comprometían el uso del tracto reproductivo en un porcentaje elevado de hembras.

Por esta razón, el capítulo 5 de la tesis se centró en el desarrollo de otro tipo de modelo animal como alternativa a los dos anteriores. Primero, se evaluó la habilidad del adhesivo tisular de cianoacrilato para bloquear el oviducto antes de la ovulación. A continuación, se evaluó la capacidad de fertilización *in vivo* de ovocitos frescos tras cerrar el oviducto una vez realizada la transferencia intraoviductal de los mismos. Finalmente, se transfirieron ovocitos congelados para evaluar la tasa de nacimientos. Los resultados mostraron que este adhesivo era efectivo a la hora de bloquear el oviducto, ya que no se recuperó ningún embrión de los oviductos bloqueados seis días después de la inseminación artificial (IA). Además, este método permitía la fertilización tanto

de óvulos frescos como congelados, obteniendo una tasa de nacimientos mayor que la obtenida con los dos modelos de receptoras anteriores. Estos resultados sugirieron que el ambiente *in vivo* podría ayudar a mejorar los resultados de la crioconservación ovocitaria.

Por ello, este fue el método empleado en el último capítulo de esta tesis para generar por primera vez, descendencia viva a partir de ovocitos vitrificados de coneja. Los resultados obtenidos revelaron que no existen diferencias en la tasa de nacimientos entre los óvulos transferidos vitrificados y los congelados. Sin embargo, basado en los resultados obtenidos con ovocitos frescos, todavía se necesitan más experimentos si se quiere mejorar la eficiencia de la técnica.

RESUM

L'objectiu general d'aquesta tesi va ser l'optimització de les actuals metodologies de crioconservació ovocitaria per a obtindre descendència viva a partir d'òvuls crioconservats de conilla.

En el capítol 1 es va avaluar la configuració del fus meiòtic, la distribució dels gràNuls corticals (GCs) i la capacitat de desenvolupament dels ovòcits després de la seua crioconservació amb els procediments actuals de congelació i vitrificació. L'organització del fus meiòtic va ser alterada dràsticament independentment del mètode utilitzat. No obstant això, l'alteració de la distribució dels GCs va ser més evident en els ovòcits vitrificats que en els congelats i la tasa de desenvolupament fins l'estadi de blastocist després de l'activació partenogenota només es va obtindre utilitzant el mètode de congelació. D'aquest capítol pot concloure's que ambdós mètodes afecten per igual l'estructura de l'ovòcit però el mètode de congelació pareix l'opció més recomenable en el conill com a conseqüència de la sensibilitat que presenta aquesta espècie a les altes concentracions de crioprotectors.

L'objectiu dels dos següents capítols va ser l'optimització dels procediments de crioconservació mitjançant l'ús de diferents estratègies per a modificar els ovòcits i fer-los així més crio-resistents.

En el capítol 2, es van emprar el Taxol i el Cytochalasin B per a estabilitzar el sistema del citoesquelet durant la vitrificació. L'efecte d'aquestes dues molècules sobre la configuració del fus meiòtic i els cromosomes i el desenvolupament fins blastocisto després de l'activació partenogenota van ser

també avaluats. No es van observar diferències en l'estructura entre els grups vitrificats. Quant a la divisió i la capacitat de desenvupament, no es van trobar diferències significatives entre el grup vitrificat no tractat i el grup d'ovòcits tractats amb Taxol, però cap dels ovòcits tractats amb Cytochalasin B va aconseguir-ho. Per tant, la configuració estructural i la capacitat de desenvolupament dels ovòcits vitrificats no van millorar després d'aquest tractament. Inclús el tractament amb Cytochlasin B va parèixer produir un efecte perjudicial en la capacitat de desenvolupament d'aquestos ovòcits.

En el capítol 3, els ovòcits van ser tractats amb ciclodextrines carregades amb colesterol (CLC) per a incrementar la fluïdesa i l'estabilitat de la membrana i millorar així la capacitat de desenvolupament dels ovòcits crioconservats després de l'activació partenogeonta o la injecció intracitoplasmàtica d'espermatozous (ICSI). La incorporació de colesterol i la seua permanència després de la crioconservació va ser avaluada utilitzant microscòpia confocal. Els resultats van mostrar que el colesterol era incorporat dins de l'ovòcit i que romania encara que en menor quantitat després dels procediments de crioconservació. No obstant això, no es van obtindre millores en la capacitat de desenvolupament després de l'activació partenogenota o la ICSI.

En els últims tres capítols d'aquesta tesi, el principal objectiu va ser el desenvolupament d'una tècnica fiable que permetera obtindre descendència viva a partir d'aquestos ovòcits crioconservats. Per a aquest objectiu, es va emprar la fecundació *in vivo* utilitzant la transferència intraoviductal d'ovòcits assistida per mitjà de laparoscòpia com una bona alternativa per a sobrepassar la deficiència de les tècniques de fertilització *in vitro* en l'espècie cunícola.

En el capítol 4, es van emprar dos models de femelles receptores (ovariectomitzades o l'oviducte de les quals va ser lligat immediatament després de la transferència) per a comparar l'habilitat dels ovòcits frescos de ser fertilitzats *in vivo*. Este primer treball va mostrar que en tots els grups transferits la taxa de recuperació embrionària va disminuir significativament, però en el grup de femelles receptores l'oviducte de les quals va ser lligat, es van obtindre millors resultats comparats amb les femelles ovariectomitzades. Per aquesta raó, el model de receptores amb els oviductes lligats després de la transferència van ser emprades per a obtindre descendència a partir d'òvuls congelats. Els resultats obtinguts en aquest capítol van suggerir que era possible obtindre descendència a partir d'aquestos òvuls. No obstant això, aquestos tipus de models animals comprometien l'ús del tracte reproductiu en un percentatge elevat de femelles.

Per aquesta raó, el capítol 5 es va centrar en el desenvolupament d'un altre tipus de model animal com a alternativa als dos anteriors. Primer, es va avaluar l'habilitat de l'adhesiu tisular de cianoacrilato per a bloquejar l'oviducte abans de l'ovulació. A continuació, es va avaluar la capacitat de fertilització *in vivo* d'ovòcits frescos després de tancar l'oviducte una vegada realitzada la transferencia intraoviductal dels mateixos. Finalment, es van transferir ovòcits congelats per a avaluar la taxa de naixements. Els resultats van mostrar que aquest adhesiu era efectiu a l'hora de bloquejar l'oviducte, ja que no es va recuperar cap embrió dels ovidcutes bloquejats sis dies després de la inseminació artificial (IA). A més, aquest mètode permetia la fertilització tant d'òvuls frescos com de congelats, obtenint una tassa de naixements major que l'obtinguda amb els models de receptores anteriors. Aquest estudi va suggerir

que l'ambient *in vivo* podría ajudar a millorar els resultats de la crioconservació ovocitària.

Per això, aquest va ser el mètode emprat en l'últim capítol d'aquesta tesi per a generar, per primera vegada, descendència viva a partir d'ovòcits vitrificats de conilla. Els resultats obtinguts revelaren que no hi havia diferències en la tassa de naixements entre els òvuls transferits vitrificats i congelats. No obstant això, basant-nos en els resultats obtinguts amb ovòcits frescos, encara es necessiten més experiments si es vol millorar l'eficiència de la tècnica.

ABBREVIATIONS

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µg microgram

µL microliters

6-DMAP 6-dimethylaminopurine

AI Artificial insemination

ART Assisted reproductive technologies

ATP Adenosine triphosphate

BM Base medium

BSA Bovine serum albumin

$CaCl_2$ Calcium chloride

Cai intracellular calcium

CCD Charged-couple device

CLC Cholesterol-loaded-cyclodextrin

cm centimeter

CO_2 Carbon dioxide

DMSO Dimethyl sulphoxide

DNA Deoxyribonucleic acid

DPBS Dulbecco's phosphate-buffered saline without calcium chloride

EG Ethylene glycol

ET Embryo transfer

FBS Foetal bovine serum

FITC Fluorescein isothiocyanate

FITC-LCA Lens culinaris agglutinin labelled with fluorescein isothiocyanate

GCs Cortical granules

GLM General Linear Model

GV Germinal vesicle

h hour

Hoechst 33342 *2'- (4- Ethoxyphenyl)- 5- (4- methyl- 1- piperazinyl) - 2,5'- bi- 1H-benzimidazol trihydrochloride*

ICSI Intracytoplasmic sperm injection

IVF *In vitro* fertilisation

kV kilovolt

LN_2 Liquid nitrogen

M molar

$MgSO_4$ Magnesium sulphate

MII Metaphase II

min minute

mL millilitres

mM millimolar

MβCD methyl-β-cyclodextrin

NBD-CLC 22- N-(7-nitrobenz-2-oxa-1,3-diazol-4yl) amino-23,24 bisnor-5-cholen-3b-ol labelled cholesterol

PI Propidium iodide

PROH 1.5 M 1,2-propanediol

PVP K12 Polyvinylpyrrolidone of Mr 5000 Da

s second

S.E.M. Means±standard error of means

t10, c12 CLA trans- 10, cis-12 octadecadienoic acid

TCM-199 Tissue culture medium 199

v/v volume/volume

w/v weight/volume

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1. GENERAL INTRODUCTION

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1. GENERAL INTRODUCTION

1.1. APPLICATIONS OF OOCYTE CRYOPRESERVATION

Cryopreservation of embryos and gametes in animal species is considered an important tool in reproductive biotechnology to preserve selected lines from pathogens, to evaluate the genetic improvement, minimise the impact of genetic drift and facilitate the diffusion of the lines to different countries, avoiding animal transportation and its sanitary risks (Leibo 1992; Whittingham and Carroll 1992; Lavara *et al*., 2011). Moreover, these techniques allow us to conserve and spread the biodiversity of animal genetics and preserve endangered species to maintain biodiversity (Andrabi and Maxwell 2007; Prentice and Anzar 2010).

In the case of gamete cryopreservation, preservation of oocytes would enable a more efficient management of livestock and laboratory animal species (Díez *et al*., 2012). Oocyte banks allow female genetic material to be stored unfertilised until an appropriate male germplasm is selected, and could also preserve the genetic material from unexpectedly dead animals and facilitate many assisted reproductive technologies (ART) (Ledda *et al*., 2001; Checura and Seidel 2007; Pereira and Marques 2008). On the other hand, in human, female gamete cryopreservation provides an alternative to embryo freezing without ethical and religious problems, and can also be used to preserve fertility in patients in danger of losing ovarian function (Ledda *et al*., 2001; Nottola *et al*., 2008; Porcu *et al*., 2008). Nevertheless, gamete cryopreservation presents the disadvantage that only the haploid genotype is conserved and if a

population is required in the future, the appropriate sperm would also have to be available (Glenister and Thornton 2000), so it is recommended to combine at least two types of samples in the formation of a bank (Boettcher *et al*., 2005).

1.2. OOCYTE CRYOPRESERVATION

Oocyte cryopreservation has more than 40 years of history and over these last four decades, significant advances have been made. Despite all these efforts, advances are rather slow, the main problems being the lack of consistency of the results among groups (Liebermann and Tucker 2002), as well as differences in survival rates after warming between species and development stages that were cryopreserved (Pereira and Marques 2008).

The first method to be developed was the slow-freezing technique in 1958, when the possibility of survival of unfertilised mouse oocytes after freezing and thawing was demonstrated (Sherman and Lin 1958). However, it was not until 1977 when the first successful *in vitro* fertilisation (IVF) and live offspring from slow-frozen mouse oocytes were reported (Whittingham 1977). Since then, relatively successful cryopreservation of oocytes has been achieved for several other species including the hamster, rabbit, pig, cat, sheep, horse, cow and human (Al-Hasani *et al*., 1989; Vincent *et al*., 1989; Schroeder *et al*., 1990; Fuku *et al*., 1992; George and Johnson 1993; Luvoni and Pellizzari 2000; Stachecki *et al*., 2002; Sakamoto *et al*., 2005; Ambrosini *et al*., 2006; Prentice and Anzar 2010). Although slow-freezing continues to be the most widely used technique of cryopreservation for *in vivo* and *in vitro* produced embryos, in the last decade vitrification has been tested in different species with good results (Vajta *et al*.,

1998; Berthelot *et al*., 2000; Lavara *et al*., 2011).

Vitrification emerged as an alternative method for oocyte cryopreservation in 1985 (Rall and Fahy 1985). This technique uses an ultra-rapid cooling rate, eliminating the need for programmable freezing equipment and prevents intracellular ice crystal formation by using high concentrations of cryoprotectants resulting in a glass transition state. Initial studies reported success in germinal vesicle-stage mouse oocytes (Van Blerkom 1989) and the first live young from mature mouse oocytes was achieved in 1989 (Nakagata 1989). Since then, this technique has become a viable and promising alternative to traditional approaches (Kuwayama 2007) and significant progress has been made in laboratory animals (Nakagata 1989; Nakagata, 1992; Shaw *et al*., 2000; Vajta, 2000; Stachecki and Cohen, 2004; Cai *et al*., 2005), farm animals (Martino *et al*., 1996; Otoi *et al*., 1996; Vajta *et al*., 1998; Maclellan *et al*., 2002; Abe *et al*., 2005; Albarracin *et al*., 2005; Cetin and Bastan, 2006; Succu *et al*., 2007; Liu *et al*., 2008), non-human primates (Parks and Ruffing 1992) and humans (Lucena *et al*., 2006; Antinori *et al*., 2007; Kuwayama 2007). Specifically, the greatest progress was achieved in the bovine species, whose blastocyst rates after fertilisation and *in vitro* culture were found to be similar to those of non-cryopreserved control oocytes (Martino *et al*., 1996; Vajta *et al*., 1998; Papis *et al*., 1999; Mavrides and Morroll 2002).

Despite all these breakthroughs, no general protocol has yet been established (Nottola *et al*., 2008; Pereira and Marques 2008; Noyes *et al*., 2010) and procedures developed for one species are difficult to adapt to another species, mainly because of differences in size, properties and sensitivity to cooling and cryoprotectants (Paynter *et al*., 1999:2001). For this reason,

subsequent progress is still limited and live offspring have only been obtained in a few species, such as mouse (Whittingham 1977), human (Chen 1986), rabbit (Al-Hasani *et al*., 1989), cattle (Fuku *et al*., 1992), rat (Nakagata 1992), horse (Maclellan *et al*., 2002), cat (Gómez *et al*., 2008) and recently, pig (Somfai *et al*., 2013). Moreover, results remain low, and pregnancy rates remain higher using cryopreserved embryos. A meta-analysis on slow freezing of human oocytes showed that clinical pregnancy rate per thawed oocyte was only 2.4% (95/4000) and only 1.9% (76/4000) resulted in live birth (Oktay *et al*., 2006). Nevertheless, the literature reports a great variability between both methods and the results obtained are different depending on the species, laboratories and cryopreservation protocol (Table 1.1).

While numerous reports designed to investigate oocyte cryopreservation in some species have been published (Mullen 2007), few works have been done in rabbit (Diedrich *et al*., 1988; Al-Hasani *et al*., 1989; Vincent *et al*., 1989; Siebzehnruebl *et al*., 1989; Cai *et al*., 2005; Salvetti *et al*., 2010; Wang *et al*., 2010) and only one recent work compared slow-freezing and vitrification methods (Salvetti *et al*., 2010). All these studies showed that rabbit is highly sensitive to low temperatures and high levels of cryoprotectants (Diedrich *et al*., 1988, Vincent *et al*., 1989, Cai *et al*., 2005, Salvetti *et al*., 2010) and, consequently, blastocyst production after warming is very low (Diedrich *et al*., 1988, Al-Hasani *et al*., 1989, Siebzehnruebl *et al*., 1989, Vincent *et al*., 1989, Cai *et al*., 2005, Salvetti *et al*., 2010, Wang *et al*., 2010). Moreover, to our knowledge, only one work done in 1989 obtained live offspring from slow-freezing oocytes (Al-Hasani *et al*., 1989) and another study, also done in 1989, showed unborn offspring at day 25 of gestation (Vincent *et al*., 1989).

Table 1.1. Pregnancy rate per embryo transfer derived from cryopreserved oocytes in different species.

Specie	Author	Year	Cryopreservation method	Maturation stage	Method of fertilization	Birth/embryo transferred (%)
Human	Virant-Klun *et al.,*	2011	Slow-freezing	MII	ICSI	2/7 (28.6)
	Kuwayama	2007	Vitrification	MII	ICSI	11/29 (37.9)
	Fadini	2009	Slow-freezing	MII	ICSI	12/224 (5.3)
			Vitrification			3/55 (5.4)
Horse	Maclellan *et al.,*	2002	Vitrification	MII	*In vivo*	2/9 (22.2)
Bovine	Kubota *et al.,*	1998	Slow-freezing	GV	IVF	2/6 (33.3)
				MII		2/12 (16.7)
	Vieira *et al.,*	2002	Vitrification	GV	IVF	3/11 (27.2)
Cat	Pope *et al.,*	2012	Vitrification	MII	ICSI	4/43 (9.3)
Rabbit	Al-Hasani *et al.,*	1989	Slow-freezing	MII	IVF	4/53 (7.5)
Rat	Nakagata	1992	Ultrarapid-	MII	IVF	28/150 (18.7)
Mouse	Aono *et al.,*	2005	Vitrification	GV	IVF	4/40 (10.0)
	Endoh *et al.,*	2007	Vitrification	MII	ICSI	36/310 (11.6)
	Eroglu *et al.,*	2009	Slow-freezing	MII	IVF	4/21 (19.0)
	Kohaya *et al.,*	2011	Vitrification	MII	IVF	92/134 (68.7)
		2013	Vitrification	MII	IVF	51/90 (56.7)
Pig	Somfai *et al.,*	2013	Vitrification	GV	IVF	10/43 (23.3)

MII: nuclearly mature oocyte at the metaphase of the second meiotic division; GV: inmature oocyte at the germinal vesicle; ICSI: intracytoplasmic sperm injection; IVF: *in vitro* fertilization.

1.3. DIFFICULTIES TO OOCYTE CRYOPRESERVATION

Oocytes are particularly difficult to cryopreserve successfully, resulting in low rates of blastocyst production after thawing, fertilisation and culture. In general, the low efficiency might be due to both morphological and biophysical factors (Ledda *et al*., 2007). The complex structure of the oocyte and the differences in membrane permeability, as well as differences in physiology, could be the cause of the differences between embryos and oocytes (Gardner *et al*., 2007).

Cryopreservation induces several types of undesirable damage by mechanical, thermal or chemical factors (Shi *et al*., 2006; Morato *et al*., 2008). The main biophysical factors that contribute to cellular injury and death during cryopreservation are intracellular and extracellular ice formation and osmotic injury. Most of the components present in the oocyte are particularly sensitive to these factors (Figure 1.1).

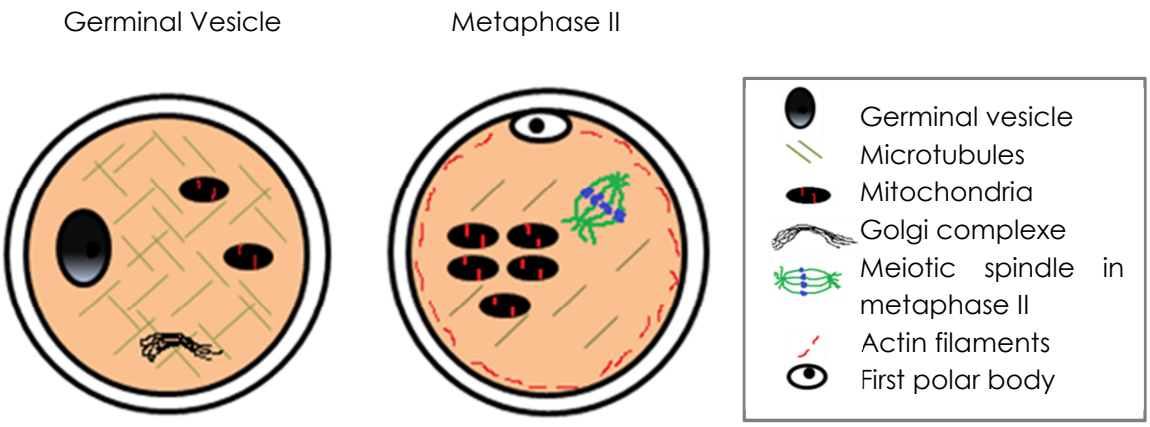

Figure 1.1: Diagram of an oocyte at germinal vesicle and metaphase II stage. (Adapted from Ferreira *et al.,* 2009)

The damage that an oocyte might suffer during cryopreservation could be as a consequence of their large size of and the greater volume to surface ratio,

make it more difficult for water and cryoprotectants to move across the cell (Fabbri *et al*., 2000). Moreover, during cryopreservation, the oocyte undergoes dramatic volume changes due to different osmotic pressures between the intracellular and extracellular solutions. These changes in cell volume affect the integrity of the oolemma as well as subcellular structures (Ambrosini *et al*., 2006).

On the other hand, the plasma membrane of an oocytes at the second metaphase stage has a low permeability coefficient, making the movement of cryoprotectants and water slower (Ruffing *et al*., 1993). Additionally, the lower amounts of sub-membranous actin microtubules make it less robust (Gook *et al*., 1993). Oocytes are also surrounded by zona pellucida, which acts as an additional barrier for the movement of water and cryoprotectants into and out of it (Saragusty and Arav 2011).

If oocytes are cryopreserved after maturation, they present the second meiotic spindle, which is essential for completion of meiosis and to ensure the correct complement of genetic material of the oocyte. Cooling, cryoprotectants and cryopreservation have all been shown to induce microtubule depolymerisation leading to an abnormal spindle configuration (Rojas *et al*., 2004; Succu *et al*., 2007), which can cause chromosome abnormalities, increasing the incidence of aneuploidies (Luvoni 2000). The use of germinal vesicle (GV) stage oocytes avoids this problem, as their chromosomes are surrounded by a nuclear membrane (Parks *et al*., 1992; Cooper *et al*., 1998; Isachenko *et al*., 1999). However, the difficulties associated with the *in vitro* maturation and culture might counteract the potential benefits (Cooper *et al*., 1998). On the other hand, the cytoskeletal disorganisation could also cause an altered distribution or exocytosis of cortical granules increasing polyspermy or, on the contrary,

zona pellucida hardening impairing fertilisation (Mavrides and Morroll 2005; Morato *et al*., 2008). Frequently, oocytes also present zona pellucida or cytoplasmic membrane fracture and altered mitochondrial organisation and activity after cryopreservation (Rho *et al*., 2002 ; Pereira and Marques 2008; Wu *et al*., 2006; Cuello *et al*., 2007; Shi *et al*., 2007; Zhou and Li 2009).

Cytoplasmic lipid content and membrane lipid composition is another aspect of oocyte composition that influences its cryotolerance because their lipids undergo phase transition (Ghetler *et al*., 2005). The lipid composition of the membrane strongly influences its properties and its resistance to thermal stress (Arav *et al*., 2000; Zeron *et al*., 2001, 2002). Species whose oocytes have extremely high lipid contents (bovine and porcine) are more sensitive to chilling injury (Arav *et al*., 1996; Naghasima *et al*., 1996; Otoi *et al*., 1997; Ledda *et al*., 2000; Park *et al*., 2005; Somfai *et al*., 2009; Gupta *et al*., 2010; Zhou and Lin 2013).These problems and their effects are summarised in Table 1.2.

Otherwise, cryopreservation could contribute to cellular apoptosis, toxicity, calcium imbalance, yielding of free radicals and general metabolism disturbance (Shaw *et al*., 1999; Mazur *et al*., 2005).

Table 1.2: Problems and effects associated with chilling and freezing of oocytes.

Alteration	Effect
Meiotic spindle depolymerisation Chromosome abnormality	Increase polyploidy and aneuploidy
Disorganize cytoskeleton	Premature cortical granule exocytosis inducing zona pellucida hardening
Microtubule damage	Abnormal mitochondria distribution
Cytoplasmic membrane alteration	Viability reduction
Citoplasmic lipid content alteration	Higher number of small lipid drops
Cellular toxicity	Increase of antioxidant compounds consumption (GSH)

1.4. STRATEGIES TO REDUCE CHILLING INJURY

To overcome the problems associate with the cryopreservation procedures, different strategies have been developed to improve the results. The most common approach is to modify cryopreservation procedures (Seidel 2006), for example by reducing container volumes, varying concentration and types of cryoprotectants or supplementation with various additives. Nevertheless, modifying the cells themselves to make them more cryopreservable has emerged as an alternative (Seidel 2006). Among these strategies are included the addition of molecules to stabilise the plasma membrane or the cytoskeleton, or modification of the oocyte composition.

1.4.1. Reducing container volumes

The smaller the volume is, the higher the probability of vitrification. Smaller volumes allow better heat transfer, which facilities higher cooling rates. In recent years, different carrier tools have been applied to minimise the volume and therefore increase the cooling rate which could allow a moderate decrease in cryoprotectants concentration, thus minimising its toxic and hazardous osmotic effects (Vajta *et al*., 2000; Sagarusty and Arav 2011).

1.4.2. Modification of cryopreservation solution

Use of novel macromolecules and synthetic polymers holds potential for improving oocyte cryopreservation. Studies examining use of Ficoll (Checura and Seidel 2007), fetuin (Horvath and Seidel 2008), hyaluronan (Lane *et al*., 2003) or "Ice Blockers" (SuperCool X-1000 and SuperCool Z-100, Marco-Jiménez *et al*., 2012) have all shown promise in improving cryopreservation outcomes.

In other studies, the addition of linoleic acid albumin to culture media improved cryosurvival of enucleated oocytes (Hochi *et al*., 2000).

On the other hand, modifications of ion levels in extracellular media used to cryopreserve the oocytes, such as removing sodium and replacing it with choline or removal of calcium (Ca_2^+), could facilitate IVF and posterior development (Larman *et al*., 2006) and pregnancy rates of cryopreserved mouse oocytes (Stachecki *et al*., 1998, 2002).

1.4.3. Modification of plasma membrane

There have been many attempts to change plasma membrane composition to improve cryopreservation of embryos and gametes (Arav *et al*., 2000; Zeron *et al*., 2002; Seidel 2006; Pereira *et al*., 2008). The plasma membrane can be enriched with unsaturated fatty acids or cholesterol. The addition of unsaturated fatty acids to ewe and bovine oocytes by electrofusion of liposomes with their plasma membrane decreased their sensitivity to chilling (Zeron *et al*., 2002). On the other hand, adding cholesterol via cyclodextrin may be worth pursuing (Horvath and Seidel 2006). These modifications of lipid membrane composition led to significant improvements in post-thaw oocyte viability and early cleavage, but blastocyst rates still remain lower than those obtained from non-cryopreserved oocytes (Ledda *et al*., 2007). Modification of the lipid phase transition temperature following phosphatidylcholine or dipalmitoylphosphatidylcholine transfer to matured oocytes also reduced chilling sensitivity (Zeron *et al*., 2002). Other authors (Pereira *et al*., 2008) proposed the possibility of direct incorporation of the conjugated isomer of linoleic acid, the trans- 10, cis-12 octadecadienoic acid (t10, c12 CLA) into the

embryo membranes during *in vitro* culture contributing to an increased membrane fluidity (unsaturation level) and so improving embryo resistance to cryopreservation.

Another area worth investigating is the stabilising of cell membranes with trehalose, a compound that many organisms in nature use naturally to increase cryotolerance (Crowe *et al*., 1992; Potts 1994). One issue is how to transfer the compound to the cytoplasm of the cell where it normally functions, as mammalian cell membranes are practically impermeable to sugars. In recent years, several groups have overcome the permeability barrier using different approaches, such as thermotropic lipid-phase transition (Beatti *et al*., 1997), reversible poration by a genetically engineered protein (Ergoglu *et al*., 2000) transfection (Guo *et al*., 2000), ATP poration (Elliott *et al*., 2006) and microinjection techniques to introduce trehalose into individual oocytes (Eroglu *et al*., 2002). The protective actions of trehalose can be attributed to their high glass transition temperature compared to conventional penetrating cryoprotectants and their stabilising effect on lipid membranes as a result of direct interaction with polar head groups (Crowe *et al*., 1993a,b, 1994). Moreover, trehalose protects against osmotic, chemical, and hypoxic stresses (Chen and Haddad, 2004) caused during cryopreservation procedures. Earlier studies using mouse oocytes and zygotes showed that microinjected trehalose at its effective concentrations (0.15M) was non-toxic and quickly eliminated during embryonic development (Eroglu *et al*., 2003, 2005). Furthermore, healthy offspring were obtained from cryopreserved mouse oocytes (Eroglu *et al*., 2009).

Kim *et al*., (2005) used another approach by modifying red blood cells with phosphoenolpyruvate to decrease membrane fragility. A protective effect on the membrane stability and reduction of chilling injury during the cooling rate has been obtained after the addition of anti-oxidant molecules (Zeron *et al*., 1997) or other macromolecules (Hochi *et al*., 2000, Yang *et al*., 2000).

On the other hand, changes in membrane composition by increasing aquaporin 3 expression via injection of cRNA increases water permeability in mouse oocytes and appeared to increase water permeability and improve embryo development following vitrification (Yamaji *et al*., 2011).

1.4.4. Cytoskeleton stabilizing agents

Conditions during cryopreservation can cause irreversible damage to meiotic spindle microtubules (Vincent *et al*., 1990; Rho *et al*., 2002; Mullen *et al*., 2004) and although it may re-polymerise after thawing or warming if temperature recovers, consequences on their function may result (Díez *et al*., 2012). One possible way to enhance the cryotolerance of oocytes and improve the post-thaw survival and subsequent development of vitrified oocytes or embryos may be the use of cytoskeleton stabilising agents such as Cytochalasin B (CB) or Taxol (Pereira and Marques 2008; Chang *et al*., 2011).

CB is a cytoskeletal relaxant considered to make the cytoskeletal elements less rigid (Fujihira *et al*., 2004). The CB effects in oocyte vitrification are controversial and may depend on the species and procedures used. In mature oocyte, CB reduced damage to microtubules and may enhance stabilisation of spindle microtubules during vitrification. In the case of GV oocytes, as no organised meiotic spindle is present, the relaxant effect of CB may preserve the function

of gap junctions between oocyte and granulosa cells, allowing a better penetration of cryoprotectants (Vieira *et al*., 2002). Studies on the effect of pre-treatment with CB on the vitrification of pig (Fujihira *et al*., 2004) and sheep (Silvestre *et al*., 2006) oocytes have been reported.

Taxol™ (paclitaxel) is a diterpenoid taxane used as an antineoplastic agent in patients diagnosed with ovarian cancer, metastatic breast carcinoma and non-small cell lung carcinoma (Pereira and Marques 2008). Taxol interacts with microtubules and increases the rate of polymerisation by reducing the critical concentration of tubulin needed for polymerisation. The cytoskeleton stabiliser was first used to improve cryopreservation of porcine embryos (Dobrinsky *et al*., 2000). Since then, the addition of Taxol to the vitrification solution improves the post-warming development of human (Fuchinoue *et al*., 2004), mouse (Park *et al*., 2001), ovine (Zhang *et al*., 2009), porcine (Shi *et al*., 2006) and bovine (Morato *et al*., 2008) oocytes.

1.4.5. Modification of lipid content

Certain factors such as oocyte and embryo origin (*in vivo* or *in vitro*), species, breed, physiologic state and nutrition affect lipid content (McEvoy *et al*., 2000; Zeron *et al*., 2001, 2002; Genicot *et al*., 2005). Recently, new strategies have been used to reduce intracellular lipid content in porcine and bovine embryos and therefore increase their tolerance to cryopreservation (Nagashima *et al*., 1994; Ushijima *et al*., 1999; Kawakami *et al*., 2008). Mechanical delipidation, through polarisation of the cytoplasmic lipid droplets by centrifugation and physical removal of excess lipid, has been applied to oocytes and embryos from porcine and bovine species (Nagashima *et al*., 1996; Ogawa *et al*., 2010;

Otoi *et al*., 1997; Ushijima *et al*., 1999; Diez *et al*., 2001). In all cases sensitivity to chilling was reduced, increasing their cryopreservation. However, besides being an invasive and extremely labour intensive method, mechanical delipidation increases the potential of pathogen transmission because of the damage inflicted upon the zona pellucida (Somfai *et al*., 2012) and also may alter the developmental potential of the delipidated blastocysts after transfer to recipient heifers (Diez *et al*., 2001). Chemical delipidation has also been studied. Forskolin, a lipolytic agent capable of stimulating lipolysis of triacylglycerols was used (Men *et al*., 2006; Somfai *et al*., 2011). This agent promoted the cryosurvival of porcine IVP embryos after partial delipidation through chemical stimulation of intracellular lipolysis. In recent times, Hara *et al*., (2005) have reported a novel lipid removal method with improved cryotolerance without losing mitochondria from the cytoplasm of porcine GV stage oocytes by being centrifuged under hypertonic conditions in medium containing 0.27M glucose.

1.4.6. Induced resistance

Recently, a novel approach to improve cryotolerance in mammalian embryos and gametes has been introduced by Pribenszky *et al*., (2005). The principle of the method consists of inducing stress resistance in cells by applying a non-lethal stress, such a high hydrostatic pressure (HHP), osmotic, heat or oxidative stress to them. The hypothesis suggests that sub-lethal stress determines stressful conditions leading to cell production and accumulation of chaperone proteins such as heat shock proteins. These proteins could be beneficial to the cells during cryopreservation, which is also a stress inducing procedure (Pribenszky *et al*., 2010). Recently, the application of HHP treatment to porcine oocytes was

reported to benefit their cryosurvival (Du *et al*., 2008; Pribenszky *et al*., 2008). On the other hand, Sakatani *et al*., (2013) have demonstrated that heat stress could induce thermotolerance. This is due in part to the activation of the embryonic genome to allow stimulation of cellular pathways in order to protect the cell, for example by preventing the accumulation of denatured proteins and free radicals. Thus, exposure to a mild heat shock could make cells largely resistant to a subsequent, more severe heat shock.

1.5. HOW TO EVALUATE THE CRYOPRESERVATION PROCEDURE?

There are many systems, both invasive and non-invasive, that have been employed to test the extent of chilling injury of the oocyte after cryopreservation. Some of them have tended to focus on ultrastructural modifications whereas others have examined functional or viability endpoints.

1.5.1. Ultrastructural criteria

Selection of live oocytes by simple observation under a stereomicroscope (shape, colour, aspect of the cytoplasm, polar body, perivitelline space and zona pellucida) is the first non-invasive method which allows us to evaluate oocyte integrity (Somfai *et al*., 2012). On the other hand, plasma membrane integrity has been assessed using different probes as vital stains (trypan blue and fluorescein diacetate, FDA) (Didion *et al*., 1990; Arav *et al*., 2000; Miyake *et al*.,1993; Somfai *et al*., 2012). It has been observed that this test does not seem to compromise the developmental competence of porcine oocytes (Shi *et al*., 2006). Structural evaluation of the meiotic spindle and cytoskeleton have been performed with cell fixation and fluorescent staining (Pickering and Johnson 1987; Diedrich *et al*., 1988; Mandelbaum *et al*., 2004; Ciotti *et al*., 2009; Rojas *et*

al., 2004; Succu *et al*., 2007; Salvetti *et al*., 2010) but this could also be done without requiring cell fixation by using polarised light microscopy, without compromising oocyte viability (Gardner *et al*., 2007; Ledda *et al*., 2007). Moreover, chromosome, cortical granule and mitochondria distribution was analysed to determine the extent of damage after oocyte cryopreservation (Hochi *et al*., 1996; Luvoni 2000; Rho *et al*., 2002; Mavrides and Morroll 2005; Shi *et al*., 2007; Morato *et al*., 2008; Fu *et al*., 2009).

1.5.2. Functional criteria

There are many novel methods that can be used for assessing the functionality of oocytes after the cryopreservation process. These include molecular and biochemical markers. Methods such as quantification of intracellular calcium (Cai), metabolomics, ATP levels, proteomics or analysis of epigenetic modification have all been employed recently (Clark and Swain 2013).

During cryopreservation, oocytes are exposed to conventional permeating cryoprotectants, which leads to an increase in Cai. This increase could initiate oocyte activation, inducing cortical granule release leading to zona hardening (Matson *et al*., 1997; Larman *et al*., 2006; Gardner *et al*., 2007). Analysis of Cai can be essential to the diagnosis of cellular trauma (Jones *et al*., 2004). Its oscillations can be quantified using fluorescent calcium indicators, such as Indo-1, and the resulting changes can be quantified using an intensified charged-coupled device (CCD) camera or photomultiplier tube-based detection systems.

The examination of oocyte metabolome, such as pyruvate uptake, may be useful to determine stress induced by cryopreservation (Lane and Gardner

2001). In the same way, the appearance of lactate dehydrogenase (LDH) in the media surrounding the oocyte could be useful as an indicator of cryo-induced membrane damage (Borini *et al*., 2009).

The energy status, for example ATP content, of oocytes is critical for their maturation and has been suggested as an indicator for the developmental potential of human (Van Blerkom *et al.,* 1995) mouse (Leese *et al*., 1984) and bovine oocytes (Stojkovic *et al*., 2001). A correlation has been observed between ATP content and total cell numbers of blastocysts (Stojkovic *et al*., 2001).

On the other hand, the development of mass spectrometry techniques has allowed us to assess specific protein expression patterns, or the proteomes, through protein chips, in gametes under different conditions (Shau *et al*., 2003; Röcken *et al*., 2004; Gardner *et al*., 2007).

Finally, epigenetic modifications, such as DNA methylation or histone modifications, can be used to assess changes due to cryopreservation, as these modifications alter the functional state of chromatin and trigger or repress gene activation (Clark and Swain 2013).

1.5.3. Viability criteria

Among the methods employed to assess the viability of oocytes after cryopreservation, parthenogenetic activation emerged as an alternative tool to assess *in vitro* developmental rates into blastocysts (Salvetti *et al*., 2010; Naturil-Alfonso *et al.,* 2011). However, parthenogenesis is used in studies where pregnancy rates are not needed (Salvetti *et al*., 2010). The possibility of fertilising

cryopreserved oocytes has been tested by *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) (Ledda *et al*., 2000). Nevertheless, IVF has not been successful in rabbit and a repeatable IVF technique has not yet been developed, possibly due to the lack of an efficient *in vitro* capacitation system for rabbit spermatozoa linked to the poor permeability of sperm plasma membrane (Curry *et al*., 2000). Similarly, ICSI has been widely used in rabbit to study oocyte fertilisation and embryo development (Keefer 1989, Zheng *et al*., 2004). However, this technique is difficult to carry out because rabbit oocytes have rough, dark granules in the plasma and easily lyse and die after the ICSI process (Cai *et al*., 2005), and the success of the process is still very limited (Deng and Yang 2001, Li *et al*., 2001). Thus, *in vivo* fertilisation emerged as an alternative to bypass the inadequacy of conventional *in vitro* fertilisation techniques (Overstreet and Bedford 1974; Motlík and Fulka 1981; Bedford and Dobrenis 1989; Carnevale *et al*., 2005; Deleuze *et al*., 2009). Nevertheless, the best test to determine the viability of these oocytes is to evaluate their capacity to generate viable offspring (Hamano *et al*., 1992).

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2. OBJECTIVES

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2. OBJECTIVES

The aim of this thesis was to optimize the methodologies of oocyte cryopreservation studying the effects of the procedures using the rabbit as an animal model. To this end, the specific objetives of the thesis were as follows:

In chapter 1, the objective was to evaluate the effects of the current methods for the cryopreservation of rabbit oocytes in terms of meiotic spindle configuration, cortical granule distribution and their developmental competence after the procedure.

The objectives in chapter 2 and 3 were to improve the oocyte survival after cryopreservation using different methods to modify the oocytes to make them more cryopreservable. Chapter 2 aimed to assess the effect of the addition of two molecules to stabilize the cytoskeleton, Taxol and Cytochalasin B, on oocyte vitrification analyzing the meiotic spindle configuration, chromosome structure and viability after cryopreservation. Whereas chapter 3 was conducted to determinate if cholesterol could be incorporated into oocyte and to assess the effect of this treatment before cryopreservation on the cleavage rate and subsequent embryonic development.

The three last chapters of this thesis were focused on developing a reliable and reproducible technique to generate live offspring from cryopreserved, slow-frozen and vitrified, rabbit oocytes.

3. CHAPTER I

Effects of Cryopreservation on the Meiotic Spindle, Cortical Granule Distribution and Development of Rabbit Oocytes

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3. CHAPTER I

Abstract

Although much progress has been made in oocyte cryopreservation since 1971, live offspring have only been obtained in a few species and in rabbits. The aim of our study was to evaluate the effect of vitrification and slow freezing on the meiotic spindle, cortical granule (CG) distribution and their developmental competence. Oocytes were vitrified in 16.84% ethylene glycol, 12.86% formamide, 22.3% dimethyl sulphoxide, 7% PVP and 1% of synthetic ice blockers using Cryotop as device or slow freezing in 1.5 M PROH and 0.2 M sucrose in 0.25 ml sterile French mini straws. Meiotic spindle and CG distribution were assessed using a confocal laser-scanning microscope. To determine oocyte competence, *in vitro* development of oocytes from each cryopreservation procedure was assessed using parthenogenesis activation. Our data showed that oocytes were significantly affected by both cryopreservation procedures. In particular, meiotic spindle organization was dramatically altered after cryopreservation. Oocytes with peripheral CG distribution have a better chance of survival in cryopreservation after slow-freezing procedures compared to vitrification. In addition, slow freezing of oocytes led to higher cleavage and blastocyst rates compared to vitrification. Our data showed that, in rabbits, structural alterations are more evident in vitrified oocytes than in slow-frozen oocytes, probably as a consequence of sensitivity to high levels of cryoprotectants. Slow-freezing method is currently the recommended option for rabbit oocyte cryopreservation.

3.1. Introduction

Cryopreservation of embryos and oocytes in animal species is considered an important tool in reproduction biotechnology. As Whittingham (1971), successfully froze mouse embryos, cryopreservation methodology and devices have progressed to increase the number of lines, breeds and species that can be embryo cryostored to preserve animal models or biodiversity or improve the reproductive rate. Although several breakthroughs have been made in oocyte cryopreservation since 1971, live offspring have only been obtained in a few species, such as mouse (Whittingham 1977), human (Chen 1986), rabbit (Al-Hasani *et al.,* 1989), cattle (Fuku *et al.,* 1992), rat (Nakagata 1992), horse (Hochi *et al.,* 1994) and cat (Gómez *et al.,* 2008). Moreover, procedures developed for one species are difficult to adapt to another (Paynter *et al.,* 1999, 2001; Nottola *et al.,* 2008; Pereira and Marques 2008; Noyes *et al.,* 2010).

In general, the low efficiency in oocyte cryopreservation might be due to the complex structure of the oocyte and differences in membrane permeability and physiology with respect to the embryos (Gardner *et al.,* 2007). Most of the components present in oocytes are particularly sensitive to temperature and osmotic pressure. During cooling to ultralow temperatures, cells are exposed to a series of stresses, such as ice formation and dehydration, increasing solute and ionic concentration and viscosity, which contribute to cell damage, for example disassembly of the meiotic spindle apparatus (Rojas *et al.,* 2004; Succu *et al.,* 2007), chromosome and DNA abnormalities (Luvoni 2000) or premature cortical granule (CG) exocytosis leading to zona pellucida hardening (Mavrides and Morroll 2005; Morato *et al.,* 2008). In consequence, the number of births per oocyte cryopreserved is very low.

Recently, most studies have focused on freezing and vitrification (Ledda *et al.,* 2007; Loudrati *et al.,* 2008; Keskintepe *et al.,* 2009; Vutyavanich *et al.,* 2009; Martinez- Burgos *et al.,* 2011), and the results are different depending on the species. In human, vitrification of oocytes shows better results than slow freezing (Fadini *et al.,* 2009), but in rabbit, slow freezing shows higher survival than vitrification (Salvetti *et al.,* 2010). In human, Fadini *et al.,* (2009) drew a comparison of the outcomes obtained using both methods in several studies, and the births per oocyte cryopreserved showed that this rate ranged from 0.9% to 1.4% for slow freezing and vitrification, respectively. In other species, such as bovine, the birth rate ranged from 0.6% to 0.8% (Suzuki *et al.,* 1996; Kubota *et al.,* 1998; Vieira *et al.,* 2002); in mouse, it ranged between 0.8% and 7.6% (Bos-Mikich *et al.,* 1995; Aono *et al.,* 2005; Lee *et al.,* 2010), and in rabbits, using only slow-freezing method, a total of 0.8% resulted in live offspring (Al-Hasani *et al.,* 1989).

In this context, the low competence of cryopreserved oocytes in rabbit is not fully understood. This study focuses on the effects of vitrification and slow freezing for the cryopreservation of rabbit oocytes in terms of meiotic spindle configuration, CG distribution and developmental competence by their parthenogenetic activation.

3.2. Materials and Methods

All chemicals were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain) unless stated otherwise. VM3 medium and Ice blockers SuperCool X-1000 and SuperCool Z-1000 were purchased from 21st Century Medicine Inc. (Fontana, CA, USA).

3.2.1. Oocyte recovery

New Zealand White females were induced to ovulate by intramuscular dose of 1 µg of Busereline acetate. Oocytes were collected from the oviducts 14–15 h after induction by flushing each oviduct with Dulbecco's phosphate-buffered saline without calcium chloride (DPBS) and supplemented with 0.1% (w/v) of bovine serum albumin (BSA). Finally, oocytes were treated for 15 min at room temperature with 0.1% (w/v) hyaluronidase in DPBS, and cumulus cells were removed by mechanical pipetting.

3.2.2. Cryopreservation procedures

The slow-freezing procedure was adapted from previously described methods (Siebzehnruebl *et al.,* 1989). Briefly, oocytes were incubated for 15 min in a solution containing 1.5 M 1,2-propanediol (PROH) in BM. Oocytes were then placed for 10 min in the freezing solution composed of 1.5 M PROH and 0.2 M sucrose in BM and mounted between two air bubbles in 0.25-ml sterile French mini straws (IMV Technologies. L'Aigle, France) sealed by a sterile plug. The straws were then placed in a programmable freezer (Cryologic, CL-8800) for the freezing process. Temperature was lowered from 20ºC to -7ºC at a rate of -2ºC/ min. Manual seeding was performed at -7ºC. Temperature was then lowered to -30ºC at a rate of -0.3ºC/ min. Finally, straws were directly plunged into liquid nitrogen (LN_2) and stored for later use. For thawing, the straws were taken out from the LN_2 into ambient temperature for 10–15 s and plunged into a 20ºC water bath. Oocytes were transferred stepwise into decreasing sucrose solutions (0.5, 0.3 and 0.1 M sucrose in BM) for 5 min before being equilibrated for 10 min in TCM-199 containing 20% FBS. After thawing, the oocytes were

incubated 2 h in medium TCM-199 containing 20% FBS at 38.5ºC and 5% CO_2 in humidified atmosphere.

Vitrification was performed following the minimum essential volume (MEV) method, using Cryotop as device (Kuwayama *et al.,* 2005) and VM3 as vitrification solution (Fahy *et al.,* 2004). Oocytes were first exposed for 3 min to equilibration solution containing 1.7% (w/v) ethylene glycol (EG), 1.3% (w/v) formamide, 2.2% (w/v) dimethyl sulphoxide (DMSO), 0.7% (w/v) PVP K12 (polyvinylpyrrolidone of Mr 5000 Da) and 0.1% (w/v) final concentrations of commercially available SuperCool X-1000 and SuperCool Z-1000 (ice blockers) in base medium (BM: DPBS + 20% foetal bovine serum, FBS). Then, the oocytes were exposed for 1 min to solution containing 4.7% (w/v) EG, 3.6% (w/v) formamide, 6.2% (w/v) DMSO, 1.9% (w/v) PVP K12 and 0.3% (w/v) final concentrations of ice blockers in BM. Finally, the oocytes were transferred to vitrification solution consisting of 16.84% (w/v) EG, 12.86% (w/v) formamide, 22.3% (w/v) DMSO, 7% (w/v) PVP K12 and 1% (w/v) final concentrations of ice blockers in BM before being loaded onto Cryotop devices and directly plunged into LN_2 within 1 min. For warming, oocytes were placed in a solution composed of 1.25 M sucrose in BM for 1 min and later transferred stepwise into 200 µl drops of decreasing sucrose solutions (0.6, 0.3 and 0.15 M sucrose in BM) for 30 s before being equilibrated for 10 min in TCM-199 containing 20% FBS at 38ºC. As with the slow-frozen group, after warming, the oocytes were incubated for 2 h in medium TCM-199 containing 20% FBS at 38.5ºC and 5% CO_2 in humidified atmosphere.

3.2.3. Meiotic spindle immunostaining

Structural evaluation of spindles was performed in the three experimental groups: fresh, vitrified and slow-frozen oocytes. Oocytes were fixed in 4% (w/v) paraformaldehyde in DPBS for 45 min at 38.5ºC and permeabilized for 30 min at 38.5ºC using 0.1% (v/v) Triton X-100 in DPBS. Mouse anti-a-tubulin monoclonal antibody was incubated with fixed oocytes overnight at 4ºC. Samples were then washed three times in a blocking solution (DPBS supplemented with 0.1% (w/v) BSA). Then, oocytes were labelled with fluorescein isothiocyanate (FITC)-conjugated Donkey anti-mouse antibody (Jackson ImmunoResearch) diluted by a ratio of 1:200 for 45 min at 38.5ºC in darkness. After extensive washing, DNA of samples was counterstained with propidium iodide (PI). Finally, samples were mounted (Vectashield Hardset Mounting Medium; Vector Laboratories, Barcelona, Spain) between a coverslip and a glass slide and stored at 4ºC and protected from the light until they were examined. The localizations of meiotic spindle and chromosomes were assessed using a confocal microscope (TCS SL; Leica, Mannheim, Germany). When FITC fluorescence was monitored, the excitation light wavelength was 488 nm and emission light wavelength was 515–535 nm. When PI fluorescence was monitored, the excitation light wavelength was 543 nm and emission light wavelength was 590–630 nm. The meiotic spindle was classified as normal when the classic symmetrical barrel shape was observed, with organized microtubules traversing from one pole to another and the chromosomes were arranged on a compact metaphase plate along the equatorial plane, whereas abnormal spindles showed disorganized, clumped, dispersed or unidentifiable spindle elements with

aberration of chromatin arrangement, clumping or dispersal from the spindle centre. Details of normal and abnormal spindle morphology are shown in Figure 3.1.

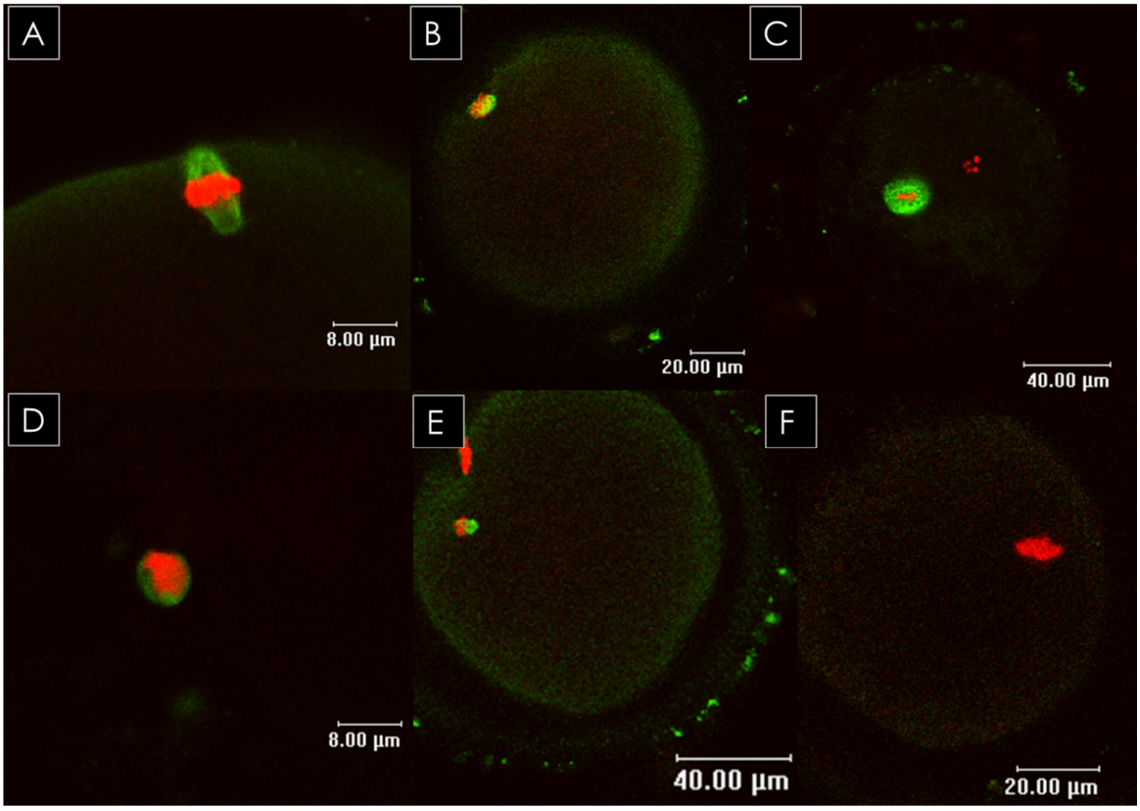

Figure 3.1: Patterns of meiotic spindle of rabbit cryopreserved oocytes. (A) Normal meiotic spindle with chromosomes arrayed at the metaphase plate. (B-E) Abnormal meiotic spindle configuration. (F) Absence of meiotic spindle.

3.2.4. Cortical granule staining

Fresh, vitrified and slow-frozen oocytes were treated with 0.5% (w/v) pronase to digest the zona pellucida. Samples were fixed in DPBS containing 4% (w/v) buffered neutral paraformaldehyde solution for 45 min at 38.5ºC. Then, oocytes were incubated for 30 min at 38.5ºC with permeabilization solution (0.02 % (v/v) Triton X-100). Finally, samples were incubated for 15 min at 38.5ºC in the dark with 100 µg/ml lens culinaris agglutinin labelled with fluorescein isothiocyanate (FITC-LCA) for CG staining. The oocytes were then washed with blocking

solution (7.5% (w/v) BSA), mounted (Vectashield Hardset Mounting Medium; Vector Laboratories) between a coverslip and a glass slide and examined under a confocal laser-scanning microscope (TCS SL; Leica). Cortical granule distribution was classified as peripheral when CGs were adjacent to the plasma membrane, whereas in abnormal oocytes most of the CGs were spread throughout the cortical area in a non-homogeneous, anomalous distribution or CGs were absent because of a premature exocytosis. Details of normal and abnormal CG distribution are shown in Figure 3.2.

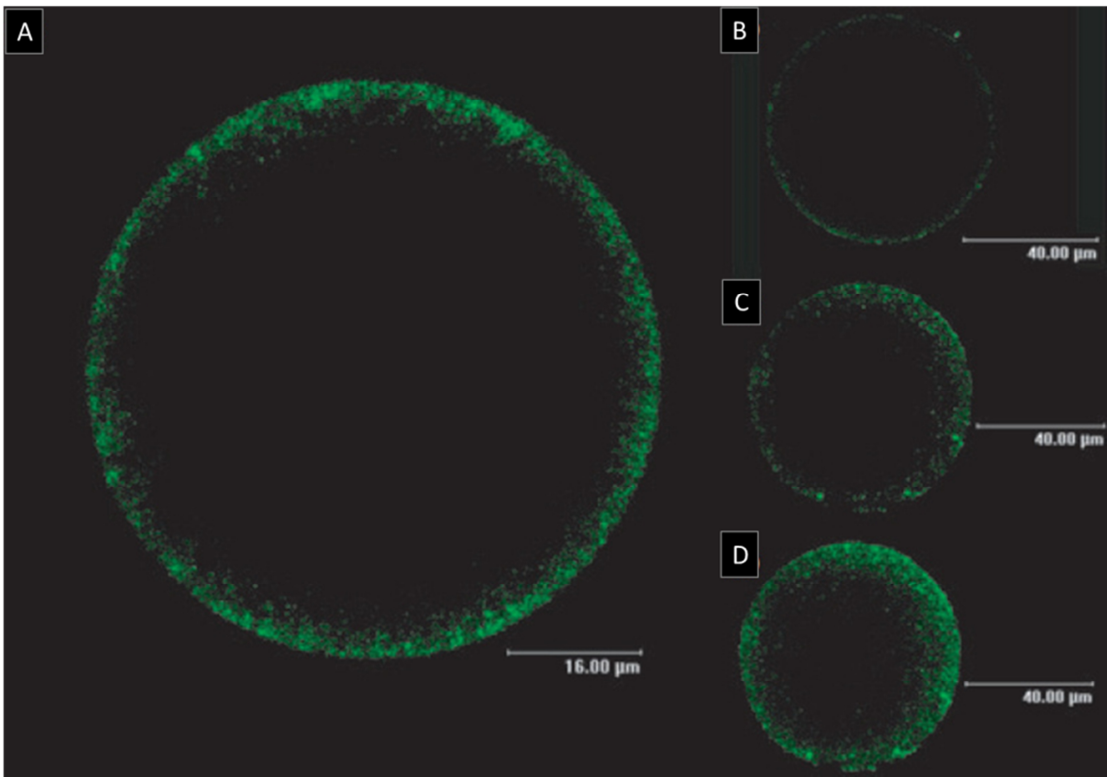

Figure 3.2: Patterns of distribution of cortical granule (CG) of rabbit cryopreserved oocytes. (A) Normal peripheral CG distribution. (B) Exocytosis. (C) Exocytosis and non-homogeneous distribution of CG throughout the cytoplasm. (D) Non-homogeneous distribution of CG throughout the cytoplasm.

3.2.5. Parthenogenetic activation

Oocytes from each experimental group were induced to parthenogenesis with two sets 1 h apart from two DC electrical pulses of 3.2 kV/cm for 20 µs at 1 s apart in an activation medium (0.3 M mannitol supplemented with 100 µM $MgSO_4$ and 100 µM $CaCl_2$), followed by 1 h exposure in TCM-199 medium supplemented with 5 µg/µl of cycloheximide and 2 mM of 6-dimethylaminopurine (6-DMAP). Parthenotes were cultured in 500 µl of TMC-199 supplemented with FBS and layered under paraffin oil at 38.5ºC in 5% CO_2 and saturated humidity. Cleavage rate was recorded at 24 h after *in vitro* activation, and the blastocyst development rate was assessed at 102 h after oocyte activation (Figure 3.3).

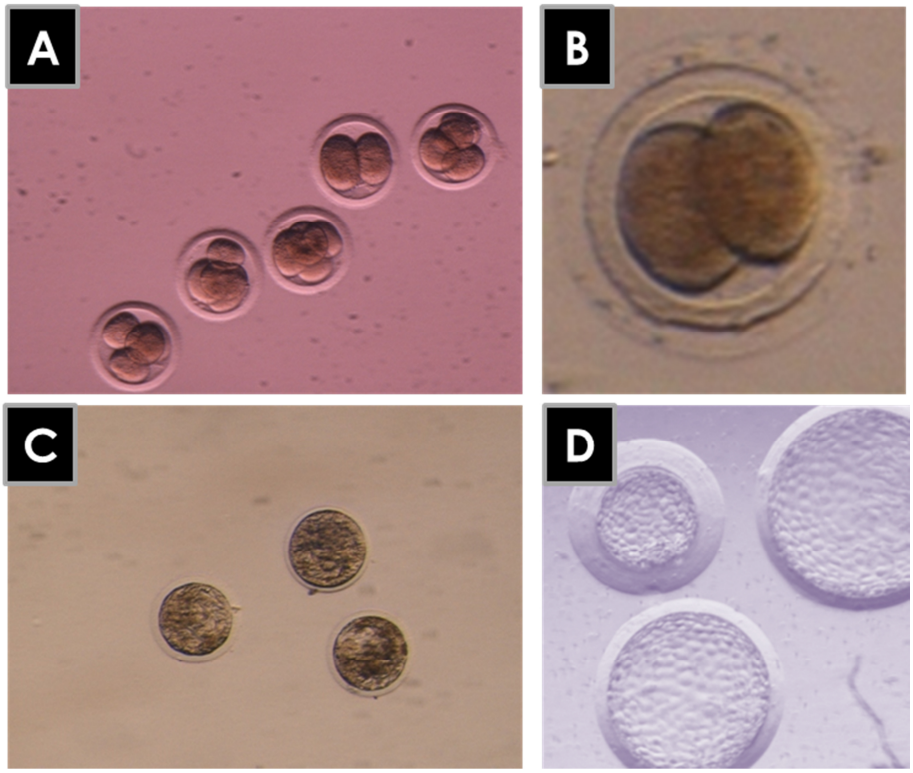

Figure 3.3: *In vitro* (A, C) and *in vivo* (B, D) developmental competence of rabbit oocytes. (A) Cleavage at 24 hours and (C) blastocyst at 102 hours after oocyte activation. (C) Two cels embryos at 24 hours and (D) blastocyst at 102 hours after *in vivo* fertilization.

3.2.6. Experimental design

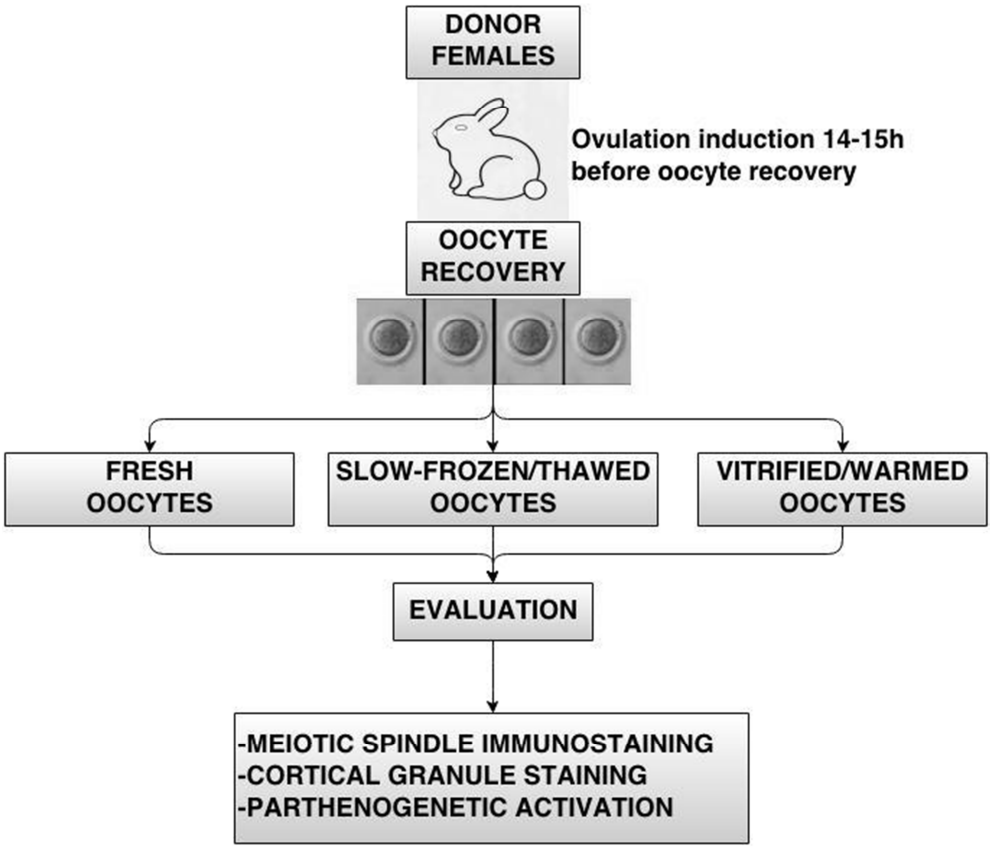

Figure 3.4: Experimental design to study the effects of slow-freezing and vitrification in terms of meiotic spindle configuration, cortical granules (CGs) distribution and developmental competence by their parthenogenetic activation.

3.2.7. Statistical analysis

All data were analysed using the generalized linear model with cryopreservation procedure (fresh, slow-freezing and vitrification) as a fixed factor and replicate and the cryopreservation procedure by replicate interaction as random factors. The replicate and interaction were non-significant and were removed from the model. The error was designated as having a binomial distribution, and the probit link function was used. Binomial data (meiotic spindle, CG distribution, cleavage and blastocyst development) for each oocyte or embryo were assigned a 1 if it had achieved the desired stage or development or a 0 if it had not. All analyses were performed using

SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA, 2002). p values ≤ 0.05 were considered significant. Means are presented ± SEM.

Results

Effect of cryopreservation method on the meiotic spindle

The spindle morphology was assessed in a total of 258 oocytes in 10 sessions. The proportion of meiotic spindle to a normal shape decreased from 89.7% for fresh oocytes to 21.8% after slow freezing and to 18.2% after vitrification (Table 3.1). Differences between the two cryopreservation methods were not significant.

Table 3.1: Proportion of fresh, slow-frozen and vitrified rabbit metaphase II oocytes with normal meiotic spindle organization.

Type	n	Meiotic spindle (%)
Fresh oocytes	29	89.7[a]
Slow-Frozen oocytes	119	21.8[b]
Vitrified oocytes	110	18.2[b]

n: Number of oocytes. Different superscripts represent significant difference (P ≤ 0.05)

Effect of cryopreservation method on cortical granule distribution

The CG distribution analysis was assessed in a total of 149 oocytes in five sessions. Table 3.2 shows the percentage of oocytes showing different types of CG distribution in fresh, slow-frozen and vitrified groups. Some 95.2% of fresh oocytes presented normal peripheral CG distribution. Cryopreservation had a major influence on the normal CG distribution, decreasing it significantly to 34.9% after slow-freezing and 14.5% after vitrification. The difference between the two cryopreservation methods was significant.

Table 3.2: Percentage of fresh, slow-frozen and vitrified metaphase II rabbit oocytes with peripheral cortical granules migration.

Type	n	Peripheral CG migration (%)
Fresh oocytes	21	95.2[a]
Slow-Frozen oocytes	66	34.9[b]
Vitrified oocytes	62	14.5[c]

n: Number of oocytes. CG: Cortical granule. Different superscripts represent a significant difference ($P \leq 0.05$).

Effect of cryopreservation method on development after parthenogenetic activation

Parthenogenetic activation was assessed in a total of 346 oocytes in seven sessions. Table 3.3 shows the developmental rates of fresh, slow-frozen and vitrified oocytes at 24 and 102 h after parthenogenetic activation. Twenty-four hours after parthenogenetic activation, 79.3% of fresh oocytes cleaved. Cryopreservation had an influence on the cleavage rates, which decreased to 32.1% after slow-freezing and 18.7% after vitrification. Statistical difference was observed between the cryopreservation methods. One hundred and two hours after parthenogenetic activation, the proportion of fresh oocytes that developed until blastocyst stage was 26.9%. Once again, the cryopreservation process had a substantial influence on the developmental ability of slow-frozen oocytes, with 4.2% of activated ova developing into blastocysts, while no vitrified oocyte reached this stage.

Table 3.3: Parthenogenetic development rate at 24 hours and 102 hours after activation of fresh, slow-frozen and vitrified oocytes.

Type	n	Cleavage rate (%)	Blastocyst rate (%)
Fresh oocytes	121	79.3 [a]	26.9 [a]
Slow-Frozen oocytes	118	32.1 [b]	4.2 [b]
Vitrified oocytes	107	18.7 [c]	-

n: Number of oocytes. Different superscripts represent a significant difference ($P < 0.05$).

Discussion

Rabbit has been used as a model organism to study mammalian reproduction for over a century (Heape 1891; Pincus 1939; Chang *et al.,* 1970). However, while numerous reports of studies designed to investigate oocyte cryopreservation have been published in some species (Mullen 2007), few works have been performed in rabbit (Diedrich *et al.,* 1988; Al-Hasani *et al.,* 1989; Siebzehnruebl *et al.,* 1989; Vincent *et al.,* 1989; Cai *et al.,* 2005; Salvetti *et al.,* 2010; Wang *et al.,* 2010) and only one recent work compared slow-freeze and vitrification methods (Salvetti *et al.,* 2010). Moreover, live offspring were obtained only in one report, using slow-freezing method (Al-Hasani *et al.,* 1989).

The impaired meiotic spindle and peripheral CG competence and drastic reduction in development up to the blastocyst stage observed in our study for both cryopreserved methods could result from the exposure of oocytes to low temperatures and high cryoprotectant concentrations. The spindle in mammalian oocytes is highly sensitive to cryoprotectants and low temperatures (Johnson and Pickering 1987; Pickering and Johnson 1987; Mandelbaum *et al.,* 2004; Ciotti *et al.,* 2009). Rabbit oocytes are not very sensitive to low temperatures but present particularly sensitivity to high levels of cryoprotectants, and this has been shown to have a dramatic effect on the

meiotic spindle configuration (Diedrich *et al.,* 1988; Vincent *et al.,* 1989; Cai *et al.,* 2005; Salvetti *et al.,* 2010). Therefore, the high concentration of cryoprotectants required to achieve vitreous state may exert a highly detrimental effect on spindle configuration. The results suggest that meiotic spindle of rabbit oocytes is highly sensitive to cryopreservation process; however, in this study, difference was not observed between vitrified and frozen oocytes in terms of spindle integrity. These discrepancies may be attributed to the differences in vitrification protocols. Salvetti *et al.,* (2010) used EG, DMSO, trehalose and Ficoll combined with open-pulled straws, while our experiment used the VM3 solution because it was previously designed to present low toxicity (Fahy *et al.,* 2004; Checura and Seidel 2007), following the minimum essential volume method, using Cryotop as device, which allowed high cooling rate, minimizing the toxic and osmotic effects (Vajta and Kuwayama 2006; Yavin *et al.,* 2009).

Inappropriate conditions of exposure to cryoprotectants and cooling may induce exocytosis and disorder of CGs after vitrification of the oocytes (Bernard and Fuller 1996). In our study, the CG distribution generally appeared to be altered after cryopreservation, especially after vitrification. To our knowledge, no previous studies of distribution after cryopreservation have been reported in rabbit. In other species, it was reported that cryopreservation has an effect on CG exocytosis as a result of disruption of the cytoskeleton that might lead to premature release of CGs and zona pellucida hardening (Vincent *et al.,* 1990; Ghetler *et al.,* 2006; Morato *et al.,* 2008; Nottola *et al.,* 2009; Tan *et al.,* 2009; Coticchio *et al.,* 2010). Induced zona pellucida hardening blocks sperm binding and penetration (Coticchio *et al.,* 2001; Mavrides and Morroll 2005; Tian *et al.,*

2007). High rates of abnormal CG distribution suggest that our vitrification protocol induces damage on cytoskeleton microfilaments causing exocytosis of CG, although it may not lead to spindle alteration. Further studies are needed to clarify the effects of cryopreservation on sperm penetration in rabbits.

Parthenogenesis activation may be an appropriate tool to assess *in vitro* rates of development into blastocysts of cryopreserved rabbit oocytes, because *in vitro* fertilization (IVF) is not well established in rabbit species, partly because problems are encountered with the capacitation of semen (Brackett *et al.,* 1982). Better results are obtained today when semen is capacitated *in vivo*. Parthenogenesis appears to be an interesting, quick and efficient tool to assess *in vitro* the developmental rates to blastocyst stage of rabbit oocytes in preliminary studies, when pregnancy rates are not needed (Salvetti *et al.,* 2010).

Thus, the cryoprotectants and low temperatures lead to depolymerization of microtubules and disrupt the network of the meiotic spindle and CGs in rabbit oocytes regardless of the cryopreservation procedure. Abnormal spindle and dispersed chromosomes have been related to poor rates of fertilization and development (Chen *et al.,* 2003; Magli *et al.,* 2010). The cleaved and blastocyst rates of fresh oocytes in this study were higher than for cryopreserved oocytes. Nevertheless, the development rate of vitrified oocytes was lower than in slow-freezing procedure. This latter result could confirm that rabbit oocytes are very sensitive to high concentration of cryoprotectants (Diedrich *et al.,* 1988; Vincent *et al.,* 1989; Cai *et al.,* 2005; Salvetti *et al.,* 2010). However, the developmental rate to blastocyst stage was only obtained using slow-freezing method after parthenogenesis activation. Our developmental rate to blastocyst stage was

similar to those previously described (Salvetti *et al.,* 2010) and similar to those obtained after IVF (Al-Hasani *et al.,* 1989) ICSI (Cai *et al.,* 2005; Wang *et al.,* 2010) or *in vivo* fertilization (Vincent *et al.,* 1989).

Our data showed that structural alterations are more evident in vitrified than in slow-frozen rabbit oocytes, probably as a consequence of sensitivity to high cryoprotectant levels. High rates of structural damage in cryopreserved oocytes have also been associated with reduced developmental competence after parthenogenetic activation. Considering the results presented in this work, slow-freezing method seems to be a valuable option for rabbit oocyte cryopreservation, although both methods need more studies to clarify cellular mechanisms associated with cryoinjury and ensure better outcomes.

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4. CHAPTER II

Post-warming competence of *in vivo* matured rabbit oocytes treated with cytoskeletal stabilization (Taxol) and cytoskeletal relaxant (Cytochalasin B) before vitrification

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4. CHAPTER II

Abstract

The aim of this study was to investigate the effect of Taxol and Cytochalasin B (CB) on the spindle, chromosome configuration and development to blastocyst stage after parthenogenesis activation of *in vivo* matured rabbit oocytes after vitrification. Oocytes were randomized into four groups: oocytes treated with Taxol or CB before vitrification, oocytes without treatment before vitrification and fresh oocytes. Oocytes were vitrified using Cryotop method, and meiotic spindle and chromosomal distribution were assessed with a confocal laser scanning microscopy. To determine oocyte competence, *in vitro* development of oocytes was assessed with parthenogenesis activation. There were no significant differences in the frequencies of normal spindle (33.0%, 31.0% and 32.6%, for non-treated, Taxol-treated and CB-treated oocytes, respectively) and chromosome (48.3%, 46.6% and 34.8%, for non-treated, Taxol-treated oocytes and CB-treated oocytes respectively) in vitrified groups, but significantly lower than those of fresh group (89.7% and 90.2%, for normal spindle and chromosome organization, respectively). No statistical differences were found in the cleavage and blastocyst development rates between non-treated and Taxol-treated oocytes (7.7% and 1.5% and 13.7% and 4.6%, for non-treated and Taxol-treated oocytes, respectively), although they were significantly lower than in the fresh group (42.3% and 32.1%, for cleavage and blastocyst development, respectively). Oocytes treated with CB failed to reach blastocyst stage. Normal spindle, chromosome configuration and blastocyst development of *in vivo* matured rabbit oocytes were damaged in vitrification, which was not improved by Taxol and CB pre-treatment before vitrification. Moreover, a detrimental effect on blastocyst development of CB pre-treatment before vitrification was observed.

4.1. Introduction

Although many breakthroughs have been made in oocyte cryopreservation in recent years, no general protocol has yet been established (Nottola *et al.,* 2008; Pereira and Marques 2008; Noyes *et al.,* 2010). Oocytes are particularly difficult to cryopreserve successfully, which may be due to the complex structure and biological processes of the oocyte (Gardner *et al.,* 2007). Cryopreservation induces several types of undesirable damage by mechanical, thermal or chemical factors (Shi *et al.,* 2006; Morato *et al.,* 2008a). During cryopreservation, an abnormal spindle configuration has been observed, mainly due to the disorganization or disassembly of meiotic microtubules (Rojas *et al.,* 2004; Succu *et al.,* 2007). Moreover, this cytoskeletal disorganization could cause chromosome abnormalities (Luvoni 2000), an altered distribution or exocytosis of cortical granules (Mavrides and Morroll 2005; Morato *et al.,* 2008a) and cytoplasmic membrane fracture (Pereira and Marques 2008; Wu *et al.,* 2006; Cuello *et al.,* 2007; Zhou and Li 2009). Negative effects of chilling sensitivity and reactive oxygen species inherent to the cryopreservation process have also been described (Ruffing *et al.,* 1993; Gupta *et al.,* 2010).

Stabilizing the cytoskeleton system during vitrification has been posited as a possible way to improve the cryotolerance of oocytes and improve post-warm survival and subsequent development (Morato *et al.,* 2008b). Among the molecules currently used to reduce cytoskeletal injury, Taxol (cytoskeletal stabilizers) and Cytochalasin B (cytoskeletal destabilizers) have been used as an alternative to optimize oocyte cryopreservation protocols (Shi *et al.,* 2006; Dobrinsky *et al.,* 2000; Park *et al.,* 2001; Fujihira *et al.,* 2004; Silvestre *et al.,* 2006).

Taxol is a diterpenoid that interacts with microtubules and increases the rate of polymerization by reducing the critical concentration of tubulin needed for polymerization (Mailhes *et al.,* 1999). The addition of Taxol to the vitrification solution improves post-warming development of vitrified mouse, human, porcine and bovine oocytes (Shi *et al.,* 2006; Morato *et al.,* 2008a,b; Park *et al.,* 2001; Fuchinoue *et al.,* 2004). Cytochalasin B (CB) is a cell-permeable mycotoxin that acts as a cytoskeletal relaxant, inhibiting microfilament synthesis and making the cytoskeletal elements less rigid (Fujihira *et al.,* 2004). Thus, CB can reduce damage to microtubules and enhance spindle microtubule stabilization during vitrification (Dobrinsky *et al.,* 2000; Fujihira *et al.,* 2004; Isachenko *et al.,* 1998; Rho *et al.,* 2002).

While numerous reports describe the effect of cytoskeleton stabilizers in vitrified oocytes in some species (Shi *et al.,* 2006; Morato *et al.,* 2008a,b; Park *et al.,* 2001; Fujihira *et al.,* 2004; Silvestre *et al.,* 2006; Zhang *et al.,* 2009), as far as we know, no reports are available in rabbit. Rabbit has been used as a model organism to study the mammalian reproduction for over a century (Heape 1891; Pincus 1939; Chang *et al.,* 1970). Nevertheless, a few works have been carried out in rabbit oocyte cryopreservation (Diedrich *et al.,* 1988; Al-Hasani *et al.,* 1989; Vincent *et al.,* 1989; Siebzehnruebl *et al.,* 1989; Cai *et al.,* 2005; Salvetti *et al.,* 2010; Wang *et al.,* 2010; Jiménez-Trigos *et al.,* 2011). Moreover, live offspring have been only obtained in rabbit using slow-freezing method (Al-Hasani *et al.,* 1989).

The aim of this study was thus to assess the effect of Taxol and CB on *in vivo* mature rabbit oocyte vitrification, analysing the meiotic spindle configuration, chromosome structure and viability by parthenogenetic activation.

4.2. Materials and Methods

All chemicals were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain) unless stated otherwise.

4.2.1. Oocyte recovery

New Zealand White females were induced to ovulate by a 1µg intramuscular dose of buserelin acetate. Does were slaughtered 14–15 h post-induction of ovulation, and the reproductive tract was immediately removed. Oocytes were recovered by perfusion of each oviduct with 5 ml of pre-warmed Dulbecco's phosphate-buffered saline without calcium chloride (DPBS) supplemented with 0.1% (w/v) of bovine serum albumin (BSA) (Naturil-Alfonso *et al.*, 2011) (Figure 4.1). Finally, oocytes were treated for 15 min at room temperature with 0.1% (w/v) hyaluronidase in DPBS, and cumulus cells were removed by mechanical pipetting.

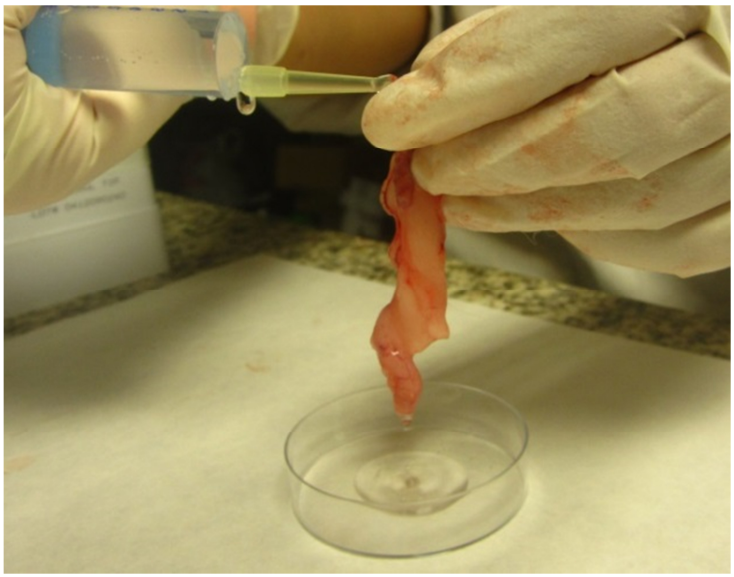

Figure 4.1: Oocyte recovery by perfusion of each oviduct with 5mL of Dulbecco's phosphate-buffered saline without calcium chloride (DPBS) supplemented with bovine serum albumin (BSA).

4.2.2. Cytoskeleton treatment

Before vitrification, oocytes were randomly distributed in two groups corresponding to the incubation in TCM-199 supplemented with 20% (v/v) foetal bovine serum (FBS) with 1 µmol of Taxol or with 7.5 µg / ml of CB, for 30 min at 38°C (Fujihira *et al.,* 2004, 2005).

4.2.3. Vitrification procedure

The vitrification protocol with Cryotop device and solution has been described by Kuwayama *et al.,* (2005). Oocytes were first exposed for 3 min to equilibration solution containing 3.75% (w/v) ethylene glycol (EG), 3.75% (w/v) dimethyl sulphoxide (DMSO), in base medium (BM: TCM-Hepes + 20% (v/v) serum substitute supplement, SSS™ (Irvine Scientific, County Wicklow, Ireland). Then, the oocytes were exposed for 3 min to solution containing 5% (w/v) EG, 5% (w/v) DMSO in BM, after which the oocytes were placed for 9 min in solution containing 7% (w/v) EG and 7% (w/v) DMSO in BM. Finally, the oocytes were transferred to vitrification solution consisting of 15% (w/v) EG, 15% (w/v) DMSO and 0.5 M sucrose in BM before being loaded onto Cryotop devices (Figure 4.2) and directly plunged into liquid nitrogen (LN_2) within 1 min.

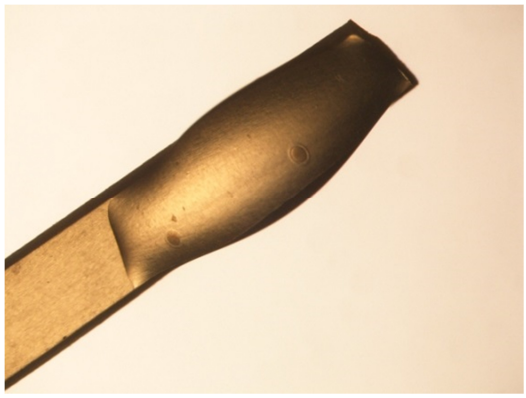

Figure 4.2: Oocytes loaded onto Cryotop device before plunged into liquid nitrogen (LN_2).

For warming, oocytes were transferred stepwise into decreasing sucrose solutions (1 M for 1 min and 0.5 M for 3 min) and then washed twice in BM for 5 min. After warming, the oocytes were incubated for 2 h in medium TCM-199 containing 20% (v/v) FBS at 38.5ºC and 5% CO_2 in humidified atmosphere.

4.2.4. Meiotic spindle immunostaining and chromosome staining

Structural evaluation of spindles was performed in the four experimental groups: fresh, vitrified, vitrified plus Taxol and vitrified plus Cytochalasin B group. After incubation, oocytes were fixed in 4% (w/v) paraformaldehyde in DPBS for 45 min at 38.5ºC and permeabilized for 30 min at 38.5ºC using 0.1% (v/v) Triton X-100 in DPBS. Mouse anti-a-tubulin monoclonal antibody was incubated with fixed oocytes overnight at 4ºC. Samples were then washed thrice in a blocking solution (DPBS supplemented with 0.1% (w/v) BSA). Then, oocytes were labelled with fluorescein isothiocyanate (FITC)-conjugated Donkey anti-mouse antibody (Jackson Immunoresearch) diluted by a ratio of 1:200 for 45 min at 38.5ºC in darkness. After extensive washing, DNA of samples was counterstained with propidium iodide (PI). Finally, samples were mounted (Vectashield Hardset Mounting Medium; Vector Laboratories, Barcelona, Spain) between a coverslip and a glass slide and stored at 4ºC and protected from the light until they were examined. The localizations of meiotic spindle and chromosomes were assessed with a confocal microscope (TCS SL; Leica, Mannheim, Germany). When FITC fluorescence was monitored, the excitation light wavelength was 488 nm and emission light wavelength was 515–535 nm. When PI fluorescence was monitored, the excitation light wavelength was 543 nm and emission light wavelength was 590–630 nm. The meiotic spindle was classified as normal when

the classic symmetrical barrel shape was observed, with organized microtubules traversing from one pole to another and the chromosomes were arranged on a compact metaphase plate along the equatorial plane, whereas abnormal spindles showed disorganized, clumped, dispersed or unidentifiable spindle elements with aberration of chromatin arrangement, clumping or dispersal from the spindle centre (pattern of classification according with Jiménez-Trigos *et al.,* 2011). Chromosomal organization was classified as normal when the structure presented condensed chromosomes aligned in the metaphase plate at the middle of the meiotic spindle and abnormal when the chromosomes were dispersed or with an aberrant less condensed appearance or absent (pattern of classification according with Salvetti *et al.,* 2010).

4.2.5. Parthenogenetic activation

After incubation, oocytes from each experimental group were induced to parthenogenesis with two sets 1 h apart of two DC electrical pulses of 3.2 kv/cm for 20 µs at 1 s apart in an activation medium (0.3 M mannitol supplemented with 100 µM $MgSO_4$ and 100 µM $CaCl_2$), followed by 1-h exposure in TCM-199 medium supplemented with 5 µg/µl of cycloheximide and 2 mM of 6-dimethylaminopurine (6-DMAP). Parthenotes were cultured in 500 µl of TMC-199 supplemented with 20% (v/v) FBS and layered under paraffin oil at 38.5ºC in 5% CO_2 and saturated humidity. Cleavage rate was recorded at 24 h after *in vitro* activation, and the blastocyst development rate was assessed at 102 h after oocyte activation.

4.2.6. Experimental design

In vivo matured oocytes were randomly classed into the following four groups (Figure 4.3):

1. Fresh control. No treatment was performed.

2. Vitrified control. Oocytes were vitrified /warmed as in the Cryotop vitrification procedure mentioned above.

3. Taxol plus vitrification. Oocytes pre-treated with Taxol and vitrified / warmed as in the Cryotop vitrification procedure.

4. CB plus vitrification. Oocytes pretreated with Cytochalasin and vitrified /warmed as in the Cryotop vitrification procedure.

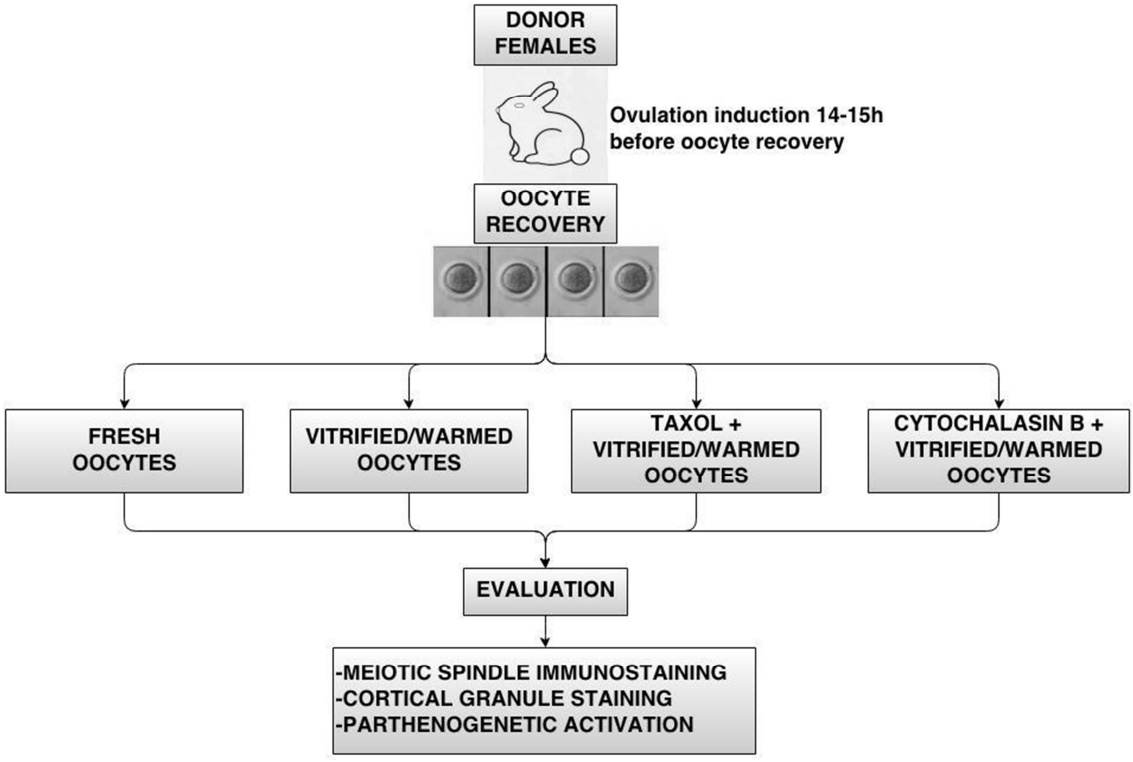

Figure 4.3: Experimental design to study the effect of Taxol and Cytochalasin B pre-treatment before vitrification on normal spindle, chromosome configuration and cleavage and blastocyst development after parthenogenetic activation.

4.2.7. Statistical analysis

All data were analysed using the generalized linear model with experimental group (fresh, vitrified, Taxol and CB pre-treatment) as a fixed factor, and replicate and the experimental group by replicate interaction as random factors. The replicate and interaction were nonsignificant and were removed from the model. The error was designated as having a binomial distribution, and the probit link function was used. Binomial data (meiotic spindle, chromosomal status, cleavage and blastocyst development) for each oocyte or embryo were assigned a one if it had achieved the desired stage of development or a 0 if it had not. All analyses were performed with SPSS 16.0 software package (SPSS Inc., Chicago, Il, USA, 2002). p values ≤ 0.05 were considered significant. Means are presented ± SEM.

4.3. Results

4.3.1. Analysis of meiotic spindle

The spindle morphology was assessed in a total of 325 oocytes in 10 sessions. Table 4.1 shows the percentage of oocytes displaying the normal meiotic spindle configuration in fresh control, vitrified control, Taxol and CB groups. The proportion of meiotic spindle with a normal shape fell from 89.7% for fresh control oocytes to 33.0%, 32.6% and 31.0% for vitrified control, CB and Taxol groups, respectively. Differences between the vitrification method with and without cytoskeleton stabilizer treatment were not significant.

Table 4.1: Meiotic spindle morphology observed in metaphase II rabbit oocytes after vitrification with or without Taxol and CB pre-treatment.

Type	n	Meiotic spindle (%)
Fresh oocytes	29	89.7[a]
Vitrified non-treated oocytes	91	33.0[b]
Vitrified Taxol-treated oocytes	116	31.0[b]
Vitrified CB-treated oocytes	89	32.6 [b]

n: Number of oocytes. CB: Cytochalasin B. Different superscripts represent significant difference ($p < 0.05$).

4.3.2. Chromosome analysis

Chromosome status was assessed in a total of 325 oocytes in 10 sessions. Table 4.2 shows the percentage of oocytes presenting the normal chromosomal structure in fresh control, vitrified control, Taxol and CB groups. Some 70% of fresh oocytes presented normal chromosome alignment. Vitrified oocytes (treated or untreated) had significantly lower percentages of compact chromosomes than the fresh controls, but no significant differences were observed between these groups.

Table 4.2: Chromosome alignment observed in metaphase II rabbit oocytes after vitrification with or without Taxol and CB pre-treatment.

Type	n	Chromosome (%)
Fresh oocytes	29	90.2[a]
Vitrified non-treated oocytes	91	48.3[b]
Vitrified Taxol-treated oocytes	116	46.6[b]
Vitrified CB-treated oocytes	89	34.8 [b]

n: Number of oocytes. CB: Cytochalasin B. Different superscripts represent significant difference ($p < 0.05$).

4.3.3. Analysis of development after parthenogenetic activation

Parthenogenetic activation was assessed in a total of 351 oocytes in 10 sessions. Table 4.3 shows the developmental rates of fresh control and vitrified (treated or untreated) oocytes at 24 and 102 h after parthenogenetic activation. Twenty-four hours after parthenogenetic activation, 42.3% of fresh oocytes cleaved. Vitrification had an influence on the cleavage rates, which decreased to 7.7% after vitrification and 13.7% and 1.2% when oocytes were treated with Taxol and CB, respectively. Statistical difference was observed between treatment groups but not between Taxol and vitrified control groups. One hundred and two h after parthenogenetic activation, the proportion of fresh oocytes that developed until blastocyst stage was 32.1%. Vitrification also had a substantial influence on the developmental ability of non-treated oocytes, with 1.5% and 4.6% of vitrified control and Taxol group, respectively, developing into blastocysts, while no oocyte pre-treated with CB reached this stage.

Table 4.3: Parthenogenetic development rate at 24 and 102 h after activation of fresh, vitrified with or without Taxol or CB oocytes.

Type	n	Cleavage rate (%)	Blastocyst rate (%)
Fresh oocytes	137	42.3 [a]	32.1 [a]
Vitrified non-treated oocytes	65	7.7 [b]	1.5 [b]
Vitrified Taxol-treated oocytes	66	13.7 [b]	4.6 [b]
Vitrified CB-treated oocytes	83	1.2 [c]	-

n: Number of oocytes. CB: Cytochalasin B. Different superscripts represent a significant difference ($p < 0.05$).

4.4. Discussion

Oocytes are particularly difficult to cryopreserve successfully resulting in low blastocyst production rates after warming, fertilization and culture (Vajta *et al.,* 1998; Mavrides and Morroll 2005). One of the main impacts on metaphase II vitrified oocytes is meiotic spindle disorganization followed by microtubule depolymerization (Shaw *et al.,* 2000; Men *et al.,* 2002; Rojas *et al.,* 2004). It is known that appropriate organization of spindle microtubules is essential for correct alignment and segregation of chromosomes (Eroglu *et al.,* 1998). Rabbit oocytes present extremely sensitivity to high concentration of cryoprotectants required to achieve vitreous state, and this has been shown to have a dramatic effect on the meiotic spindle configuration (Diedrich *et al.,* 1988; Vincent *et al.,* 1989; Cai *et al.,* 2005; Salvetti *et al.,* 2010; Jiménez-Trigos *et al.,* 2011).

In the present study, we studied the effects of vitrifying rabbit oocytes by Cryotop method using a cytoskeleton stabilizer and cytoskeleton relaxant before the cryopreservation. The results for post-warm meiotic spindle, chromosome configuration and development after parthenogenesis activation showed that competence of vitrified rabbit oocytes was not improved by pre-treatment of Taxol and CB before vitrification. Instead, in other species (porcine and bovine), Taxol or CB have been shown to reduce microtubular injury in oocytes during vitrification (Isachenko *et al.,* 1998; Dobrinsky *et al.,* 2000; Rho *et al.,* 2002; Fujihira *et al.,* 2004; Shi *et al.,* 2006; Morato *et al.,* 2008a,b), although the beneficial effect remains controversial. Some studies with CB in bovine and sheep immature oocytes did not observe any improvement (Mezzalira *et al.,* 2002; Silvestre *et al.,* 2006). To our knowledge, this is the first study where both agents have been applied in rabbit oocyte vitrification.

The results demonstrate that pre-treatment with Taxol (cytoskeleton stabilizers) and CB (cytoskeleton relaxant) did not improve the normal spindle and chromosome configuration and the development to blastocyst stage after parthenogenesis activation. Moreover, the developmental rate to blastocyst stage was only obtained using Taxol. Structural alterations in rabbit oocytes after cryopreservation have been reported previously, with results coinciding with the data presented here (Salvetti *et al.,* 2010; Jiménez- Trigos *et al.,* 2011). No beneficial effects were obtained when using Taxol and CB at the concentration and equilibration time used in this experiment, so the conditions used may be not optimum in rabbit.

Schmidt *et al.,* (2004) and Zhang *et al.,* (2009) reported that the embryo developmental capacity of vitrified oocytes improved by CB treatment in some species. In contrast, the ability of Taxol to improve embryo development of cryopreserved oocyte is moot, as a positive effect was observed by Park *et al.,* (2001) in mouse, Schmidt *et al.,* (2004) in bovine, Shi *et al.,* (2006) and Ogawa *et al.,* (2010) in porcine and Zhang *et al.,* (2009) in ovine oocytes. However, no improvements in post-warming developmental capacity were observed in other studies (Mezzalira *et al.,* 2002; Vieira *et al.,* 2002). Fujihira *et al.,* (2005) showed that the addition of Taxol did not improve the post-warming developmental capacity of *in vitro* matured porcine oocytes. In our study, no significant difference was observed in the cleavage and blastocyst rates between the vitrified and Taxol-treated oocytes before vitrification. However, in the group treated with CB, none of the oocytes reached the blastocyst stage. The reason for this was not apparent. It may have been caused by an excess of CB making the cytoskeletal elements less rigid and more elastic and perhaps inducing a translocation of organelles (Sun *et al.,* 2001; Kabashima *et al.,* 2007). Suzuki *et al.,* (2006) proposed that altered distribution of mitochondria may be

one of the reasons for the low developmental ability of embryos cultured *in vitro*. More research is needed into the efficiency of CB, including the dosage and animal species applied.

Our results showed that the use of these stabilizer agents had no positive effect on the meiotic spindle, chromosome configuration and development capacity of vitrified rabbit oocytes. The cytoskeleton stabilizer effects are controversial, and more studies are needed to enhance cryopreservation procedures.

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5. CHAPTER III

Treatment with cholesterol-loaded methyl-β-cyclodextrin increased the cholesterol in rabbit oocytes, but did not improve developmental competence of cryopreserved oocytes

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5. CHAPTER III

Abstract

Membrane cholesterol:phospholipids ratio is an important determinant of cell chilling sensitivity. At low temperatures, major membrane destabilisation occurs when the membrane undergoes a phase transition. To increase membrane fluidity and stability during cooling and thus increase oocyte cryoresistance, cholesterol has been added to the plasma membrane. This study was conducted to determine if cholesterol could be incorporated into rabbit oocytes by incubation with cholesterol-loaded methyl-β-cyclodextrin (CLC) and if added cholesterol could improve the developmental ability of cryopreserved oocytes after parthenogenetic activation or intracytoplasmic sperm injection. Fresh, frozen and vitrified oocytes incubated with CLC containing 20% NBD-labelled cholesterol (NBD-CLC) were evaluated using confocal microscopy. Fluorescence intensity was higher in fresh oocytes than in cryopreserved ones. Pre-treating rabbit oocytes with 1 mg of NBD-CLC/mL did not improve cleavage and developmental rates after cryopreservation. Results showed that treatment with CLC increased the cytoplasmic cholesterol content, but did not improve cleavage rate and developmental competence of cryopreserved oocytes.

5.1. Introduction

Cryopreservation of oocytes is a promising technique for longterm preservation of female genetic material in several mammalian species. Oocyte cryopreservation could be very useful for many assisted reproductive technologies and the production of animals in breeding programmes. Nevertheless, oocytes have not been successfully cryopreserved and this technique should continue to be considered experimental (Jain and Paulson 2006). The main structure affected during the cryopreservation process is the plasma membrane (Horvath and Seidel Jr. 2006). Cell membranes are composed mainly of phospholipids, cholesterol, other lipids and proteins and this membrane phospholipid composition strongly influences its properties and its resistance to osmotic and thermal stress (Seidel Jr. 2006). The greatest membrane destabilization occurs when the membrane undergoes a phase transition from a liquid to a gel state (Horvath and Seidel Jr. 2006; Seidel Jr. 2006). During this lipid phase transition, phospholipids are lost from the plasma membrane resulting in an increase in membrane permeability, membrane disruption and even cell death (Moore *et al.,* 2005). Membrane cholesterol:phospholipids ratio is an important determinant of the fluidity and stability of a membrane during cryopreservation (Horvath and Seidel Jr. 2006). Low ratios are associated with less successful cryopreservation (Seidel Jr. 2006). It has been demonstrated that some kinds of cells tolerate thermal stresses better than others, and modifying the cells themselves can make them more cryotolerant (Seidel Jr. 2006). Recently, the cholesterol content of cell membranes has been modified by using cyclodextrins (Horvath and Seidel Jr. 2006; Buschiazzo *et al.,* 2013). Methyl-β-cyclodextrin is a hydrophilic molecule

with a hydrophobic centre that can encapsulate hydrophobic compounds such as cholesterol (Moore *et al.,* 2005). Incubation of cells with cholesterol-loaded-cyclodextrin (CLC) has been proposed to incorporate cholesterol to cell membranes and has previously been used to improve oocyte cryosurvival (Horvath and Seidel Jr. 2006; Srícigo *et al.,* 2012). This study was undertaken to determinate whether cholesterol could be incorporated into oocyte and to assess the effect of pre-treatment with cholesterol before cryopreservation on the cleavage rate and subsequent embryonic development.

5.2. Materials and Methods

5.2.1. Experiment 1: Determination of cholesterol incorporation

Fourteen–Fiveteen hours after ovulation induction (1 µg of buserelin acetate), oocytes were recovered and treated for 15 min with 0.1% (w/v) hyaluronidase for cumulus cell removal. In order to determine the incorporation of the cholesterol into the oocytes, they were incubated with 1 mg or 2 mg of cholesterol containing 20% (w/v) 22- N-(7-nitrobenz-2-oxa-1,3-diazol-4yl) amino-23,24 bisnor-5-cholen-3b-ol labelled cholesterol (NBD-CLC) in TCM-199 supplemented with 20% (v/v) foetal bovine serum (FBS) for 1 h. NBD-CLC was prepared as described by Horvath and Seidel Jr. (2006). After 1 h, oocytes were washed in Dulbecco's phosphate-buffered saline without calcium chloride (DPBS) and 0.1% (w/v) of bovine serum albumin (BSA), mounted (Vectashield Hardset Mounting Medium; Vector Laboratories, Barcelona, Spain) and examined using a confocal microscope (TCS SL: Leica, Mannheim, Germany). The incorporation of cholesterol was measured using ImageJ (Figure 5.1).

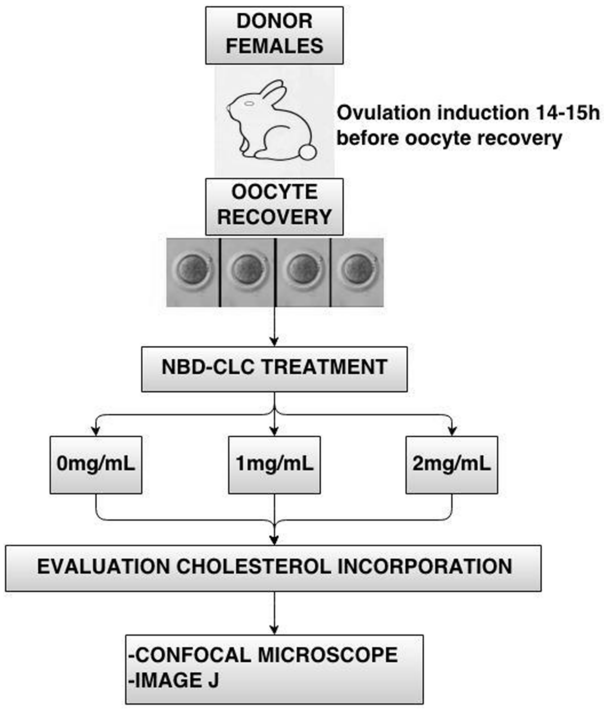

Figure 5.1: Experimental design to determine the incorporation of cholesterol into the oocytes after 1h of incubation with 0 mg, 1 mg or 2 mg/mL of cholesterol containing 20% (w/v) 22- N-(7-nitrobenz-2-oxa-1,3-diazol-4yl) amino-23,24 bisnor-5-cholen-3b-ol labelled cholesterol (NBD-CLC). Oocytes were examined using a confocal microscope and the incorporation of cholesterol was measured using ImageJ.

5.2.2. Experiment 2: Determination of the presence of cholesterol after cryopreservation procedures

Oocytes were cryopreserved using two different cryopreservation procedures: slow-freezing or vitrification. For slow-freezing procedure, oocytes were incubated for 15 min at room temperature in a solution containing 1.5 M 1,2-propanediol (PROH) in DPBS and 20% of foetal bovine serum (FBS). Oocytes were then placed for 10 min in the freezing solution composed of 1.5 M PROH and 0.2 M sucrose in DPBS and 20% FBS and mounted between two air bubbles in 0.25 ml sterile French mini straws (IMV Technologies. L'Aigle, France) sealed by a sterile plug. The straws were then placed in a programmable freezer (Cryologic, CL-8800) for the freezing process. Temperature was lowered from 20ºC to -7ºC at a rate of 2ºC/min. Manual seeding was performed at -7ºC.

Temperature was then lowered to -30ºC at a rate of 0.3ºC/min. Finally, straws were directly plunged into liquid nitrogen (LN_2) and stored for later use. For thawing, the straws were taken out of the LN_2 into ambient temperature for 10–15 s and plunged into a 20ºC water bath. Oocytes were transferred stepwise into decreasing sucrose solutions (0.5, 0.3 and 0.1 M sucrose in TCM-199 with 20% FBS) for 5 min before being equilibrated for 10 min in TCM-199 containing 20% (v/v) FBS. After thawing, the oocytes were incubated for two hours in medium TCM-199 containing 20% (v/v) FBS at 38.5ºC and 5% CO_2 in humidified atmosphere. The vitrification protocol was carried out following the Cryotop method. Oocytes were first exposed for 3 min to equilibration solution at room temperature, containing 3.75% (w/v) ethylene glycol (EG), 3.75% (w/v) dimethyl sulphoxide (DMSO) in base medium (BM: TCM-199- Hepes and 20% (w/v) serum substitute supplement, SSS™ (Irvine Scientific, County Wicklow, Ireland). Then, the oocytes were exposed for 3 min to solution containing 5% (w/v) EG, 5% (w/v) DMSO in BM, after which the oocytes were placed for 9 min in solution containing 7% (w/v) EG and 7% (w/v) DMSO in BM. Finally, the oocytes were transferred to vitrification solution consisting of 15% (w/v) EG, 15% (w/v) DMSO and 0.5 M sucrose in BM before being loaded onto Cryotop devices and directly plunged into LN_2 within 1 min. For warming, oocytes from LN_2 were transferred stepwise into decreasing sucrose solutions (1 M for 1 min and 0.5 M for 3 min) and then washed twice in BM for 5 min at room temperature. As with the slow-frozen group, the oocytes were incubated for two hours in medium TCM-199 containing 20% (v/v) FBS at 38.5ºC and 5% CO_2 in humidified atmosphere. To examine the cholesterol presence after cryopreservation, oocytes were incubated 1h with 1 mg of NBD-CLC/mL, as previously (Figure 5.2).

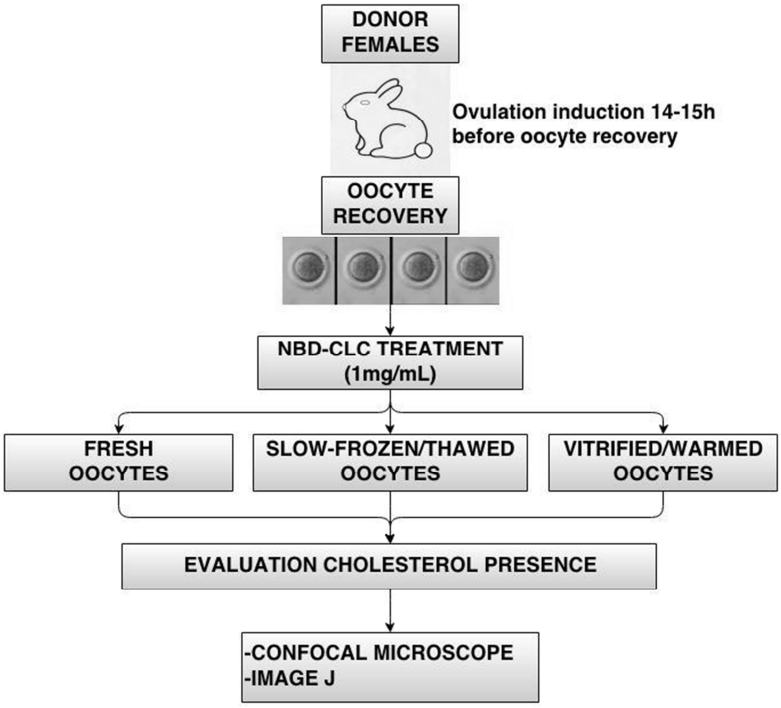

Figure 5.2: Experimental design to examine the cholesterol presence after cryopreservation. Oocytes were incubated 1h with 1 mg/mL of cholesterol containing 20% (w/v) 22- N-(7-nitrobenz-2-oxa-1,3-diazol-4yl) amino-23,24 bisnor-5-cholen-3b-ol labelled cholesterol (NBD-CLC) before cryopreservation procedures.

5.2.3. Experiment 3: Effect of NBD-CLC treatment on parthenogenetic development or ICSI fertilisation after cryopreservation

For developmental competence test, parthenogenetic activation and intracytoplasmic sperm injection (ICSI) procedures were performed (Figure 5.3). Oocytes from each experimental group were induced to parthenogenesis with two sets 1 h apart of two DC electrical pulses of 3.2 kv/cm for 20 µs at 1 s apart in an activation medium (0.3 M mannitol supplemented with 100 µM $MgSO_4$ and 100 µM $CaCl_2$), followed by 1 h exposure in TCM-199 medium supplemented with 5 µg/µl of cycloheximide and 2 mM of 6-dimethylaminopurine (6-DMAP). Parthenotes were cultured in 500 µl of TMC-199 supplemented with 20% (v/v) FBS and layered under paraffin oil at 38.5ºC in 5% CO_2 and saturated humidity. ICSI was performed with a PMM-150 FU piezo-

impact unit (Prime Tech, Japan) and Eppendorf micromanipulators using a blunt-ended, mercury-containing pipette (inner diameter, 6–7 µm). Oocytes were injected in groups of 10. After 15 min, surviving oocytes were returned to mineral oil-covered TCM-199 supplemented with 20% (v/v) FBS and cultured at 38.5ºC in an atmosphere of 5% CO_2 air and saturated humidity. After 24 and 102 h of *in vitro* culture, cleavage and blastocyst rates were recorded.

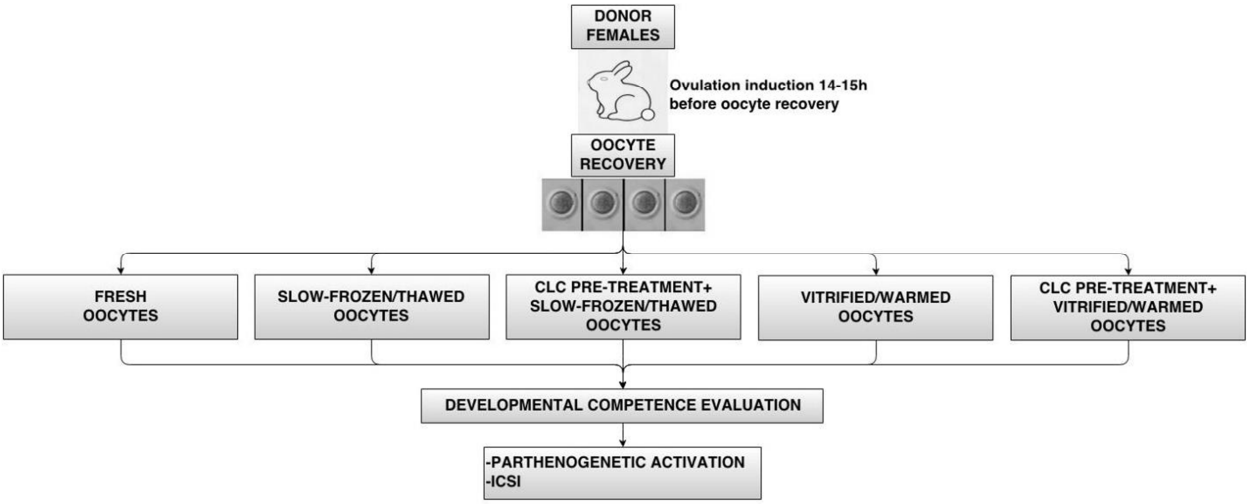

Figure 5.3: Experimental design to examine the effect of cholesterol-loaded-cyclodextrin (CLC) pre-treatment and cryopreservation procedure on cleavage and blastocyst development by parthenogenetic activation and ICSI.

5.2.5. Statistical analysis

The incorporation and the cholesterol presence after cryopreservation was analysed by General Linear Model (GLM) with NBD-CLC concentration and cryopreservation procedure as a fixed factors. Cleavage and blastocyst rates were also analysed using GLM with cryopreservation procedure (fresh, slow-frozen, NBD-CLC plus slow-frozen, vitrified and NBD-CLC plus vitrified) as a fixed factor and replicate and the cryopreservation procedure by replicate interaction as random factors. The replicate and interaction were non-significant and were removed from the model. For this analysis, the error was

designated as having a binomial distribution and the probit link function was used. Binomial data (cleavage and blastocyst development) for each embryo were assigned a 1 if it had achieved the desired stage of development or a 0 if it had not. Differences were considered significant at a level of $p < 0.05$. Data were expressed as least squares means ± standard error of the least squares means. All analyses were performed using the SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA, 2002).

5.3. Results and Discussion

Both NBD-CLC concentrations exhibited greater fluorescence than control oocytes (Figure 5.4). Moreover, 2 mg NBD-CLC/mL resulted in higher corrected total cell fluorescence than 1 mg NBD-CLC/mL (11.3 ± 0.56 vs. 7.9 ± 0.63, arbitrary units, respectively).

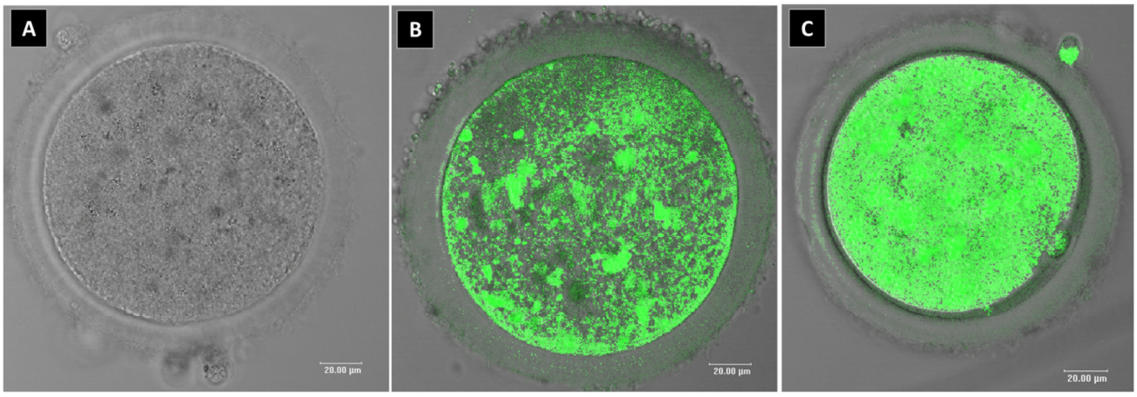

Figure 5.4: Fluorescent confocal image with the details of cholesterol incorporation into fresh oocytes incubated without (A), 1 mg/mL (B) and 2 mg/mL (C).

These results indicate that the cholesterol was incorporated into the oocyte cytoplasm. Previously, Horvath and Seidel (2006) showed that cholesterol diffused into oocytes, although they did not know exactly where the cholesterol was located within the oocytes. Our results show that NBD-CLC diffuses through the zona pellucida and plasma membrane incorporated the exogenous cholesterol into the cytoplasm, but we were unable to observed cholesterol

inclusion to the plasma membrane. However, recently Buschiazzo *et al.*, (2013) demonstrated that cholesterol was incorporated into plasma membrane of the mouse oocyte. Cryopreserved oocytes resulted in lesser fluorescence than non-cryopreserved oocytes (10.6 ± 0.48 vs. 1.9 ± 0.26 and 1.3 ± 0.33 of arbitrary units, for non-cryopreserved, frozen and vitrified oocytes, respectively), according with Kim *et al.*, (2001). Moreover, both slow-freezing and vitrification resulted in a similar loss of cytoplasmic cholesterol (Figure 5.5). In oocytes, lesser phospholipid content after freezing and thawing has been proposed as the main reasons for the low survival and development rates (Parks and Ruffing 1992).

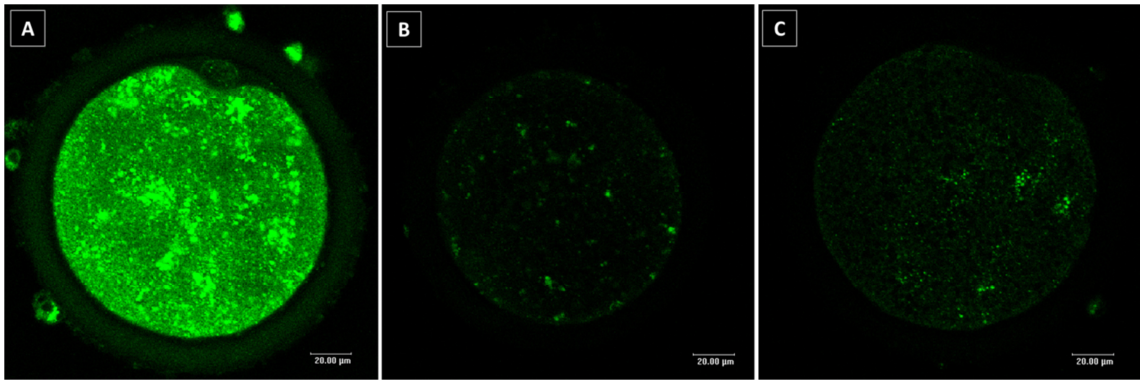

Figure 5.5: Loss of cholesterol after slow-freezing (B) or vitrification (C) compared with non vitrified oocyte (A).

The cleavage rates after parthenogenic activation and ICSI differ significantly between fresh and cryopreserved groups (Table 5.1). There were no differences among cryopreservation treatments, but none of the oocytes pre-treated with NBD-CLC and slow-frozen cleaved after parthenogenetic activation. When we tested whether embryo development could be improved by incubation of oocytes with CLC prior to slow-freezing or vitrification, no vitrified oocytes developed to the blastocyst stage, either with or without cholesterol treatment.

After slow-freezing, ICSI oocytes developed to the blastocyst stage irrespective of the NBD-CLC treatment, but their rates were low.

Table 5.1: Developmental rate at 24 and 102 h after parthenogenetic activation or intracytoplasmic sperm injection (ICSI) of fresh and cryopreserved rabbit oocytes treated or not with 1mg of cholesterol-loaded methyl-β.cyclodextrin per mL prior to cryopreservation.

Type	Parthenogenetic			ICSI		
	n	Cleavage (%)	Blastocyst (%)	n	Cleavage (%)	Blastocyst (%)
Fresh oocytes	159	72.3[a]	11.3[a]	18	61.1 [a]	50.0 [a]
Slow-frozen oocytes	33	3.0[c]	0.0[b]	37	24.3 [b]	2.7 [b]
CLC+Slow-frozen oocytes	34	0.0[d]	0.0[b]	42	26.2 [b]	2.3 [b]
Vitrified oocytes	32	15.6[b]	0.0[b]	64	28.1 [b]	0.0[c]
CLC+Vitrified oocytes	36	5.6[bc]	0.0[b]	52	26.9 [b]	0.0[c]

CLC: Cholesterol-loaded methyl-β-cyclodextrin. n: Number of oocytes. a,b,c,d: Data in the same column with uncommon letters are different ($p < 0.05$).

Cleavage and blastocyst development rates obtained in the present study are similar than some previously reported in rabbit (Cai *et al.,* 2005; Salvetti *et al.,* 2010). In bovine, although it seems to be beneficial in vitrified oocytes with cleavage to the eight-cell stage, it did not improve blastocyst rates (Horvath and Seidel Jr. 2006; Srícigo *et al.,* 2012). This discrepancy in results could arise from within-species differences in cryoresistance. We have found no previously published information regarding the incorporation of exogenous cholesterol into the cytoplasm before slow-freezing on oocytes cryoresistance.

We suggest that the absence of effect observed in the present study may arise from the amount of cholesterol used. However, we consider our results to be preliminary and need further study for application of this methodology.

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6. CHAPTER IV

Live birth from slow-frozen rabbit oocytes after *in vivo* fertilisation

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6. CHAPTER IV

Abstract

In vivo fertilisation techniques such as intraoviductal oocyte transfer have been considered as alternatives to bypass the inadequacy of conventional *in vitro* fertilisation in rabbit. There is only one study in the literature, published in 1989, that reports live offspring from cryopreserved rabbit oocytes. The aim of the present study was to establish the *in vivo* fertilisation procedure to generate live offspring with slow-frozen oocytes. First, the effect of two recipient models (i) ovariectomised or (ii) oviduct ligated immediately after transfer on the ability of fresh oocytes to fertilise were compared. Second, generation of live offspring from slow-frozen oocytes was carried out using the ligated oviduct recipient model. Throughout the experiment, recipients were artificially inseminated 9 hours prior to oocyte transfer. In the first experiment, two days after unilateral transfer of fresh oocytes, oviducts and uterine horns were flushed to assess embryo recovery rates. The embryo recovery rates were low compared to control in both ovariectomised and ligated oviduct groups. However, ligated oviduct recipient showed significantly ($P<0.05$) higher embryo recovery rates compared to ovariectomised and control-transferred. In the second experiment, using bilateral oviduct ligation model, all females that received slow-frozen oocytes became pregnant and delivered a total of 4 live young naturally. Thus, *in vivo* fertilisation is an effective technique to generate live offspring using slow-frozen oocytes in rabbits.

6.1. Introduction

Storage below the critical temperature of -130 ºC allows the preservation of cells and tissues after a long storage in liquid nitrogen (LN_2) without any decrease in viability (Coticchio *et al.,* 2004; Lavara *et al.,* 2011). There are currently two methods for gamete and embryo cryopreservation: slow-freezing and vitrification. The first to be developed was slow-freezing (Whittingham 1971). In this technique, germplasm is gradually exposed to low concentrations of cryoprotectants, in combination with very slow cooling rates, which leads to crystallization of extracellular water, resulting in an osmotic gradient that draws water from the intracellular compartment till intracellular vitrification occurs (Saragusty and Arav 2011).

Since Whittingham (1971) successfully froze mouse embryos, cryopreservation methodology have progressed to increase the number of lines, breeds and species that can be cryostored to preserve animal breeding and laboratory products (transgenics, clones) against loss caused by disease or hazards or improve the reproductive rate. For example, the rabbit breeding industry is increasingly using selected lines; the generation and characterisation of these lines require great effort and they must be kept in stock even if not needed for commercial use (García and Baselga 2005). From a genetic standpoint, the cryopreservation of inbred strains is useful to establish control populations to study the genetic drift and gain when selection programmes are applied (Apelo and Kanagawa 1989; García *et al.,* 2000; García and Baselga 2005). Although several breakthroughs have been made in oocyte cryopreservation since 1971, live offspring have only been obtained in a few species, such as

mouse (Whittingham 1977), human (Chen 1986), rabbit (Al-Hasani *et al.,* 1989), cattle (Fuku *et al.,* 1992), rat (Nakagata 1992), horse (Hochi *et al.,* 1994) and cat (Gómez *et al.,* 2004). Moreover, procedures developed for one specie are difficult to adapt to another (Paynter *et al.,* 1999, 2001; Nottola *et al.,* 2008; Pereira and Marques 2008; Noyes *et al.,* 2010) Specifically, few works have been carried out in rabbit (Diedrich *et al.,* 1988; Al-Hasani *et al.,* 1989; Siebzehnruebl *et al.,* 1989; Vincent *et al.,* 1989; Cai *et al.,* 2005; Salvetti *et al.,* 2010; Wang *et al.,* 2010; Jiménez-Trigos *et al.,* 2012, 2013a,b) and to the best of our knowledge, only Al-Hasani *et al*. (1989) obtained live offspring from cryopreserved rabbit oocytes.

Rabbit has been used as an animal model organism to study mammalian reproduction for over a century (Heape 1891; Chang *et al.,* 1970). To this end, different technologies for *in vitro* development of rabbit embryos have been assayed, such as *in vitro* fertilisation (IVF) (Bedford and Chang 1962; Brackett and Williams 1968), intracytoplasmic sperm injection (ICSI) (Keefer 1989; Deng and Yang 2001; Li *et al.,* 2001; Zheng *et al.,* 2004; Cai *et al.,* 2005) , and parthenogenetic activation (Ozil 1990; García-Ximenez and Escribá 2002; Salvetti *et al.,* 2010; Naturil-Alfonso 2011; Jiménez-Trigos *et al.,* 2012). Although it seems possible, IVF has not been successful in rabbit and a repeatable IVF technique has not yet been developed, possibly due to the lack of an efficient *in vitro* capacitation system for rabbit spermatozoa linked to the poor permeability of sperm plasma membrane (Curry *et al.,* 2000). Similarly, ICSI has been widely used in rabbit to study oocyte fertilisation and embryo development (Keefer 1989; Zheng 2004). However, this technique is difficult to perform because rabbit oocytes have rough, dark granules in the plasma and

easily lyse and die after the ICSI process (Cai *et al.,* 2005). The success rate of rabbit ICSI is less than 4% (Deng and Yang 2001; Li *et al.,* 2001). Some studies have employed parthenogenetic activation technique as an alternative tool to evaluate oocyte competence and *in vitro* development in rabbit (Salvetti *et al.,* 2010; Jiménez-Trigos *et al.,* 2012). However, one substantial limitation to this technique is that it cannot obtain offspring. Thus, *in vivo* techniques, such as intraoviductal oocyte transfer and intrafollicular oocyte transfer, have been considered as alternatives to bypass the inadequacy of conventional *in vitro* fertilisation techniques (Carnevale *et al.,* 2005; Deleuze *et al.,* 2009).

The aim of the present study was to develop a reliable and reproducible technique to generate live rabbit offspring with frozen oocytes.

6.2. Material and methods

All chemicals and reagents were purchased from the Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated. The study was approved by the ethical committee of the Universidad Politécnica de Valencia. All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (BOE, 2013; BOE = Official Spanish State Gazette).

6.2.1. Animals

We used New Zealand White females (5 months old) for the collection of metaphase II (MII) oocytes and New Zealand White males (8 months old) for artificial insemination (AI). All the animals used as donors and recipients in this study belonged to a selected line based on New Zealand White rabbits

selected since 1980 by a family index for litter size at weaning. The animals used came from the experimental farm of the Universidad Politécnica de Valencia. The rabbits were housed in conventional housing (with light alternating cycle of 16 light hours and eight dark hours, under controlled environmental conditions: average daily minimum and maximum temperature of 17.5 and 25.5°C, respectively), using individual cages (700×500×320 mm) with free access to a commercial diet and filtered water.

6.2.2. Animal models: unilateral ovariectomised and unilateral oviduct ligation

Unilateral ovariectomy technique: Females had surgery before puberty (at 16 weeks of age). Animals were sedated by intramuscular injection of 16 mg xylazine (Rompun, Bayer AG, Leverkusen, Germany). As surgical preparation for laparotomy, anaesthesia was performed by intravenous injection of 16-20 mg ketamine hydrochloride (Imalgene®, Merial, S.A., Lyon, France) into the marginal ear vein. During surgery, 12 mg of morphine hydrochloride (Morfina®, B.Braun, Barcelona, Spain) was administered intramuscularly. After surgery, does were treated with antibiotics (200,000 IU procaine penicillin and 250 mg streptomycin, Duphapen® Strep, Pfizer, S.L.) and buprenorphine hydrochloride (0.08 mg every 12 hours for 3 days, Buprex®, Esteve, Barcelona, Spain). Ovariectomy was performed gripping the left ovary with haemostatic tongs; blood vessels were ligated avoiding the oviduct and the ovary was removed. Abdominal wall and skin were closed using absorbable suture material (Monosyn®, B. Braun, Spain).

Ligated oviduct assisted by laparoscopy: The equipment used was a Hopkins® Laparoscope, which is a 0°-mm straight-viewing laparoscope, 30-cm in length,

with a 5-mm working channel (Karl Storz Endoscopia Ibérica S.A. Madrid). Recipients were anaesthetised as previously. The abdominal region was shaved, and the animals were then placed on an operating table in a vertical position (head down at 45-degree angle). This vertical positioning ensures that the stomach and intestines are cranially located so that the Fallopian tubes form a downwardly pointing loop between the ovaries and uterus. The endoscope trocar and traumatic forceps were inserted into the abdominal cavity. When the trocar was removed, the abdomen was insufflated with CO_2 and the endoscope was then inserted. Oviduct was closed with a non-absorbable polymer locking clip (Hem-o-lok® Ligation System), applied by laparoscopy using a 5mm automatic endoscopic locking clip applier (Reflex® Clip Applier, Conmed® Corporation, Utica, USA). Hem-o-lok product line is a ligation system that allows suture of 2 mm to 16 mm of vessel and/or tissue bundle. The clip was placed at the ampulla to prevent entry of the recipient's own oocytes (Figure 6.1).

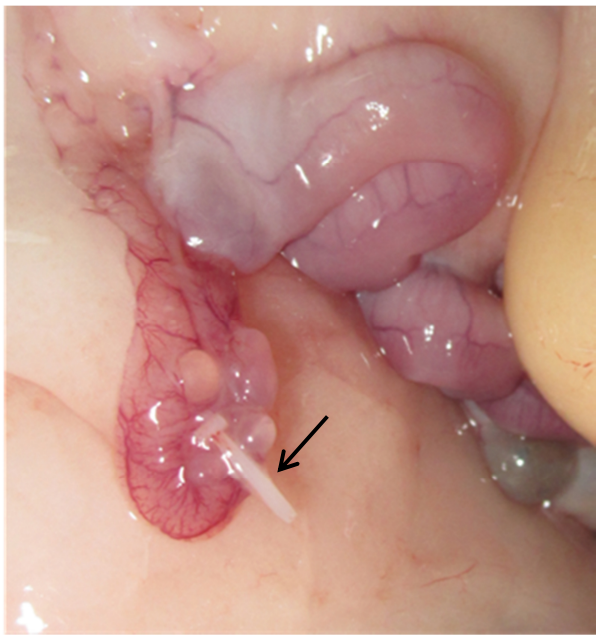

Figure 6.1: Representative oviduct ligation with a non-absorbable polymer locking clip (Hemo-o-lock® Ligation System) (arrow) after transfer (48h post ovulation induction).

6.2.3. Oocyte recovery

Donor females were induced to ovulate by intramuscular dose of 1 µg of Buserelin Acetate (Suprefact, Hoechst Marion Roussel, S.A., Madrid, Spain) (Vicente *et al.,* 2011). Oocytes were collected from the oviducts 14-15 h after ovulation induction by flushing each oviduct with Dulbecco's phosphate-buffered saline without calcium chloride (DPBS) and supplemented with 0.1% (w/v) of bovine serum albumin (BSA). Cumulus cells were removed and oocytes were incubated for 15 min at room temperature with 0.1% (w/v) hyaluronidase.

6.2.4. Intraoviductal oocyte transfer

Recipient females were inseminated 9 h prior to transfer with 0.5mL of fresh heterospermic pool semen at a rate of 40 $\times 10^6$ spermatozoa/mL in Tris-citric-glucose extender (Viudes-De-Castro and Vicente 1997). The semen collection method was carried out using an artificial vagina, as described by Vicente *et al.*, (2011). Motility was examined at room temperature under a microscope with phase-contrast optics at 40x magnitude. Only those ejaculates with >70% motile sperm (minimum requirements commonly used in artificial insemination) were pooled (Marco-Jiménez *et al.,* 2010). Immediately after insemination, ovulation was induced by an intramuscular injection of 1 µg Buserelin Acetate.

A detailed description of the technique used in rabbit was published previously (Besenfelder and Brem 1993). Laparoscopic oviductal transfer was performed as previously described, but using only the endoscope trocar. For oocyte transfer, oocytes were aspirated in an epidural catheter (Vygon corporate, Paterna, Valencia), introduced into the inguinal region with an epidural needle and then inserted in the oviduct through the infundibulum. Transfers were

always done unilaterally into the left oviduct, while the right oviduct was used as control. Prior to transfer, it was confirmed that ovulation had not yet taken place. In the ligation group, just after transfer the oviduct was ligated. Finally, the peritoneal air was removed from the abdominal cavity, the incision was sprayed with a Dermafill plastic dressing (Nobecutan, Laboratorios Inibsa, S.A. Barcelona) and antibiotic was intramuscularly administered (200,000 IU procaine penicillin and 250 mg streptomycin, Duphapen® Strep, Pfizer, S.L.).

6.2.5. Slow-freezing protocol

The slow-freezing procedure was adapted from previously described methods (Siebzehnruebl *et al.,* 1989). Briefly, oocytes were incubated for 15 min at room temperature in a solution containing 1.5 M 1,2-propanediol (PROH) in DPBS and 20% (v/v) foetal bovine serum (FBS). Oocytes were then placed for 10 min in the freezing solution composed of 1.5 M PROH and 0.2 M sucrose in DPBS and 20% FBS and mounted between two air bubbles in 0.25-ml sterile French mini straws (IMV Technologies. L'Aigle, France) sealed by a sterile plug. The straws were then placed in a programmable freezer (Cryologic, CL-8800) for the freezing process. Temperature was lowered from 20ºC to -7ºC at a rate of 2ºC/ min. Manual seeding was performed at -7ºC. Temperature was then lowered to -30ºC at a rate of 0.3ºC/ min. Finally, straws were directly plunged into LN_2 and stored for later use.

For thawing, the straws were taken out of the LN_2 into ambient temperature for 10–15 s and plunged into a 20ºC water bath. Oocytes were transferred stepwise into decreasing sucrose solutions (0.5, 0.3 and 0.1 M sucrose in TCM-199 with 20% (v/v) FBS) for 5 min before being equilibrated for 10 min in TCM-199

containing 20% (v/v) FBS. Then, the oocytes were incubated for 2 h in medium TCM-199 containing 20% (v/v) FBS at 38.5ºC and 5% CO_2 in humidified atmosphere.

6.2.6. Experimental design

6.2.6.1. Experiment 1. *In vivo* fertilisation of fresh oocytes

Females were divided into 4 groups: unilateral ovariectomy (n=4), unilateral ligated oviduct (n=8), control-transferred (n=6) and control (n=6) (Figure 6.2). The number of transferred oocytes per female varied from 10 to 20 (normal proportion of oocytes obtained after superovulation treatment with rhFSH (17.9 ± 0.8 vs. 9.7 ± 0.4)) (Cortell et al., 2010), depending on the number of oocytes available in each session (5 sessions were performed). Two days after insemination, oviducts and uterine horns were removed and each was flushed separately with 5 ml of DPBS containing 0.1% (w/v) of BSA to assess the embryo and oocyte recovery rates.

To discard any sperm effect, a sample of pooled embryos and oocytes from all transfer groups and sessions were fixed in DPBS containing 4% (w/v) buffered neutral paraformaldehyde solution for 2 h at room temperature. Then, embryos and oocytes were placed into 500 µl drops of DPBS containing Hoechst 33342 (*2'- (4- Ethoxyphenyl)- 5- (4- methyl- 1- piperazinyl) - 2,5'- bi- 1H- benzimidazol trihydrochloride*; 6 µM) and incubated for 15 min at room temperature in darkness. Embryos and oocytes were then washed twice, mounted, and the spermatozoa binding to the zona pellucida counted under a microscope equipped with ultraviolet illumination (excitation at 330–380 nm, emission at 460 nm).

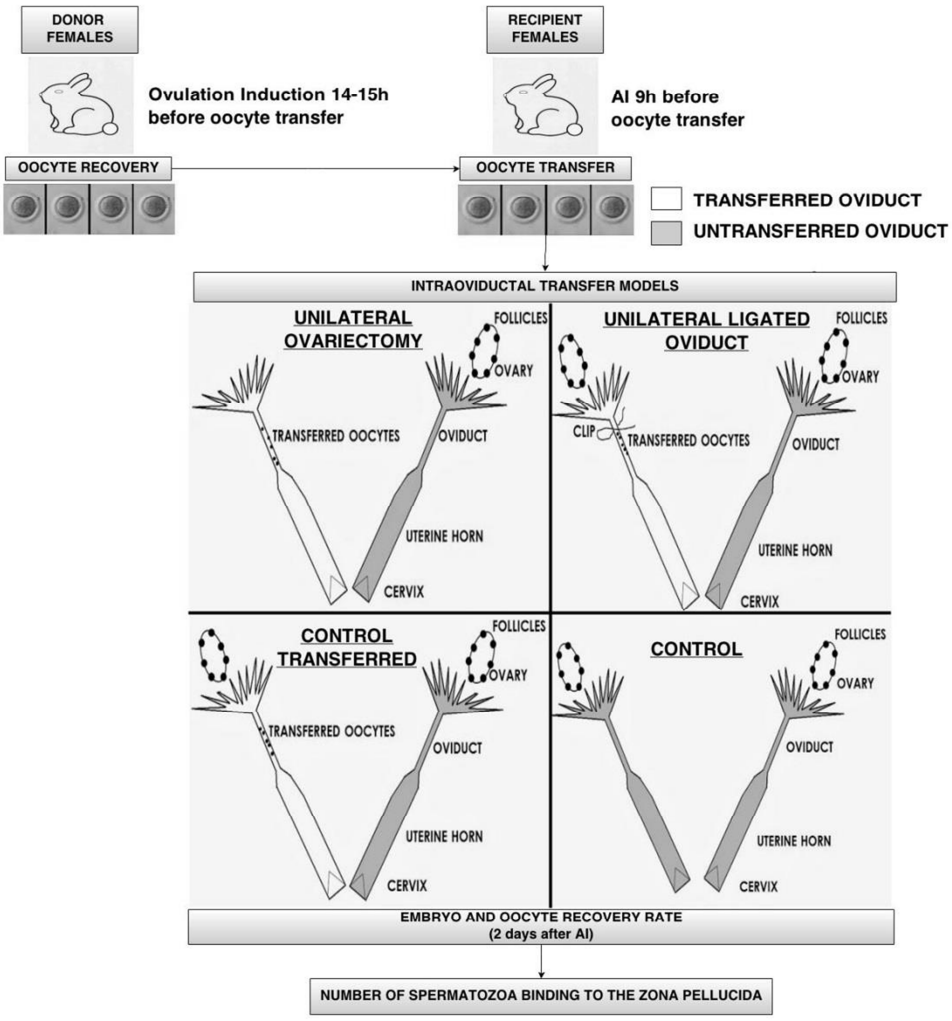

Figure 6.2: Experimental design of *in vivo* fertilisation in rabbit after intraoviductal transfer of oocytes into unilateral ovariectomised, unilateral oviduct ligated (oviduct was immediately ligated after oocytes transfer), control-transferred and control (no oocytes transferred) females. All transfers were always done unilaterally (left oviduct). Between 10 and 20 oocytes were transferred per oviduct, except to control females. AI: Artificial Insemination, h: hours.

6.2.6.2. Experiment 2. Generation of live offspring from slow-frozen oocytes

To generate live offspring, after 9 hours of insemination a total of 121 slow-frozen and thawed oocytes classified as normal (homogeneous cytoplasm, no vacuoles or granulations and an intact zona pellucid) and 38 fresh oocytes were transferred into both oviducts by laparoscopy to 6 recipient does (15 to 30 oocytes per recipient) and later oviducts were closed with a non-absorbable polymer locking clip (assessed in the results of experiment 1). Survival rates of

slow-frozen oocytes were assessed by laparoscopy in the recipient does on the basis of implantation rate (number of implanted embryos at day 14 from total oocytes transferred) and birth rate (kits born/total oocytes transferred). To prove the sterility of oviduct ligated recipients, females were inseminated at day 21 postpartum and evaluated the implantation rate fourteen days later (Figure 6.3).

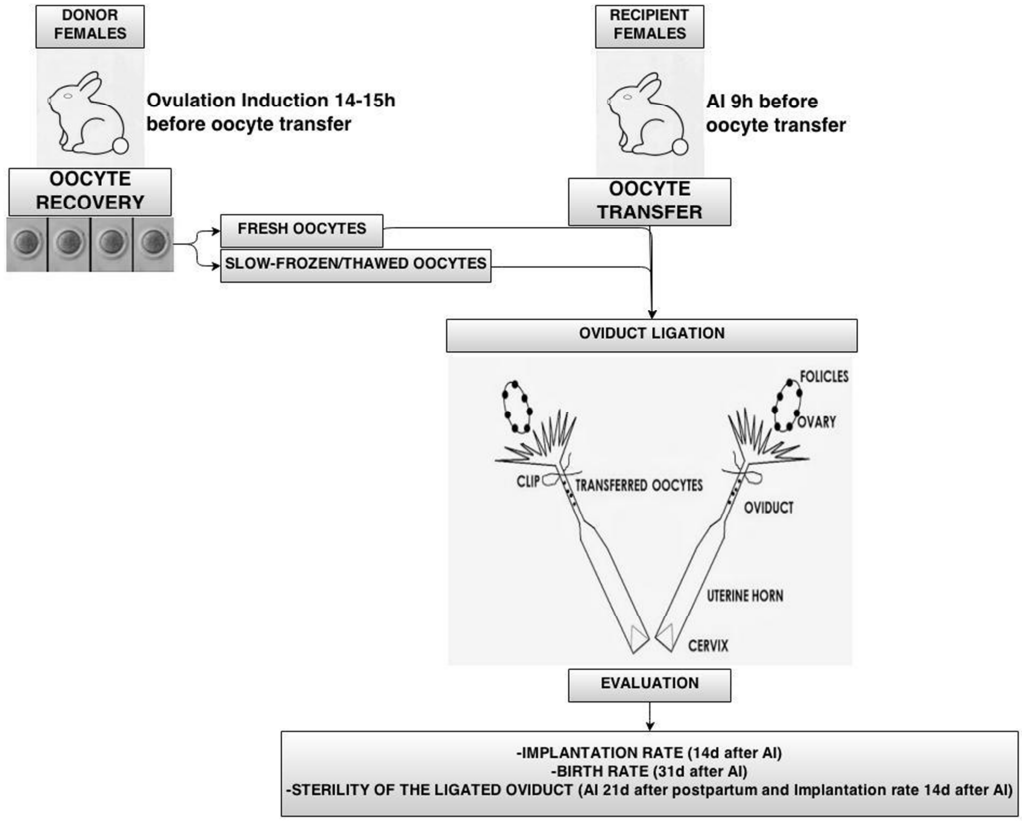

Figure 6.3: Experimental design to generate live offspring from fresh and slow-frozen oocytes after intraoviductal transfer into oviduct ligated females (oviducts were immediately ligated after oocytes transfer). All transfers were always done bilaterally. Between 15 and 30 oocytes were transferred per oviduct. h: hours. d: days. AI: Artificial Insemination.

6.2.7. Statistical analysis

To compare recovery rates and embryo recovery rates according to the intraoviductal transfer model (unilateral ovariectomised, unilateral oviduct ligation, control-transferred and control, experiment 1) as a fixed factor and the

type of oocytes (fresh and slow-frozen, experiment 2) on implantation and offspring at birth as a fixed factor, a generalised linear model was used. The error was designated as having a binomial distribution using the probit link function. Binomial data for hatching or hatched blastocyst rate were assigned a value of one if positive development had been achieved or a zero if it had not. $P<0.05$ was considered significant. Data are shown as means±standard error of means (S.E.M.). All analyses were performed with SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA, 2002).

6.3. Results

A total of 9 females were ovariectomised, but at time of transfer the epidural needle could only be inserted into the oviduct through the infundibulum of 4 females. Although 12 females underwent oviduct ligation, only 8 rabbits were included in the experiment, because after euthanasia 4 females presented tubal fluid accumulation and were rejected (Figure 6.4). Only the intact females were considered in the following analysis (4 and 8 females for ovariectomised and oviduct ligation groups, respectively).

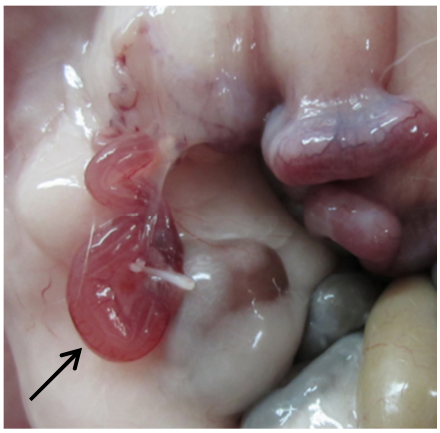

Figure 6.4: Detail of intrauterine fluid retention (arrow) after transfer and oviduct ligation (48h post ovulation induction).

All recovered embryos and oocytes were from oviducts and none were found in uterine horns. Overall recovery rates in ovariectomised and ligated females

were significantly lower than in control groups (69.0 ± 4.90% and 72.0 ± 5.50% *vs* 87.0 ± 2.90% and 94.0 ± 3.30%, for ligated and ovariectomised *vs* control-transferred and control, respectively, Figure 6.5). Likewise, an overall reduction in embryo recovery rate was observed in all transferred groups (40.0 ± 8.10%, 55.0 ± 4.50%, 59.0 ± 5.10% and 90.0 ± 3.80%, for unilateral ovariectomy, control-transferred, ligated and control, respectively, Figure 6.6). When oviducts were analysed separately, in untransferred oviduct similar recovery rates were observed in all of the experimental groups (Figure 6.5). However, in transferred oviduct, the recovery rate in ovariectomised and ligated females was significantly lower than in both control groups (34.0 ± 5.30%, 39.0 ± 5.40%, 85.0 ± 2.90% and 90.0 ± 4.90%, for ligated, ovariectomised, transferred-control and control, respectively, Figure 6.5). Likewise, embryo recovery rates in untransferred oviduct were similar for all experimental groups, except for the control-transferred group (93.0 ± 5.00%, 92.0 ± 4.20%, 88.0 ± 4.10% and 73.0 ± 6.70%, for control, ovariectomised, ligated, and transferred-control, respectively, Figure 6.6). However, in transferred oviduct, embryo recovery rates in ovariectomised, ligated and control-transferred were significantly lower than in control (3.0 ± 1.70%, 23.0 ± 4.70%, 37.0 ±4.00% and 87.0 ± 5.4%, respectively, Figure 6.6), despite the fact that embryos produced after intraoviductal transfer and the control embryos presented similar numbers of spermatozoa binding to the zona pellucida per embryo (13.7 ± 1.71 and 17.7 ± 1.58, control-transferred and control, respectively). In line with this result, oocytes that failed in fertilisation showed similar numbers of spermatozoa binding to the zona pellucida per oocyte, independently of the experimental group (4.2 ± 2.99 and 3.0 ± 8.20, for control-transferred and control, respectively).

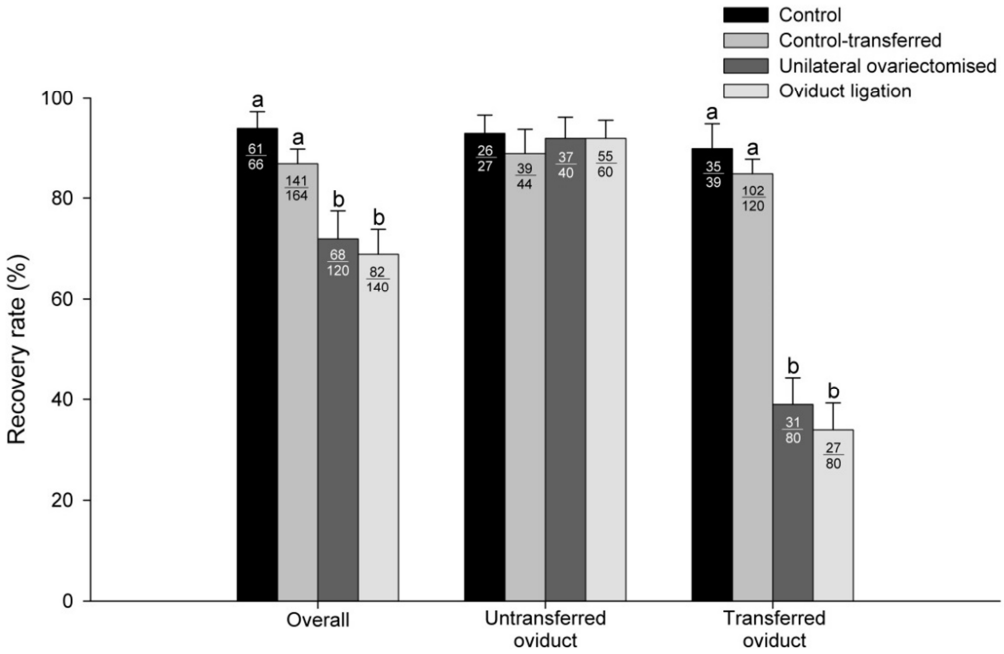

Figure 6.5: Overall recovery rate of *in vivo* fertilisation in rabbit after unilateral intraoviductal transfer of oocytes into ovariectomised, oviduct ligated, control-transferred (recovery rates calculated in excess to the number of ovulations) and control (no oocytes transferred) females. The numbers inside the bars indicate the number of oocytes and embryos recovered/total. Bars with different superscripts denote statistically significant differences between groups ($P < 0.05$). Data shown are representative of five independent sessions.

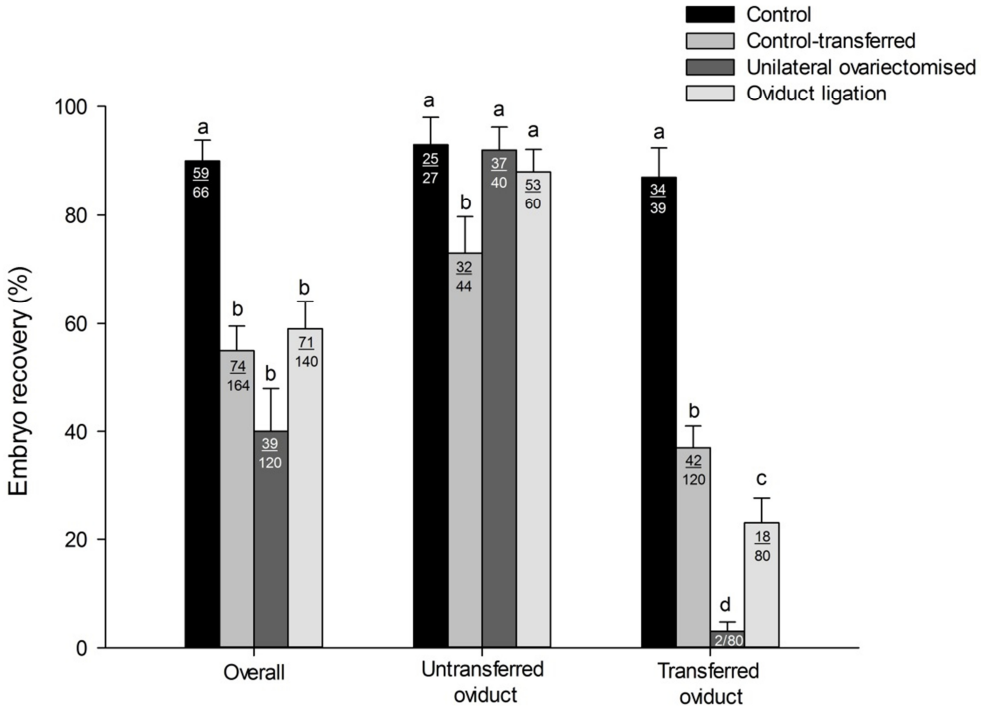

Figure 6.6: Embryo recovery rate of *in vivo* fertilisation in rabbit after unilateral intraoviductal transfer of oocytes into ovariectomised, oviduct ligated, control-transferred (recovery rates calculated in excess to the number of ovulations) and control (no oocytes transferred) females. The numbers inside the bars indicate the number of embryos recovered/total. Bars with different superscripts denote statistically significant differences between groups ($P < 0.05$). Data shown are representative of five independent sessions.

All transferred females that received cryopreserved oocytes became pregnant, and delivered a total of 4 live young naturally; 3 of these kits presented survival and growth until weaning at approximately 70 d of age (Figure 6.7). The offspring were visually normal.

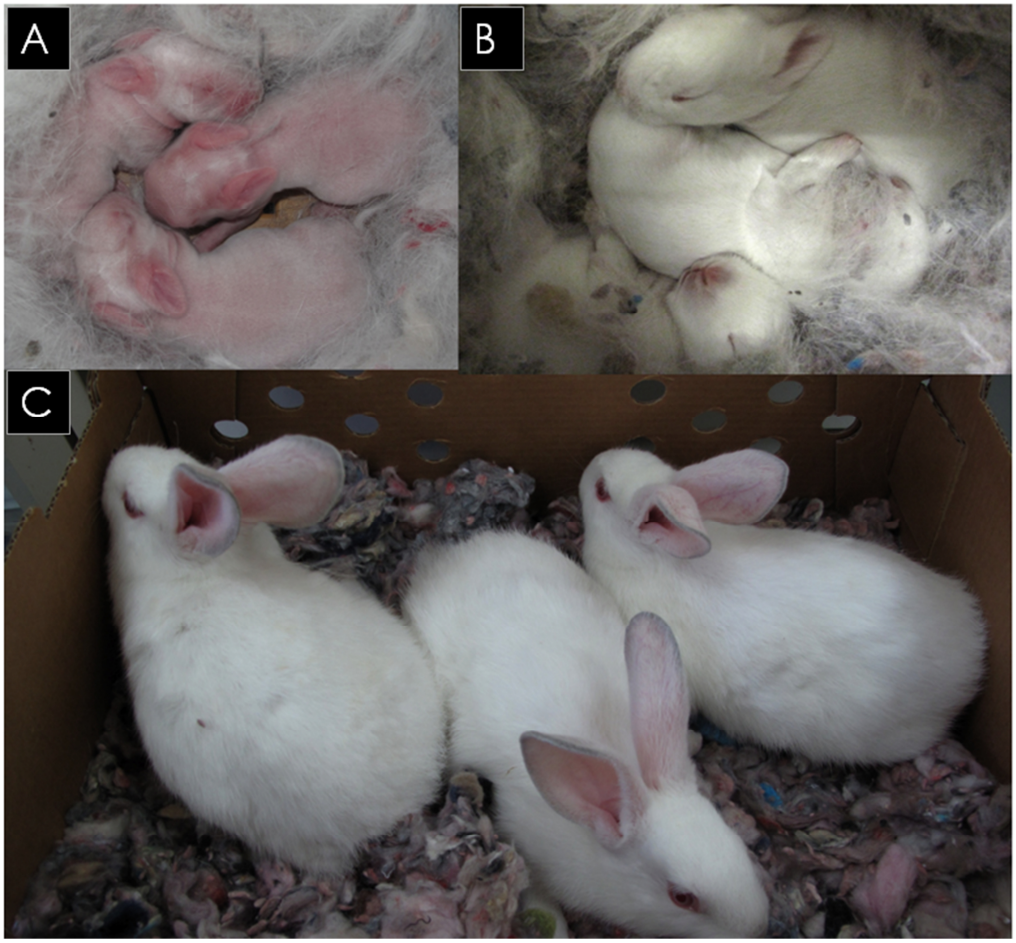

Figure 6.7: Rabbit derived from oocytes cryopreserved with slow-freezing protocol. (A) After birth (B), at 21 days old and (C) at 71 days old.

In the fresh transferred oocytes group, the implantation rate was 26.0 ± 7.1%, while in the slow-frozen oocytes group, the implantation rate was 7.0 ±2.3% (Figure 6.8). The overall rate of offspring obtained using slow-frozen oocytes was significantly lower (3.0 ± 1.6% vs. 18.0 ± 6.3% for slow-frozen vs. fresh oocytes, respectively, Figure 6.8). None of the oviduct ligated recipients inseminated at day 21 postpartum had implanted embryos.

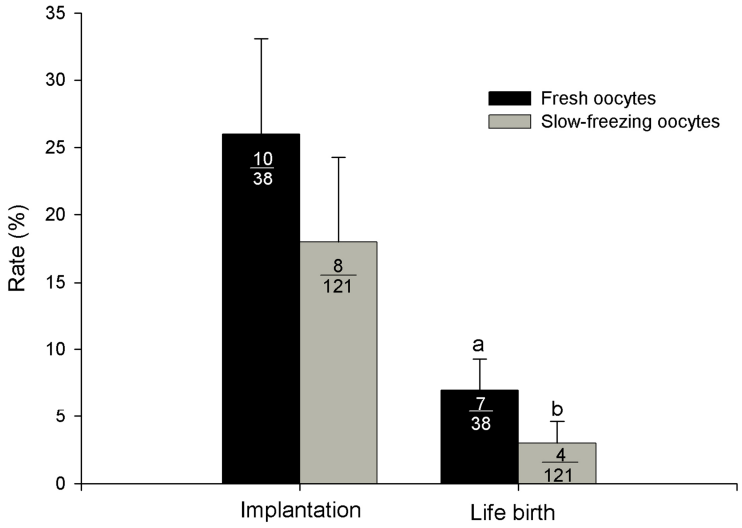

Figure 6.8: *In vivo* development of slow-frozen oocytes from rabbit. The numbers inside the bars indicate the number of oocytes transferred/total for implantation and life birth rates. Bars with different superscripts denote statistically significant differences between groups ($P < 0.05$).

6.4. Discussion

Historically, the rabbit was a 'classic' species in the early decades of embryology and reproductive biology, starting from the late 19th century (Fischer *et al.,* 2012). However, although it seems possible, the use of assisted reproductive technology has not been successful in rabbit and a repeatable IVF or ICSI techniques has not yet been developed (Keefer 1989; Curry *et al.,* 2000; Zheng *et al.,* 2004). We performed *in vivo* experiments in the rabbit for two reasons: its reproductive features allowed us to manage oocyte transfer according to the timing of gamete biological events and to mimic the uterine environment to improve fertilisation and the embryo culture systems. To date, *in vitro* conditions have been unable to mimic the dynamic changes of oviduct and uterus secretion that respond to the varying metabolism of a developing embryo (Bavister 1995; Rizos *et al.,* 2002; Fleming *et al.,* 2004; Purpera *et al.,* 2009; Saenz-de-Juando *et al.,* 2013). However, performing both animal models

affected the reproductive tract functionality of half of the females. Thus, the entrance to the infundibulum was not accessible via laparoscopy as a consequence of the surgery in ovariectomised females, whereby oviduct ligation induced an accumulation of tubal fluid. This accumulation of tubal fluid after oviduct ligation has been described previously (Guidobaldi *et al.,* 2012).

The oviduct plays a major part in different reproductive processes, providing the microenvironment for numerous steps in early embryogenesis (Besenfelder *et al.,* 2010).The overall recovery rate was similar between control-transferred and control group, in line with those reported in the literature (Mehaisen *et al.,* 2004). Nevertheless, a low recovery rate was observed after transfer into unilateral ovariectomy and ligated group. This would indicate a tubal disorder in these models. Although this hypothesis is difficult to verify, it is based on the differences observed between control-transferred group and ovariectomy and ligated females. The low recovery rate during the first day after transfer has been observed previously (Ryan and Moore 1988; Cortell *et al.,* 2010). However, as high recovery rates were obtained in our control-transferred and control groups, we ruled out a negative impact of technology transfer. Physiological properties of the oviduct involve a complex interaction of gamete transport and muscular, ciliar, secretory and adhesive functions (Ellington 1991). Nevertheless, we ruled out an altered tubal migration because all uterine horns were perfused separately and no oocytes or embryos were collected. It is known that oocyte transport occurs in the opposite direction to the oviductal fluid current, which flows towards the peritoneal cavity, coinciding with the maximum secretion of oviductal fluid during oestrogen dominance (Stone and Hamner 1975; Killian *et al.,* 1989). However, the oviduct ligated group ensures

that no loss of oocytes occurs in the peritoneal cavity. Thus, the recovery rate obtained in ovariectomised females (''open'' system) was similar to that reported in ligated does. Assuming that once oocytes are transferred to the oviduct they do not become lost in the peritoneal cavity or in the uterus, the issue remains of how they are retained by the oviduct in both models. Both groups have in common the absence of follicular fluid as opposed to control-transferred females. At least a part of this fluid must be transported into the oviduct together with the cumulus-oocyte complex (Yanagimachi 1969). Thus, the absence of the ovulation product could induce the retention oocyte in the oviduct. However, this hypothesis needs to be tested.

Overall embryo recovery rates from the untransferred oviduct for the all groups were similar. Therefore, synchronisation between artificial insemination and oocyte transfer was efficient. This conclusion is reinforced by the fact that embryos and oocytes failing to fertilise, regardless of the experimental group, presented similar numbers of sperm binding to the zona pellucida. Thus, we also confirm that anaesthetics did not affect the sperm transport (Sultan and Bedford 1996). However, in all transferred groups, embryo recovery rates decreased significantly. This result suggests that intraoviductal oocyte transfer reduced the probability of fertilisation. Some studies suggest that removal of cumulus cells prior to IVF reduced the cleavage rate through loss of a factor secreted by these cells (Fatehi *et al.,* 2002). It has also been suggested that cumulus cells continuously secrete sperm attractants (Guidobaldi *et al.,* 2012). Thus, capacitated spermatozoa are guided to reach the oocyte surface, passing through the cumulus mass. Moreover, our results demonstrated that despite similar numbers of spermatozoa binding to the zona pellucida,

successful fertilisation was not achieved. Nevertheless, this hypothesis needs to be tested.

While numerous reports of studies designed to investigate oocyte cryopreservation have been published in some species (Mullen *et al.,* 2007), few works have been performed in rabbit (Diedrich *et al.,* 1988; Al-Hasani *et al.,* 1989; Siebzehnruebl *et al.,* 1989; Vincent *et al.,* 1989; Cai *et al.,* 2005; Salvetti *et al.,* 2010; Wang *et al.,* 2010; Jiménez-Trigos *et al.,* 2012, 2013a,b) and only two recent works compared slow-freeze and vitrification methods (Salvetti *et al.,* 2010; Jiménez-Trigos *et al.,* 2012). Moreover, live offspring were obtained only in one report, using the slow-freezing method (Al-Hasani *et al.,* 1989). Rabbit oocytes are not very sensitive to low temperatures but present particularly sensitivity to high levels of cryoprotectants, and this has been shown to have a dramatic effect on the meiotic spindle configuration (Diedrich *et al.,* 1988; Vincent *et al.,* 1989; Cai *et al.,* 2005; Salvetti *et al.,* 2010; Jiménez-Trigos *et al.,* 2012,2013a). To date, there are no reports of offspring obtained from vitrified rabbit oocytes. Although several strategies have been developed to improve cryopreservation results (Ledda *et al.,* 2007), our data would clearly suggest that there is a developmental advantage for slow-frozen oocytes being transferred for *in vivo* fertilisation and *in vivo* embryo development. Following oocyte transfer, there were significant differences in births per oocyte cryopreserved between fresh and slow-frozen oocytes (18% v 3%). However, our offspring rates were similar to those reported for oocytes cryopreserved (In human (Fadini *et al.,* 2009), bovine (Suzuki *et al.,* 1996; Kubota *et al.,* 1998; Vieira *et al.,* 2002) and mouse (Bos-Mikich *et al.,* 1995; Aono *et al.,* 2005; Lee *et al.,* 2010)). Specifically, in rabbits using a slow-freezing method has resulted in live

offspring with a total of 0.8% (Al-Hasani *et al.,* 1989). Our model may therefore have an advantage, since the *in vivo* environment provides optimal conditions that an *in vitro* assay is unable to provide.

Our results indicated that oviduct manipulation to prevent the entrance of oocytes into the oviduct of the female recipient compromised the use of the reproductive tract in a high percentage of females. Taken together, our results demonstrate that we succeeded for the second time in the cryopreservation of rabbit oocytes. In conclusion, a combination of *in vivo* fertilisation and slow-frozen oocytes might be a useful approach to generate live offspring in rabbit. Nevertheless, further studies should be done to improve the recipient model.

6.5. References

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7. CHAPTER V

Generation of live birth from cryopreserved rabbit oocytes after *in vivo* fertilisation

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7. CHAPTER V

Abstract

There are only two studies in the literature, that reports live birth from cryopreserved rabbit oocytes. In this study, we used the intraoviductal transfer technique in combination with *in vivo* fertilising as an alternative method to assess live birth after transfer slow-frozen oocytes. The aims were 1) to evaluate the ability of cyanoacrylate tissue adhesive to block the oviducts after ovulation; 2) to evaluate the effect of blocked the oviducts on *in vivo* fertilising ability; and 3) to assess the live birth rate after transfer of slow-frozen oocytes. In all the experiments, recipients were artificially inseminated 9 hours prior to blocking the oviducts. First, the left oviduct was blocked with cyanoacrylate tissue adhesive, while the right one was used as control. Six days later, oviducts and uterine horns were flushed to assess embryo recovery rates. While the embryo recovery rate was 79.2% in the intact oviduct, no embryos were recovered in the blocked one. Second, fresh oocytes were transferred into both oviducts, which were immediately blocked using cyanoacrylate tissue adhesive. At day 6 after transfer, significantly fewer embryos were recovered from transferred females than from their untransferred counterparts (33.7 vs 100.0%). Nevertheless, in the last experiment, slow-frozen oocytes were transferred and the rate of live birth was 13.2±4.5%. This study shows that successful production of live rabbit offspring using slow-frozen oocytes in combination with *in vivo* fertilisation is feasible, and suggests that *in vivo* environment could help improve the results of oocyte cryopreservation.

7.1. Introduction

Preservation of female genetics can be achieved through the preservation of oocytes and embryos (Saragusty and Arav, 2011). Since Whittingham (1971) successfully froze mouse embryos, cryopreservation methodology and materials have progressed to increase the number of lines, breeds and species that can be embryo cryostored in order to preserve animal models or biodiversity or improve the reproductive rate. Oocytes are very different from sperm or embryos with respect to cryopreservation (Saragusty and Arav 2011). The first successful birth from a cryopreserved (slow-frozen) oocyte was reported in 1977 (Whittingham 1977) and although several breakthroughs have been made since then, live offspring have only been obtained in a few species, such as mouse, human, rabbit, cattle, rat, horse and cat (Jiménez-Trigos *et al.,* 2012). Specifically, in rabbit live young were not produced since 1989 and showed that the rate of live births per oocyte transferred was reported to be 7.5% (4/53) (Al-Hasani *et al.,* 1989) whereas Vincent *et al,*. (1989) showed a figure of 8.6% (9/105), but in unborn offspring at day 25 of gestation. We have recently obtained live young from slow-frozen oocytes showing an offspring rate of 3.3% (4/121) (Jiménez-Trigos *et al.,* 2013c).

Rabbit has been used as an animal model organism to study mammalian reproduction for over a century (Chang *et al.,* 1970; Heape, 1981; Fischer *et al.,* 2012). To this end, different technologies for *in vitro* production of embryos have been assayed, such as *in vitro* fertilisation (IVF) (Bedford and Chang 1962; Brackett and Williams 1968), intracytoplasmic sperm injection (ICSI) (Keefer 1989; Deng and Yang 2001; Li *et al.,* 2001; Zheng *et al.,* 2004; Cai *et al.,* 2005; Jiménez-Trigos *et al*., 2013b) and parthenogenetic activation (Ozil 1990; Salvetti

et al., 2010; Naturil-Alfonso *et al.,* 2011; Jiménez-Trigos *et al.,* 2012, 2013a, b). Although it seems possible, IVF has not been successful in rabbit and a repeatable IVF technique has not yet been developed, possibly due to the lack of an efficient *in vitro* capacitation system for rabbit spermatozoa linked to the poor permeability of sperm plasma membrane (Curry *et al.,* 2000). Similarly, ICSI has been widely used in rabbit to study oocyte fertilisation and embryo development (Keefer 1989; Zheng *et al.,* 2004). However, this technique is difficult to carry out because rabbit oocytes have rough, dark granules in the plasma and easily lyse and die after the ICSI process (Cai *et al.,* 2005). The success of ICSI in rabbit is still very limited, in the range of 2-6% live births (Deng and Yang 2001; Li *et al.,* 2001). For this reason, in recent years parthenogenesis has appeared as an interesting, quick and efficient tool to assess *in vitro* the developmental rates into blastocysts of rabbit oocytes in preliminary studies, when pregnancy rates are not needed (Salvetti *et al*. 2010, Jiménez-Trigos *et al.*, 2012, 2013a,b).

As an alternative, oocyte transfer can be use as a method to induce pregnancies in rabbits (Jiménez-Trigos *et al.,* 2013c) and in mares due the minimal success of *in vitro* fertilisation (Carnevale *et al.,* 2005, Deleuze *et al.,* 2009). Although different surgical methods such as laparotomy or laparoscopic procedures are well established in rabbit and have been used to fertilise oocytes (Motlik and Fulka 1974; Overstreet and Bedford 1974; Motlik and Fulka 1981; Bedford and Dobrenist 1989) and collect and transfer embryos (Adams 1962; Besenfelder and Brem 1993; Vicente and Garcia-Ximénez 1993; Mehaisen *et al.,* 2004; Cortell *et al.,* 2010), laparoscopic intraoviductal oocyte transfer has arised as good alternative technique to generate live births from cryopreserved

oocytes (Jiménez-Trigos *et al.,* 2013c). The *in vivo* environment could perhaps be more beneficial when oocyte quality is not optimal.

In this study, intraoviductal transfer technique was used to assess the live birth rate after transferring slow-frozen rabbit oocytes. The aims were 1) to evaluate the ability of cyanoacrylate tissue adhesive to block the entrance of the oocytes in the oviducts after ovulation; 2) to evaluate the effect on *in vivo* fertilising ability of blocking the oviducts using cyanoacrylate tissue adhesive immediately after transferring fresh oocytes; and 3) to assess the live birth rate after slow-frozen oocyte transfer and *in vivo* fertilisation.

7.2. Materials and Methods

All chemicals and reagents were purchased from the Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated.

7.2.1. Animals

All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (BOE, 2013; BOE = Official Spanish State Gazette). Ethical approval for this study was obtained from the Universidad Politécnica de Valencia Ethics Committee. New Zealand white females (n=38), 5 months old, were used as oocyte donors and recipients. The animals used came from the experimental farm of the Universidad Politécnica de Valencia. The rabbits were housed in a conventional housing (with light alternating cycle of 16 light hours and eight dark hours, and under controlled environmental conditions: average daily minimum and maximum temperature of 17.5 and 25.5°C, respectively). All rabbits had free access to fresh food and water.

7.2.2. Oocyte collection

Cumulus oocyte complexes (COCs) at the MII stage were collected from donor females induced to ovulate by an intramuscular dose of 1 µg of buserelin acetate (Suprefact, Hoechst Marion Roussel, S.A., Madrid, Spain). COCs were collected from the oviducts 14-15 h after ovulation induction by flushing each oviduct with Dulbecco's phosphate-buffered saline without calcium chloride (DPBS) and supplemented with 0.1% (w/v) of bovine serum albumin (BSA). Cumulus cells were removed and oocytes were incubated for 15 min at room temperature with 0.1% (w/v) hyaluronidase.

7.2.3. Slow-freezing of oocytes

The slow-freezing procedure was adapted from previously described methods (Siebzehnruebl *et al.,* 1989). Briefly, oocytes were incubated for 15 min at room temperature in a solution containing 1.5 M 1,2-propanediol (PROH) in DPBS and 20% (v/v) of foetal bovine serum (FBS). Oocytes were then placed for 10 min in the freezing solution composed of 1.5 M PROH and 0.2 M sucrose in DPBS and 20% (v/v) FBS and mounted between two air bubbles in 0.25-ml sterile French mini straws (IMV Technologies. L'Aigle, France) sealed by a sterile plug. The straws were then placed in a programmable freezer (Cryologic, CL-8800) for the freezing process. Temperature was lowered from 20ºC to -7ºC at a rate of 2ºC/ min. Manual seeding was performed at -7ºC. Temperature was then lowered to -30ºC at a rate of 0.3ºC/ min. Finally, straws were directly plunged into liquid nitrogen (LN_2) and stored for later use.

For thawing, the straws were taken out of the LN_2 into ambient temperature for 10–15 s and plunged into a 20ºC water bath. Oocytes were transferred stepwise into decreasing sucrose solutions (0.5, 0.3 and 0.1 M sucrose in TCM-199 with 20% (v/v) FBS) for 5 min before being equilibrated for 10 min in TCM-199 containing 20% (v/v) FBS. After that, oocytes were incubated for 2 h in medium TCM-199 containing 20% FBS at 38.5ºC and 5% CO_2 in humidified atmosphere.

7.2.4. *In vivo* fertilisation

Recipient females were inseminated 9 h prior to oocyte transfer with 0.5mL of fresh heterospermic pool semen at a rate of 40×10^6 spermatozoa/mL in Tris-citric-glucose extender (Viudes-de-Castro and Vicente 1997). Motility was examined at room temperature under a microscope with phase-contrast optics at 40x magnitude. Only those ejaculates with >70% motile sperm (minimum requirements commonly used in artificial insemination, AI) were pooled (Marco-Jiménez *et al.,* 2010). Immediately after insemination, ovulation was induced by an intramuscular injection of 1 µg buserelin acetate.

The intraoviductal oocyte transfer procedure was adapted from previously described technique used in rabbit (Besenfelder and Brem 1993). The equipment used was a Hopkins® Laparoscope, which is a 0°-mm straight-viewing laparoscope, 30-cm in length, with a 5-mm working channel (Karl Storz Endoscopia Ibérica S.A. Madrid). Recipients were sedated by intramuscular injection of 16 mg xylazine (Rompun, Bayer AG, Leverkusen, Germany). As surgical preparation for laparoscopy, anaesthesia was performed by intravenous injection of 16-20 mg ketamine hydrochloride (Imalgene®, Merial,

S.A., Lyon, France) into the marginal ear vein. During laparoscopy, 12 mg of morphine hydrochloride (Morfina®, B. Braun, Barcelona, Spain) was administered intramuscularly. First, the abdominal region was shaved, and the animals were then placed on an operating table in a vertical position (head down at 45-degree angle). This vertical positioning ensures that the stomach and intestines are cranially located so that the Fallopian tubes form a downwardly pointing loop between the ovaries and uterus. Only an endoscope trocar was inserted into the abdominal cavity. When the trocar was removed, the abdomen was insufflated with CO_2 and the endoscope was then inserted. For oocyte transfer, oocytes were aspirated in a 17-gaugue epidural catheter (Vygon corporate, Paterna, Valencia), introduced into the inguinal region with an epidural needle and then inserted in the oviduct through the infundibulum. Transfers were always done bilaterally deep in the ampulla of both oviducts. Prior to transfer, it was confirmed that ovulation had not yet taken place. Immediately after transfer, the infundibulum and the first part of the ampulla were closed with cyanoacrylate tissue adhesive (Histoacryl® Blue, B. Braun, Barcelona, Spain) applied by laparoscopy using the epidural catheter as oocyte transfer procedure to block the entrance of recipient doe oocytes (Figure 7.1). After surgery, does were treated with antibiotics (200,000 IU procaine penicillin and 250 mg streptomycin, Duphapen® Strep, Pfizer, S.L.) and buprenorphine hydrochloride (0.08 mg every 12 hours for 3 days, Buprex®, Esteve, Barcelona, Spain).

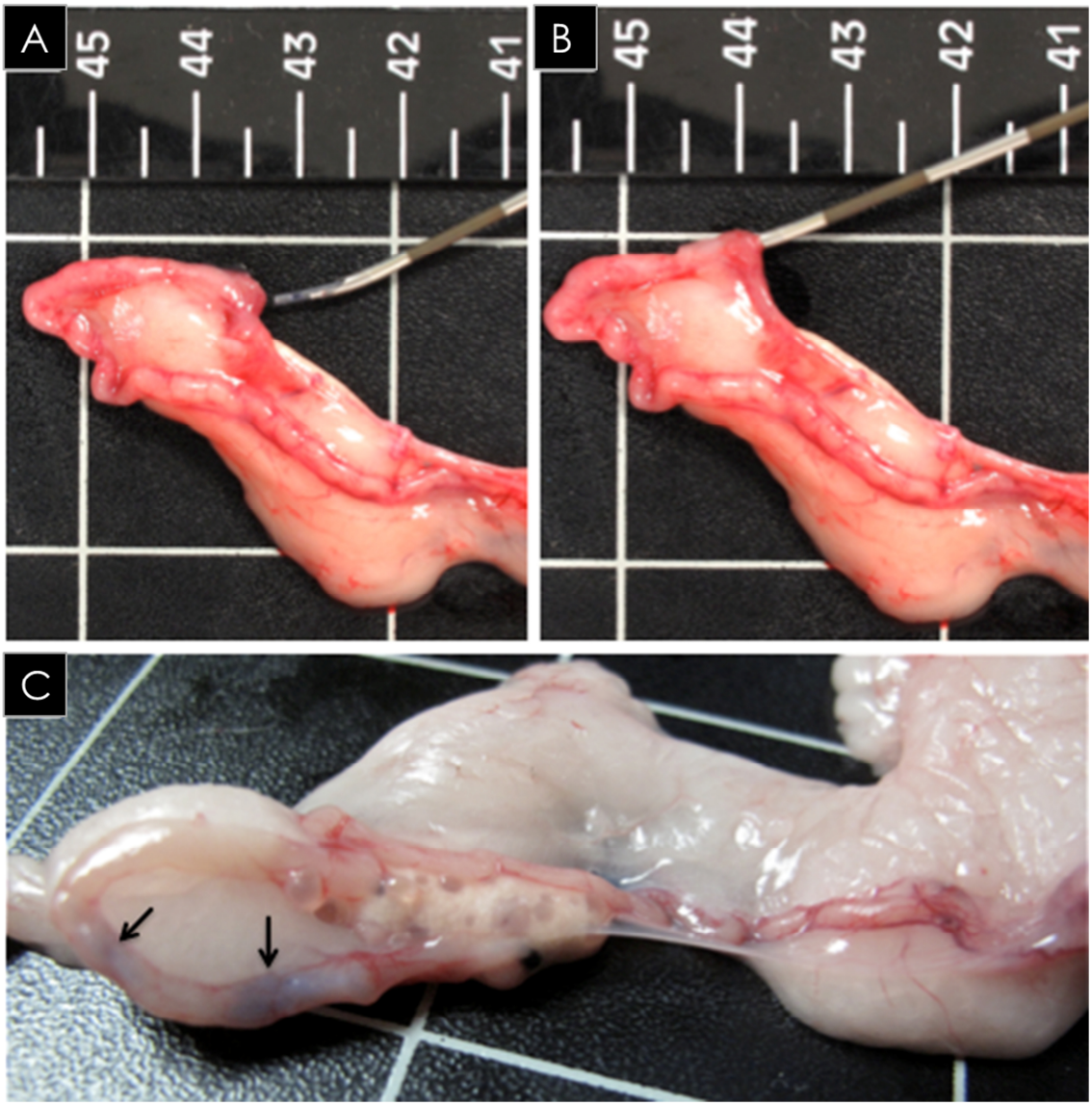

Figure 7.1: Representative images of the procedure to block the oviduct using the epidural catheter (Vygon corporate, Paterna, Valencia) to introduce the cyanoacrylate tissue adhesive (Histoacryl® Blue, B. Braun, Barcelona, Spain) (A-B). Detail of the blue colour aspect of the cyanoacrylate tissue adhesive inside the oviduct (arrows, C).

7.2.5. Experimental design

7.2.5.1. Experiment 1. Blocking the oviducts

To evaluate the ability of cyanoacrylate tissue adhesive to block the oviducts, four females were used for this experiment. After 9 h of AI, the left oviduct was closed with adhesive while the right oviduct was used as control (intact). Six

days after AI, uterine horns were removed and flushed with 10 ml of DPBS containing 0.1% (w/v) of BSA to assess the embryo recovery rates. Ovulation rate was estimated as the number of corpora lutea and embryo recovery rate by uterine horn was estimated as number of recovered embryos per uterine horn divided by ovulation rate (Figure 7.2).

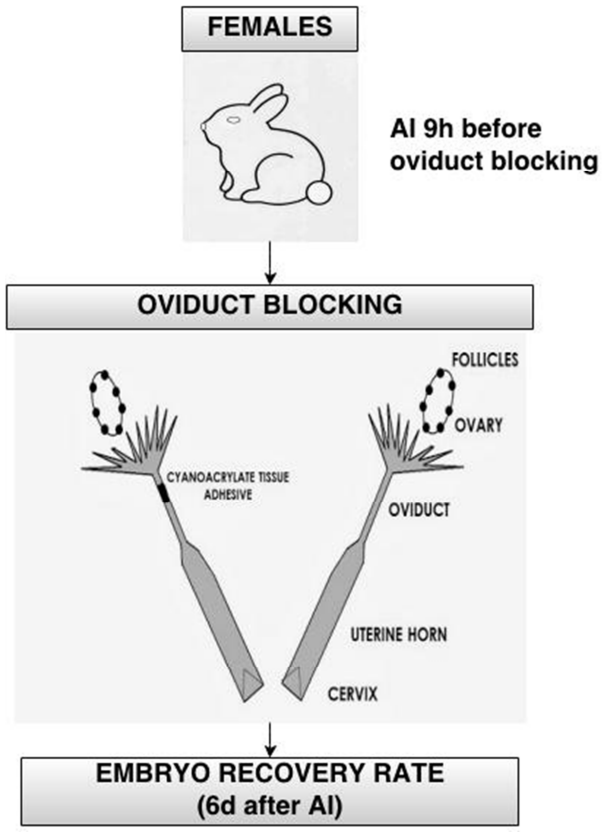

Figure 7.2: Experimental design to evaluate the ability of cyanoacrylate tissue adhesive to block the oviducts. The left oviduct was closed with adhesive while the right oviduct was used as control. AI: artificial insemination. h: hours.

7.2.5.2. Experiment 2. *In vivo* fertilisation of fresh oocytes

To evaluate the *in vivo* fertilisation, six females were used for this experiment (five used as recipients and one as control). After 9 h of AI, 10 oocytes were transferred into each oviduct and the oviducts were immediately blocked with cyanoacrylate tissue adhesive. Six days after insemination, uterine horns were

removed and flushed with 10 ml of DPBS containing 0.1% (w/v) of BSA to assess the embryo recovery rates (Figure 7.3).

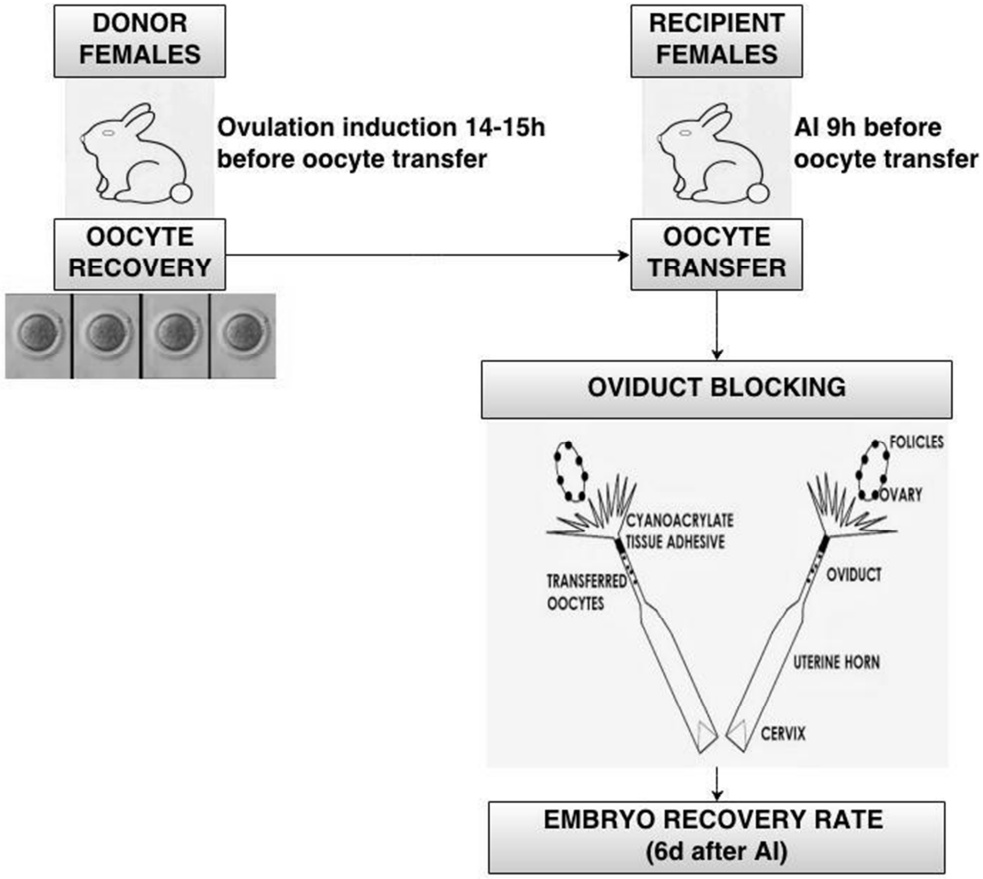

Figure 7.3: Experimental design of *in vivo* fertilisation of fresh oocytes after intraoviductal transfer. Oviducts were immediately blocked with cyanoacrylate tissue adhesive. All transfers were always done bilaterally and 10 oocytes were transferred per oviduct.

7.2.5.3. Experiment 3. Generation of live birth from slow-frozen oocytes

To generate live birth, 9 h after AI, a total of 76 slow-frozen and thawed oocytes classified as normal (homogeneous cytoplasm, no vacuoles or granulations and an intact zona pellucida) were transferred into both oviducts by laparoscopy to four recipient does (16 to 29 oocytes per recipient). Likewise, 19 fresh oocytes were also transferred to two recipient does. Later oviducts were closed with cyanoacrylate tissue adhesive. Fourteen days after insemination, females were anesthetised following the same procedure described previously and ventral

midline laparoscopy was carried out, noting the number of implanted embryos. At birth, total kits born were recorded. To prove the sterility of the oviduct blocked in the recipients, females were inseminated at day 21 postpartum and the implantation rate was evaluated fourteen days later (Figure 7.4).

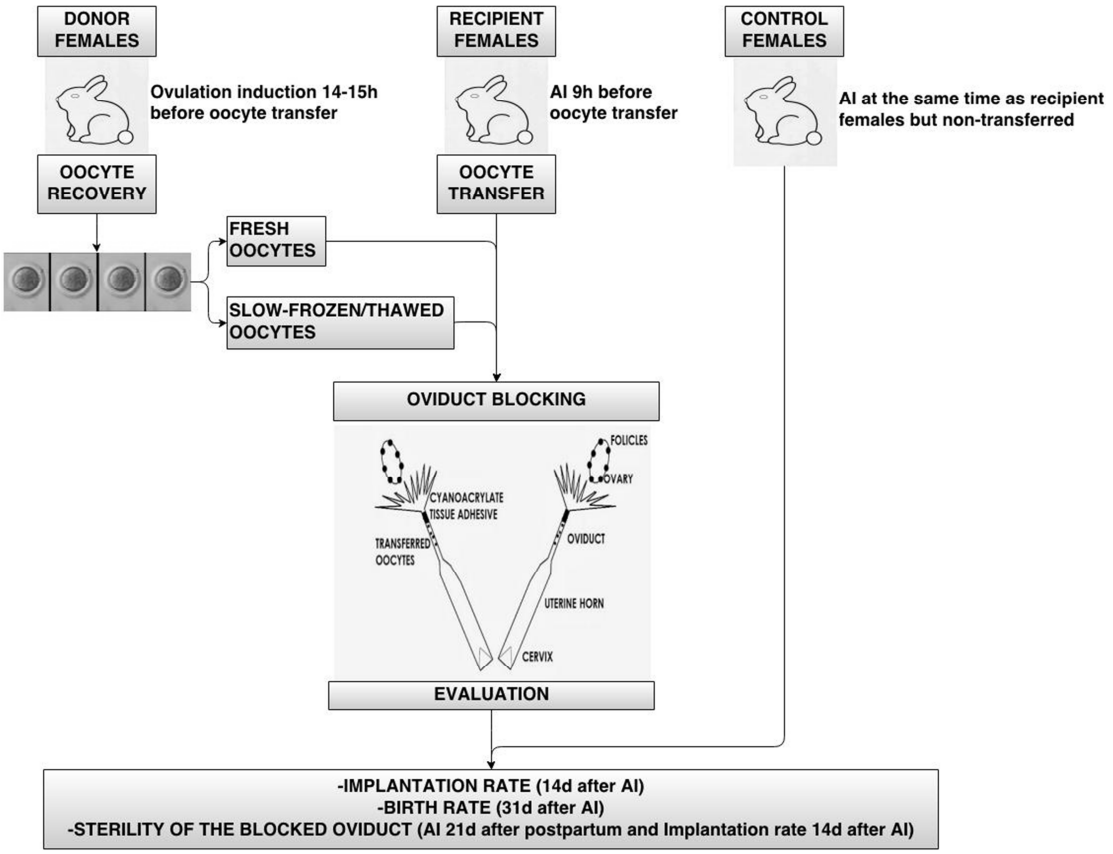

Figure 7.4: Experimental design to generate live offspring from fresh and slow-frozen oocytes after intraoviductal transfer. Oviducts were immediately blocked with cyanoacrylate tissue adhesive. All transfers were always done bilaterally. Between 16 to 29 oocytes were transferred per oviduct. h: hours. AI: artificial insemination. d: days.

7.2.6. Statistical Analyses

A generalised linear model was employed to compare embryo recovery rates and the implantation and live birth rate using the transferred oocytes or not (experiment 2) and the type of oocytes (fresh and slow-frozen, experiment 3) as a fixed factor. The error was designated as having a binomial distribution using the probit link function. Binomial data were assigned a value of one if positive

development had been achieved or a zero if it had not. P<0.05 was considered significant. Data are shown as means ± standard error of means (S.E.M.). All analyses were performed with SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA, 2002).

7.3. Results

The results of the ability of cyanoacrylate tissue adhesive to block the oviducts showed that in the unclosed oviduct, recovery rate at six days old embryos was 79.2% (19/24), while in the blocked oviduct no six day old embryos were recovered (0/16).

The *in vivo* fertilisation results after transfer are shown in Figure 7.5. An elevated rate of fertilisation was obtained in the control oocytes group (not transferred) (100%), while in the transferred oocytes group, this rate decreased (33.7%).

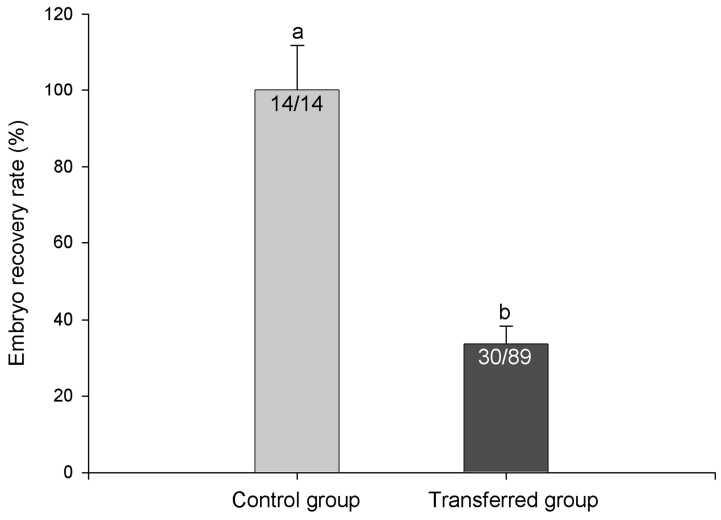

Figure 7.5: *In vivo* fertilised ability of fresh oocytes after intraoviductal transfer in transferred and control (no oocytes transferred) females. The numbers inside the bars indicate the number of embryos recovered in total. Bars with different superscripts denote statistically significant differences between groups (P < 0.05). Data shown are representative of five independent sessions.

Two transferred females that received cryopreserved oocytes became pregnant and delivered a total of 10 live young naturally and eight of these pups survived and grew until weaning (at approximately 70 d of age). All the offspring were visually normal (Figure 7.6).

Figure 7.6: Live young derived from oocytes cryopreserved with slow-freezing procedure. All the offspring were visually normal.

Implantation rate and birth rate are shown in Figure 7.7. In the fresh transferred oocytes group, the implantation rate was 37.5 ± 9.63%, while in the slow-frozen oocytes group, the implantation rate was 14.5 ± 4.42%, lower than that in the fresh oocytes group. The rate of live birth obtained using slow-frozen oocytes (13.2 ± 4.5%) was significantly lower than when using fresh oocytes (37.5 ± 9.63%), indicating that the successful slow-freezing of rabbit oocytes was achieved. None of the oviduct blocked recipients inseminated at day 21 postpartum had implanted embryos.

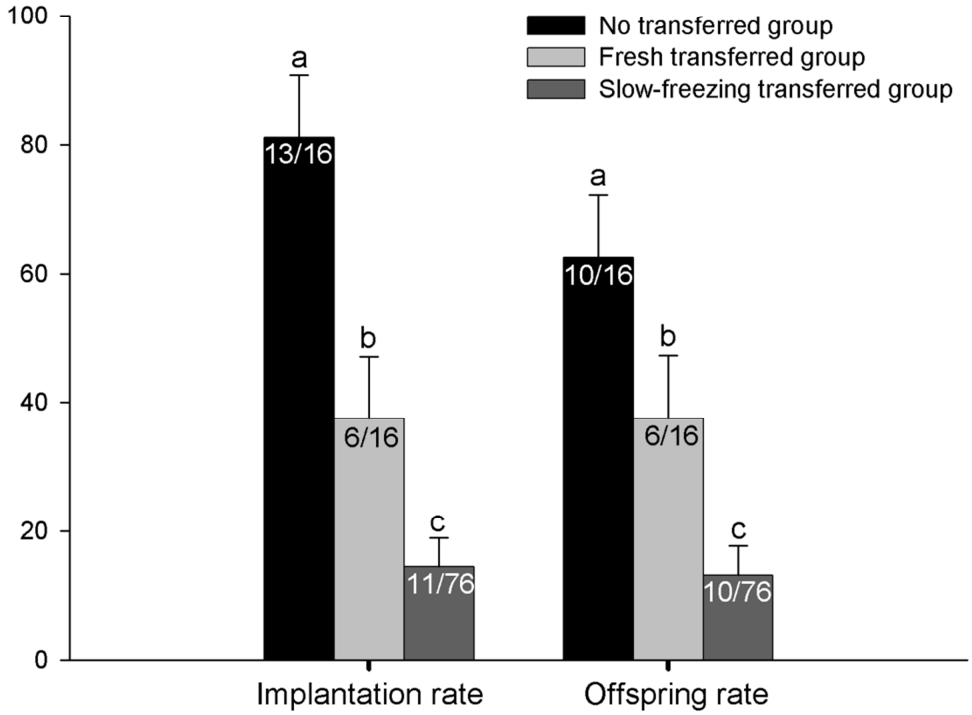

Figure 7.7: *In vivo* development of slow-frozen and fresh oocytes from rabbit after *in vivo* fertilisation. The numbers inside the bars indicate the number of implantation and live birth in total oocytes transferred. Bars with different superscripts denote statistically significant differences between groups ($P < 0.05$).

7.4. Discussion

Cryopreservation of oocytes has been described as a promising technique for long-term preservation of female genetic material (Andrabi and Maxwell 2007; Pereira and Marques 2008; Arav *et al.,* 2010). Moreover, it also could be very useful for many assisted reproductive technologies and the production of animals in breeding programmes (Ledda *et al.,* 2001; Checura and Seidel Jr 2007; Pereira and Marques 2008). While numerous studies have been published in some species (Mullen 2007), few works have been carried out in rabbit (Diedrich *et al.,* 1988; Al-Hasani *et al.,* 1989; Siebzehnruebl *et al.,* 1989; Vincent *et al.,* 1989; Cai *et al.,* 2005; Salvetti *et al.,* 2010; Wang *et al.,* 2010; Jiménez-Trigos *et al.,* 2012, 2013a,b). Furthermore, live birth was achieved only twice,

one in the 80s and the other recently, using the slow-freezing method (Al-Hasani *et al.,* 1989; Jiménez-Trigos *et al.,* 2013c). Although the use of assisted reproductive technologies for *in vitro* development of rabbit oocytes seems possible, they have not been successful developed (Keefer 1989; Curry *et al.,* 2000; Zheng *et al.,* 2004). Moreover, in cryopreserved oocytes, the rabbit species is highly sensitive to low temperatures and high levels of cryoprotectants, and cryopreservation causes damage to the organisation of the microtubules and meiotic spindle (Diedrich *et al.,* 1988; Vincent *et al.,* 1989; Cai *et al.,* 2005; Salvetti *et al.,* 2010; Jiménez-Trigos *et al.,* 2012, 2013a,b) inducing exocytosis, disorder of cortical granules (Jiménez-Trigos *et al.,* 2012) and chromosome aberration (Diedrich *et al.,* 1988; Salvetti *et al.,* 2010; Jiménez-Trigos *et al.,* 2013a). Consequently, blastocyst production after warming is very low (Diedrich *et al.,* 1988; Al-Hasani *et al.,* 1989; Siebzehnruebl *et al.,* 1989; Vincent *et al.,* 1989; Cai *et al.,* 2005; Salvetti *et al.,* 2010; Wang *et al.,* 2010; Jiménez-Trigos *et al.,* 2012, 2013a,b).

The rabbit belongs to the few species in which ovulation is induced by mating, resulting in an exactly defined pregnancy and embryonic age (Fischer *et al.,* 2012). When recipient does are induced to ovulate, sperm transport, fertilisation, and embryo development can be exactly synchronised with the donor in this reproductive model species. This study used intraoviductal transfer technique to provide the best conditions for fertilisation and early embryogenesis of cryopreserved rabbit oocytes to generate live birth. Live births achieved from slow-frozen rabbit oocytes derived from *in vivo* fertilisation have not been reported previously.

The results presented here demonstrate that the efficiency of *in vivo* fertilisation

after fresh oocyte transfer was affected in the untransferred oocytes (33.7% vs. 100%), indicating that oviduct manipulation or the oocyte handling is critical for oocyte competence and for these oocytes to fertilise successfully. The mammalian oviduct not only serves as a duct for the transport of gametes (Killian, 2001), but is also involved in several important processes that are necessary for the appropriate gamete and embryo physiology (Hunter, 1998). Ellington (1991) emphasised that the physiological properties of the oviduct are a complex interaction of gamete transport and muscular, ciliar, secretory and adhesive functions. Oviduct contractibility increases after 16h post-ovulation (Spilman *et al.,* 1978). In our study, slow-frozen oocytes were warmed and transferred into induced recipient females to ovulate 9 h before (ovulation had not yet taken place), which could lead to asynchrony in the fertilisation process. However, in rabbit the ovulation normally occurs 10–12 h after mating (Chang 1951) and this methodology was therefore adjusted to the maximum allowed for the species. Among the adverse effects attributed to oviduct manipulation are an inflammatory reaction and the release of some substances, such as catecholamines, cytokines, prostaglandins and leukotrienes, that may affect the normal oviduct function, loss of ciliary movement associated with failure of oocyte transportation and consequently failure of fertilisation, a deleterious effect on oocyte and a fertilisation rate reduction (David *et al.,* 1969; Wainer *et al.,* 1997; Tuffrey *et al.,* 1990). As for oocyte handling, oocyte manipulation itself may retard the developmental rate of transferred oocytes (Tarkowski 1959; Adams 1973). Moreover, in our study cumulus cells were removed prior to transfer to identify morphological normal oocytes which could induce an inadequate oocyte adhesion and transport. Talbot *et al.,* (2003) demonstrated that uptake of COCs into the oviduct

involves adhesion of the COCs to the oviductal epithelium. Therefore, removing cumulus cells could modify sperm-oocyte interaction affecting the fertilisation process (Van Soom *et al.,* 2002), since cumulus cells continuously secrete sperm attractants that guide capacitated spermatozoa to reach the oocyte surface, passing through the cumulus mass (Wetscher *et al.,* 2005). Therein lies the main source of this alternative method's low efficiency with fresh oocytes, which needs to be improved for it to become an effective method.

Nevertheless, the use of this method reported a rate of live births after transfer of slow-frozen rabbit oocytes of 13.2% (10/76), still a higher pregnancy rate than those obtained previously (7.5% (4/53) by Al-Hasani *et al.,* 1989 and 3.3% (4/121) by Jiménez-Trigos *et al.,* 2013c) and to those reported in other species such as human (Fadini *et al.,* 2009), bovine, (Suzuki *et al.,* 1996; Kubota *et al.,* 1998; Vieira *et al.,* 2002) and mouse (Bos-Mikich *et al.,* 1995; Aono *et al.,* 2005; Lee *et al.,* 2010). Up to now, *in vitro* conditions have been unable to mimic the dynamic changes of oviduct and uterus secretion that respond to the varying metabolism of a developing embryo (Rizos *et al.,* 2002; Saenz-de-Juano *et al.,* 2013). It is known that preimplantational embryos can develop *in vitro* and can produce normal offspring after transfer; however, their development is compromised compared with those grown *in vivo* (Avilés *et al.,* 2010). Rizos *et al*. (2002) demonstrated that deprivation of some *in vivo* produced maternal factors could be responsible for this impairment. Moreover, Fernández-González *et al.,* (2007) noted some pathological alterations associated with *in vitro* produced embryos. In our study, this protocol provides the best environmental conditions for fertilisation and embryo development of slow-frozen oocytes. However, based on the results with fresh oocytes, further experiments to

improve the efficiency of both fresh and slow-frozen oocytes are still needed, especially the establishment of successful fertilisation conditions to achieve higher success rates for future application.

In conclusion, this method allows obtaining higher live birth rates after slow-freezing and warming of rabbit oocytes in combination with *in vivo* fertilisation. It is significant that the *in vivo* environment could help to improve the results of oocyte cryopreservation.

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8. CHAPTER VI

First pregnancy and live birth from vitrified rabbit oocytes

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8. CHAPTER VI

Abstract

Intraoviductal oocyte transfer in combination with *in vivo* fertilisation has arisen as an alternative method to induce pregnancies from cryopreserved oocytes in rabbits. In this study, offspring were obtained for the first time from vitrified rabbit oocytes using this technique. In all the experiments, recipients were artificially inseminated 9 hours prior to oocyte transfer. Cryopreserved (vitrified and slow-frozen) and non-cryopreserved (fresh) oocytes were transferred into both oviducts, which were immediately closed using cyanoacrylate tissue adhesive to block the entry of the recipient's own oocytes. Three transferred females that received vitrified oocytes became pregnant and delivered a total of 9 live young naturally. The results revealed that there were no differences between vitrified and slow-frozen transferred oocytes and the live birth rate was 5.5% and 4.4% for vitrified and slow-frozen transferred oocytes, respectively. When fresh oocytes were transferred, this rate increased to 19.2%, whereas in the control females (non-transferred) the rate of offspring obtained was 71.4%. This is the first reported result of the development to term of vitrified rabbit oocytes and suggests that *in vivo* environment could help improve the results of oocyte cryopreservation.

8.1. Introduction

Female genetics cryopreservation can be achieved through the preservation of oocytes, embryos or ovarian tissue (Saragusty and Arav 2011). Current knowledge suggests that cryopreservation of unfertilised oocytes plays an essential role in different assisted reproductive technologies (ART) (Kohaya *et al.,* 2013). In livestock, it permits the preservation of valuable genetic lines until the female and the appropriate male express their genetic merit and suitable mating can be accomplished (Prentize and Anzar 2011; Díez *et al.,* 2012).

Since Whittingham (1977) successfully froze mouse oocytes, cryopreservation methodology and materials have progressed and live birth has been obtained in different species (Whittingham 1977; Chen 1986; Al-Hasani *et al.,* 1989; Fuku *et al.,* 1992; Nakagata 1992; Maclellan *et al*., 2002; Gómez *et al*., 2004; Somfai *et al*., 2013). Nevertheless, developmental rates are compromised and lower than those yielded by fresh oocytes (Lane and Gardner 2001; Shi *et al*., 2007; Morato *et al*., 2008; Fadini *et al*., 2009; Ogawa *et al*., 2010). In rabbits, to our knowledge, only four studies using slow-freezing oocytes obtained live young. Two of them were carried out in 1989 and showed that the rate of live births per oocyte transferred was reported to be 7.5% (4/53) (Al-Hasani *et al*., 1989) and 8.6% (9/105) (Vincent *et al*., 1989), but in unborn offspring at day 25 of gestation. The other two studies were done in our laboratory and showed that this rate ranged from 3.3% (4/121) to 13.2% (10/76) (Jiménez-Trigos *et al*., 2013s and personal contribution). However, to our knowledge, to date there are no reports of offspring obtained from vitrified oocytes.

Vitrification has emerged as an optimal procedure for oocyte and embryo cryopreservation (Kuwayama 2007). This method avoids intracellular ice crystallisation by supercooling the solution and transforming it into a 'vitreous', state (Kuwayama *et al*., 2005a), which could reduce oocyte damage, increasing survival rates after warming (Arav *et al*., 2002). However, a critical concentration of cryoprotectants is required for this process, which contributes to the damages associated after warming (Luvoni 2000; Rojas *et al*., 2004; Ambrosini *et al*., 2006; Morató *et al*., 2008; Pereira and Marques 2008). Rabbit oocytes are particularly sensitivity to high levels of cryoprotectants (Diedrich *et al*., 1988; Vincent *et al*., 1989; Cai *et al*., 2005; Salvetti *et al*., 2010; Jiménez-Trigos *et al*., 2012) and as a result, blastocyst rate after vitrification is compromised (Cai *et al*., 2005; Salvetti *et al*., 2010; Wang *et al*., 2010; Jiménez-Trigos *et al*., 2012, 2013a,b).

To date, different technologies to asses *in vitro* developmental ability of cryopreserved rabbit oocytes have been employed, such as parthenogenetic activation (Salvetti *et al*. 2010; Jiménez-Trigos *et al*. 2012; 2013a,b), *in vitro* fertilisation (IVF, Al-Hasani *et al*., 1989) and intracytoplasmic sperm injection (ICSI, Cai *et al*., 2005; Wang *et al*., 2010; Jiménez-Trigos *et al*., 2013b) However, they have not been successfully developed in this species (Curry *et al*., 2000; Cai *et al*., 2005; Viudes-de-Castro *et al*., 2005) and offspring were only obtained from slow-frozen oocytes using *in vivo* fertilisation (Vincent *et al*., 1989; Jiménez-Trigos *et al*., 2013c).

Intraoviductal oocyte transfer has emerged as a minimally invasive method to induce pregnancies in rabbits (Jiménez-Trigos *et al*., 2013c).and in mares (Carnevale *et al*., 2005; Deleuze *et al*., 2009). It has also been a good

alternative to evaluate the capacity of cryopreserved oocytes to generate viable offspring.

In this study, *in vivo* fertilisation after intraoviductal oocyte transfer was used to obtain live offspring from vitrified rabbit oocytes for the first time.

8.2. Materials and Methods

All chemicals and reagents were purchased from the Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated.

8.2.1. Animals

All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (BOE, 2013; BOE = Official Spanish State Gazette). Ethical approval for this study was obtained from the Universidad Politécnica de Valencia Ethics Committee. New Zealand white females (n=60), 5 months old, were used as oocyte donors and recipients. The animals used came from the experimental farm of the Universidad Politécnica de Valencia. The rabbits were kept in conventional housing (with light alternating cycle of 16 light hours and eight dark hours, and under controlled environmental conditions: average daily minimum and maximum temperature of 17.5 and 25.5 °C, respectively). All rabbits had free access to fresh food and water.

8.2.2. Oocyte collection

Cumulus oocyte complexes (COCs) at the metaphase II (MII) stage were collected from donor females induced to ovulate by an intramuscular dose of 1 µg of buserelin acetate (Suprefact, Hoechst Marion Roussel, S.A., Madrid,

Spain). COCs were collected 14-15 h after ovulation induction by flushing each oviduct with Dulbecco's phosphate-buffered saline without calcium chloride (DPBS) supplemented with 0.1% (w/v) of bovine serum albumin (BSA). Cumulus cells were removed and oocytes were incubated for 15 min at room temperature with 0.1% (w/v) hyaluronidase.

8.2.3. Vitrification procedure

The vitrification protocol with Cryotop device and solution has been described by Kuwayama *et al.,* (2005b). Oocytes were first exposed for 3 min to equilibration solution containing 3.75% (w/v) ethylene glycol (EG), 3.75% (w/v) dimethyl sulphoxide (DMSO), in base medium (BM: TCM-199 + 25mM Hepes + 20% (v/v) serum substitute supplement, SSS™ (Irvine Scientific, County Wicklow, Ireland). Then, the oocytes were exposed for 3 min to solution containing 5% (w/v) EG, 5% (w/v) DMSO in BM, after which the oocytes were placed for 9 min in solution containing 7% (w/v) EG and 7% (w/v) DMSO in BM. Finally, the oocytes were transferred to vitrification solution consisting of 15% (w/v) EG, 15% (w/v) DMSO and 0.5 M sucrose in BM before being loaded onto Cryotop devices and directly plunged into liquid nitrogen (LN_2) within 1 min. For warming, oocytes were transferred stepwise into decreasing sucrose solutions (1 M for 1 min and 0.5 M for 3 min) and then washed twice in BM for 5 min. After warming, the oocytes were incubated for 2 h in medium TCM-199 containing 20% (v/v) Foetal Bovine Serum (FBS) at 38.5ºC and 5% CO_2 in humidified atmosphere.

8.2.4. Slow-freezing procedure

The slow-freezing procedure was adapted from previously described methods (Siebzehnruebl *et al.,* 1989). Briefly, oocytes were incubated for 15 min at room

temperature in a solution containing 1.5 M 1,2-propanediol (PROH) in DPBS and 20% (v/v) FBS. Oocytes were then placed for 10 min in the freezing solution composed of 1.5 M PROH and 0.2 M sucrose in DPBS and 20% (v/v) FBS and mounted between two air bubbles in 0.25-ml sterile French mini straws (IMV Technologies. L'Aigle, France) sealed by a sterile plug. The straws were then placed in a programmable freezer (Cryologic, CL-8800) for the freezing process. Temperature was lowered from 20ºC to -7ºC at a rate of 2ºC/ min. Manual seeding was performed at -7ºC. Temperature was then lowered to -30ºC at a rate of 0.3ºC/ min. Finally, straws were directly plunged into LN_2 and stored for later use. For thawing, the straws were taken out of the LN_2 into ambient temperature for 10–15 s and plunged into a 20ºC water bath. Oocytes were transferred stepwise into decreasing sucrose solutions (0.5, 0.3 and 0.1 M sucrose in TCM-199 with 20% (v/v) FBS) for 5 min before being equilibrated for 10 min in TCM-199 containing 20% (v/v) FBS. After that, oocytes were incubated for 2 h in medium TCM-199 containing 20% (v/v) FBS at 38.5ºC and 5% CO_2 in humidified atmosphere.

8.2.5. *In vivo* fertilisation

Recipient females were artificial inseminated (AI) 9 h prior to oocyte transfer with 0.5mL of fresh heterospermic pool semen at a rate of 40 × 10^6 spermatozoa/mL in Tris-citric-glucose extender (Viudes-de-Castro and Vicente 1997). Motility was examined at room temperature under a microscope with phase-contrast optics at 40x magnitude. Only those ejaculates with >70% motile sperm were pooled. Immediately after insemination, ovulation was induced by an intramuscular injection of 1 µg buserelin acetate.

The intraoviductal oocyte transfer procedure was adapted from previously described technique used in rabbit (Besenfelder and Brem 1993). The equipment used was a Hopkins® Laparoscope, which is a 0°-mm straight-viewing laparoscope, 30-cm in length, with a 5-mm working channel (Karl Storz Endoscopia Ibérica S.A. Madrid). Recipients were sedated by intramuscular injection of 16 mg xylazine (Rompun, Bayer AG, Leverkusen, Germany). As surgical preparation for laparoscopy, anaesthesia was performed by intravenous injection of 16-20 mg ketamine hydrochloride (Imalgene®, Merial, S.A., Lyon, France) into the marginal ear vein. During laparoscopy, 12 mg of morphine hydrochloride (Morfina®, B. Braun, Barcelona, Spain) was administered intramuscularly. First, the abdominal region was shaved, and the animals were then placed on an operating table in a vertical position (head down at 45-degree angle). This vertical positioning ensures that the stomach and intestines are cranially located so that the Fallopian tubes form a downwardly pointing loop between the ovaries and uterus. Only an endoscope trocar was inserted into the abdominal cavity. When the trocar was removed, the abdomen was insufflated with CO_2 and the endoscope was then inserted. For oocyte transfer, oocytes were aspirated in a 17-gaugue epidural catheter (Vygon corporate, Paterna, Valencia), introduced into the inguinal region with an epidural needle and then inserted in the oviduct through the infundibulum. Transfers were always done bilaterally deep in the ampulla of both oviducts. Prior to transfer, it was confirmed that ovulation had not yet taken place. Immediately after transfer, the infundibulum and the first part of the ampulla were closed with cyanoacrylate tissue adhesive (Histoacryl® Blue, B. Braun, Barcelona, Spain) applied by laparoscopy using the epidural catheter as oocyte transfer procedure to block the entrance of

recipient doe oocytes (Figure 8.1). After surgery, does were treated with antibiotics (0.1mL/kg procaine penicillin, Duphapen® Strep, Pfizer, S.L.) and buprenorphine hydrochloride (0.08 mg every 12 hours for 3 days, Buprex®, Esteve, Barcelona, Spain).

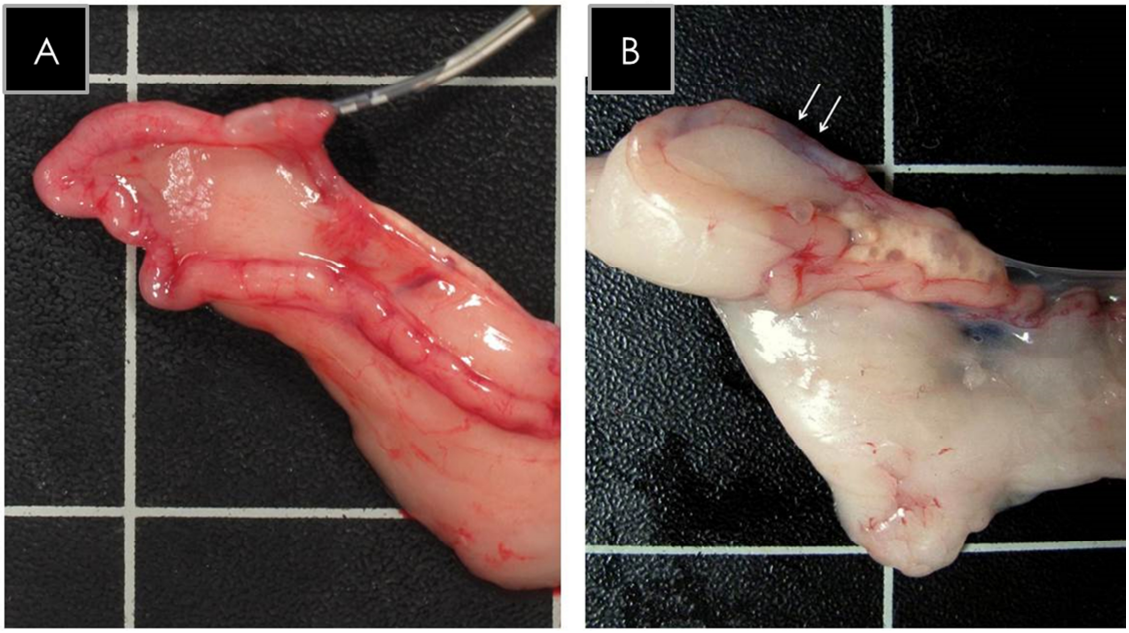

Figure 8.1: Representative images of the procedure to block the oviduct using the epidural catheter (Vygon corporate, Paterna, Valencia) to introduce the cyanoacrylate tissue adhesive (Histoacryl® Blue, B. Braun, Barcelona, Spain) (A). Detail of the blue colour aspect of the cyanoacrylate tissue adhesive inside the oviduct (arrows, B).

8.2.6. Experimental design: Generation of live birth from cryopreserved rabbit oocytes.

To generate live birth, 9 h after AI, a total of 165 vitrified and warmed oocytes and 161 slow-frozen and thawed oocytes, both types classified as normal (homogeneous cytoplasm, no vacuoles or granulations and an intact zona pellucida), were transferred into both oviducts by laparoscopy in 12 recipient does (16 to 30 oocytes per recipient). Likewise, 52 fresh oocytes were also transferred to four recipient does. Later, oviducts were closed with

cyanoacrylate tissue adhesive. On the other hand, four females were not transferred, in order to evaluate the effect of transfer procedure on *in vivo* fertilisation. Fourteen days after insemination, females were anaesthetised following the same procedure described previously and ventral midline laparoscopy was carried out, noting the number of implanted embryos. At birth, total kits born and birth weight were recorded. To prove the sterility of the oviduct blocked in the recipients, females were inseminated at day 21 postpartum and the implantation rate was evaluated fourteen days later. Experimental design to generate live offspring after intraoviductal oocyte transfer is shown in Figure 8.2.

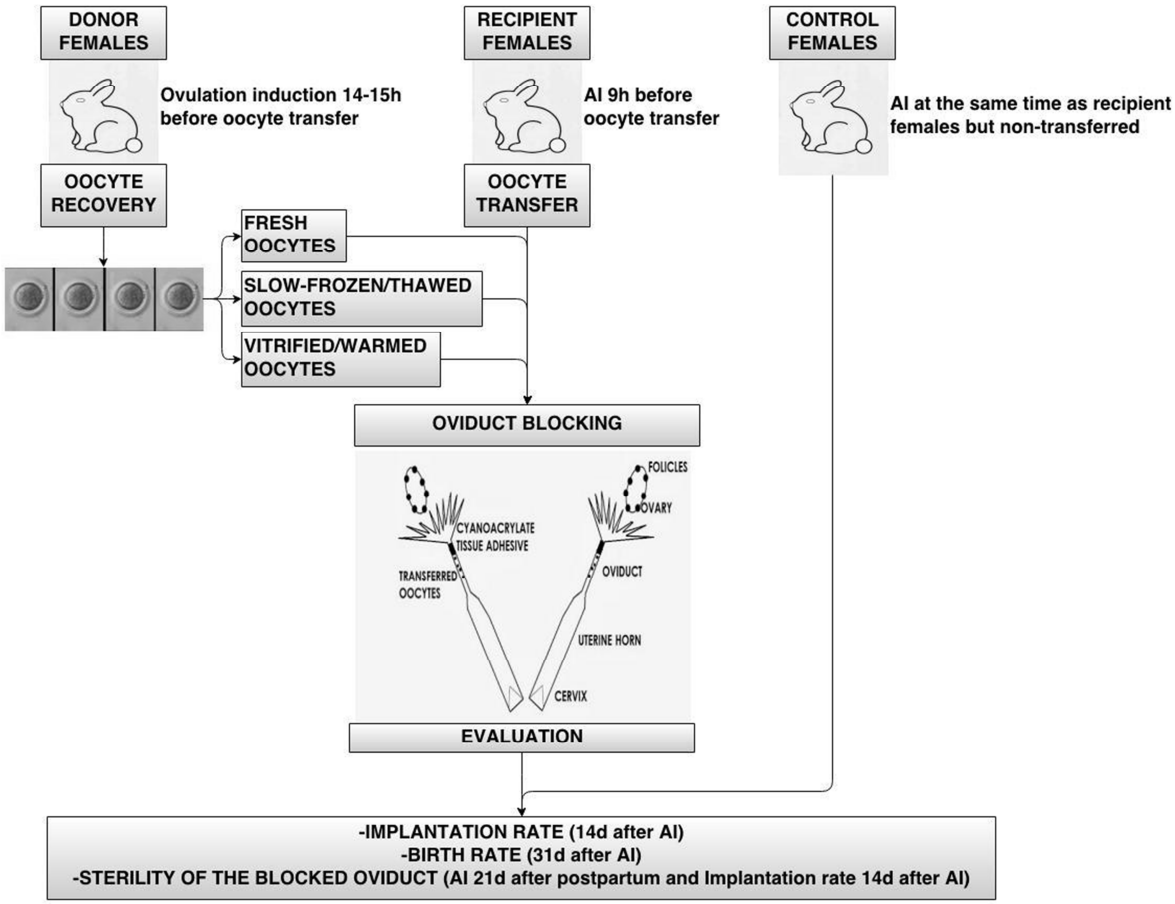

Figure 8.2: Experimental design to generate live offspring from fresh and cryopreserved oocytes after intraoviductal oocyte transfer (oviduct was immediately closed after oocytes transfer). All the transfers were done bilaterally. AI, artificial Insemination, h, hours. d, days.

8.2.7. Statistical Analyses

The general linear model was use to compare implantation and offspring rates using the type of oocyte (control, fresh-transferred, vitrified and slow-frozen) as a fixed factor. The error was designated as having a binomial distribution using the probit link function. Binomial data were assigned a value of one if positive development had been achieved or a zero if it had not. Additionally, the general linear model was employed to compare birth weight with the type of oocyte (control, fresh-transferred, vitrified and slow-frozen) as a fixed factor, and litter size mean as a covariable. $P<0.05$ was considered significant. Data are shown as least squares means ± standard error of the mean (S.E.M.). All analyses were performed with SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA, 2002).

8.3. Results

The *in vivo* fertilisation results after transfer are shown in Table 8.1.

Tabe 8.1: *In vivo* development of cryopreserved and fresh oocytes from rabbit after *in vivo* fertilisation.

Type	Recipients	Transferred oocytes	Implantation rate (n)	Birth rate (n)
Not transferred	4	49*	0.73 ± 0.063^{a} (36)	0.71 ± 0.065^{a} (35)
Fresh-transferred	4	52	0.23 ± 0.058^{b} (12)	0.19 ± 0.055^{b} (10)
Slow-frozen	6	161	0.06 ± 0.018^{c} (9)	0.04 ± 0.016^{c} (7)
Vitrified	6	165	0.06 ± 0.019^{c} (10)	0.05 ± 0.018^{c} (9)

n: number of implantated embryos or number of kits. *In control group (not-transferred), transferred oocytes refer to ovulation rate. Data are shown as least squares means ± standard error of the mean. Values a,b,c with different superscripts in the same column are statistically different ($P < 0.05$).

Implantation rate obtained 14 days after insemination showed that there were no differences between vitrified and slow-frozen transferred oocytes (6.1% and 5.6% for vitrified and slow-frozen transferred oocytes, respectively). However, this rate increased when fresh oocytes were transferred (23.1%) and it was significantly higher (73.5%) in the non-transferred control females. Regarding live birth rate, no differences were observed between vitrified transferred oocytes (5.5%) and slow-frozen ones (4.4%). However, when fresh oocytes were transferred, this rate was significantly higher (19.2%). In the non-transferred control group, this live birth reached 71.4%. None of the oviduct blocked recipients inseminated at day 21 postpartum presented implanted embryos. Three transferred females that received vitrified oocytes became pregnant and delivered a total of 9 live young naturally. Likewise, three transferred females that received slow-freezing oocytes became pregnant and delivered a total of 7 kits. All the offspring were visually normal (Figure 8.3) and no differences on birth weight were observed among groups (Table 8.2).

Table 8.2: Birth weight of young rabbits from cryopreserved and fresh oocytes after warming, transfer and *in vivo* fertilization.

Type	n	Birth Weight* (g)	Litter size
Not transferred	35	62.0 ± 1.55	9.5 ± 1.19
Fresh-transferred	10	57.9 ± 2.56	5.0 ± 1.68
Slow-frozen	7	64.9 ± 3.48	2.3 ± 1.37
Vitrified	9	58.3 ± 2.59	3.0 ± 1.37

n: number of pups. * Birth weight has been corrected by the litter size mean of each type. Data are shown as least squares means ± standard error of the mean.

Figure 8.3: Live young derived from vitrified rabbit oocytes.

8.4. Discussion

Although much progress has been made since the first live birth from cryopreserved oocytes (Whittingham 1977), few works have been carried out in rabbit (Diedrich *et al*., 1988, Al-Hasani *et al*., 1989, Siebzehnruebl *et al*., 1989, Vincent *et al*., 1989, Cai *et al*., 2005, Salvetti *et al*., 2010, Wang *et al*., 2010, Jiménez-Trigos 2012, 2013a,b,c) and live birth was achieved exclusively using the slow-freezing method (Al-Hasani *et al*., 1989; Vincent *et al*., 1989; Jiménez-Trigos *et al*., 2013). To our knowledge, this is the first report to obtain live young from vitrified rabbit oocytes.

Different assisted reproductive technologies for *in vitro* development of rabbit oocytes have been applied, although they have not been successfully developed (Keefer 1989, Curry *et al*., 2000, Zheng *et al*., 2004). Moreover, it has been posited that the suboptimal *in vitro* culture environment may lead to blastocysts of inferior quality compared to those grown *in vivo* (Rizos *et al*., 2002b; Avilés *et al*., 2010). This has been related with modifications in embryo

morphology (Fair *et al*., 2001), metabolism (Khurana and Niemann, 2000) and gene expression (Wrenzycki *et al*., 1996; Niemann and Wrenzycki, 2000; ; Saenz-de-Juano *et al*., 2011; Rizos *et al*., 2002a), resulting in low pregnancy rates (Holm *et al*., 1996; Hasler, 2000; Lonergan *et al*., 2003). Specifically, *in vitro* cultured rabbits undergo retarded development and do not form a mucin coat, which reduces pregnancy rates after embryo transfer (Seidel *et al*., 1976; Jin *et al*., 2000). Taken together, the purpose of our study was to provide the best conditions for fertilisation and embryo development in order to generate live birth from vitrified rabbit oocytes.

Results obtained showed that we generated live birth from vitrified rabbit oocytes for the first time in the world using intraoviductal oocyte transfer as a method to induce pregnancies. The efficiency of *in vivo* fertilisation after vitrified oocyte transfer (5.5% (9/165)) was similar to those obtained with slow-freezing ones (4.4% (7/161)). Moreover our offspring rates were similar to those obtained previously in our laboratory (3.3% (4/121) (Jiménez-Trigos *et al*. 2013) and by Al-Hasani *et al*., (1989) (7.5% (4/53)) using slow-freezing oocytes, and to those reported in other species such as human (Fadini *et al*., 2009; Virant-Klun *et al*., 2011), bovine, (Suzuki *et al*., 1996, Kubota *et al*., 1998, Vieira *et al*., 2002; Morató *et al*., 2008) and mouse (Aono *et al*., 2005, Lee *et al*., 2010).

It has been reported that rabbit oocytes are highly sensitive to low temperatures and high levels of cryoprotectants, and cryopreservation causes damage to the organisation of the microtubules and meiotic spindle (Diedrich *et al*., 1988, Vincent *et al*., 1989, Cai *et al*., 2005, Salvetti *et al*., 2010, Jiménez-Trigos *et al*., 2012, 2013a) inducing chromosome aberration (Diedrich *et al*., 1988, Salvetti *et al*., 2010, Jiménez-Trigos *et al*., 2013a). Based on our previous

results, only around 18-33% of cryopreserved oocytes had intact nuclei after warming. (Jiménez-Trigos *et al*., 2012, 2013a). If we consider that the efficiency of *in vivo* fertilisation after fresh oocyte transfer was 23.1%, improvements to this technique (*in vivo* fertilisation) could obtain better results after the transfer of cryopreserved oocytes. When we compared our results, we observed that after the transfer of fresh oocytes the rate of offspring decreased compared to the control (non-transferred) group (23.1% vs. 73.5%, respectively). However, no differences in birth weight were observed among groups.

The reduction of *in vivo* fertilisation after intraoviductal oocyte transfer could be related with alterations caused by oviduct manipulation or oocyte handling. First, we discard any asynchrony in the fertilisation process, because in rabbit the ovulation normally occurs 10–12 h after mating (Chang 1951) and in our study, oocytes were transferred into induced recipient females induced to ovulate 9 h beforehand (ovulation had not yet taken place). Therefore, this methodology was adjusted to the maximum allowed for the species. On the other hand, it has been noted that oviduct manipulation could give rise to some adverse effects, such as inflammatory reaction and the release of substances, such as catecholamines, cytokines, prostaglandins and leukotrienes, that may affect the normal oviduct function, loss of ciliary movement associated with failure of oocyte transportation and consequently failure of fertilisation, a deleterious effect on oocytes and a fertilisation rate reduction (David *et al*., 1969, Wainer *et al*., 1997, Tuffrey *et al*., 1990). Another hypothesis could be that the adhesive used to close the oviduct immediately after transfer could attach the oocytes after transfer and consequently block the fertilisation process. Concerning oocyte handling, oocyte manipulation

itself may also retard the developmental rate of transferred oocytes (Tarkowski 1959, Adams 1973). Therefore, although further experiments to improve the efficiency of the technique are still needed, the use of this method allowed us to obtain higher offspring rates than when using ICSI, where the birth rate using fresh oocytes ranged between 0.4-6.0% (Deng and Yang 2001; Li *et al*., 2001; Li *et al*., 2010).

Our experimental design suggests that *in vivo* environment could help improve the results of oocyte cryopreservation, as this is the first report that resulted in the development to term of vitrified rabbit oocytes in combination with *in vivo* fertilisation.

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9. GENERAL DISCUSSION

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9. GENERAL DISCUSSION

Since the middle of the last century, several assisted reproductive technologies (ART) have been developed in rabbit, including superovulation, artificial insemination (AI), *in vitro* fertilisation (IVF), intracytoplasmic sperm injection (ICSI), *in vitro* embryo culture and embryo transfer (ET). Furthermore, cryopreserved embryos, gametes (sperm and oocytes) or ovarian tissue have become an integral part of ART. Although embryo and gamete cryopreservation in animal species is considered an important tool in reproductive biotechnology, from a genetic standpoint cryopreserved selected lines constitute an important tool in livestock production, as the rabbit breeding industry is increasingly using these selected lines (García and Baselga 2002). Generation and characterisation of these lines must be kept in stock even if not needed for commercial use (Santacreu *et al.,* 2005) in order to preserve them from pathogens, to evaluate the genetic improvement, minimise the impact of genetic drift and facilitate diffusion of the genetics to different countries, avoiding animal transportation and its sanitary risks (García and Baselga 2002; Lavara *et al*., 2011). In our laboratory, establishing the rabbit embryo cryobank began in the nineties, and since then more than 10,000 embryos from different selected rabbit lines have been vitrified (Lavara *et al*., 2011). Several works involving transfers of these vitrified rabbit embryos were shown to be effective not only after a short period of cryostorage (Kasai *et al*., 1992; Vicente *et al.,* 1999; Mehaisen *et al*., 2006) but also after a long period. Vitrification for up to 15 years maintains embryonic developmental viability and could achieve good pregnancy rate, fertility and survival at birth after the transfer (Lavara *et al.,* 2011). On the other hand, generation of a gamete bank

enables us to stored unfertilised material from valuable individuals or from unexpectedly dead animals until an appropriate germplasm is selected (Ledda *et al*., 2001; Checura and Seidel 2007; Pereira and Marques 2008). Nevertheless, only the haploid genotype is conserved and if the original genetic background is required in the future, the appropriate gamete would also have to be available (Glenister and Thornton 2000). Although cryopreserved rabbit sperm is not used for commercial purposes at present, it has been used for experimental or genetic resource bank purposes with a variable fertility after vaginal AI (Mocé and Vicente 2009).

Concerning oocyte cryopreservation, it has numerous practical, economical and ethical benefits. It has proven to be very useful to preserve animal breeding and laboratory products against loss of valuable genotypes due to unexpected disease or hazards, or to improve the reproductive rate and facilitate implementation of many ART (Ledda *et al*., 2001; Woods *et al*., 2004; Checura and Seidel 2007; Pereira and Marques 2008; Prentice and Anzar 2011; Díez *et al*., 2012). Oocyte banking could provide significant additional increases in offspring potential of females. Despite all efforts, oocyte survival and development following cryopreservation remains poor, the main problems being the lack of consistency of the results among groups (Liebermann and Tucker 2002) and the differences in survival rates after warming between species, development stages that were cryopreserved and oocyte quality (Prentice *et al*., 2011). Variable developmental rate after cryopreservation has been described in cows (Vajta *et al*., 1998; Dinnyes *et al*., 2000; Schmidt *et al*., 2004), mouse (Rall and Fahy 1985; Lane and Gardner 2001; Kohaya *et al*., 2013), rat (Fujiwara *et al.,* 2010), swine (Rojas *et al*., 2004; Gupta *et al*., 2007; Zhou and

Li 2009), humans (Edgar and Gook 2012), horses (Tharasanit *et al*., 2006) or cat (Luvoni 2006). Moreover, live offspring have only been obtained in a few species, such as mouse (Whittingham 1977), human (Chen 1986), rabbit (Al-Hasani *et al.,* 1989), cattle (Fuku *et al*., 1992), rat (Nakagata 1992), horse (Maclellan *et al*., 2002), cat (Gómez *et al.,* 2008) and swine (Somfai *et al*., 2013), thus oocyte cryopreservation is still considered a developing technology.

Specifically in rabbit, oocyte developmental competence after warming remains low (Diedrich *et al.,* 1988; Al-Hasani *et al.,* 1989; Siebzehnruebl *et al.,* 1989; Vincent *et al.*, 1989; Cai *et al*., 2005; Salvetti *et al.*, 2010; Wang *et al*., 2010). Moreover, to our knowledge only two studies published in 1989 reported offspring from cryopreserved rabbit oocytes (Al-Hasani *et al.,* 1989 and Vincent *et al*., 1989). However, only one of them obtained live birth (Al-Hasani *et al*., 1989), as Vincent *et al.,* (1989) showed unborn offspring at day 25 of gestation. Rabbit animal models have been used to advance our understanding of mammalian reproduction for over a century (Heape 1891; Pincus 1939; Chang *et al.,* 1970). In the early days of cryobiology, in the 20th century, rabbit appeared as one of the species widely used (Hoagland and Pincus 1942; Emmens and Blackshaw 1950 Smith 1953; Bank and Maurer 1974; Whittingham and Adams 1974; Diedrich *et al*., 1986; Al-Hasani *et al.,* 1989; Siebzehnruebl *et al.*, 1989; Vincent *et al*., 1989). Nevertheless, few works on oocyte cryopreservation has been done since 1988 (Diedrich *et al.,* 1988; Al-Hasani *et al.*, 1989; Siebzehnruebl *et al*., 1989; Vincent *et al.,* 1989; Cai *et al*., 2005; Salvetti *et al.,* 2010; Wang *et al*., 2010) and results showed that rabbit oocytes are highly sensitive to low temperatures and high levels of cryoprotectants. All these studies showed that, during cryopreservation, oocytes suffer considerable

morphological and functional damage leading to disorganisation of microtubules and meiotic spindle (Diedrich *et al.,* 1988, Vincent *et al*., 1989, Cai *et al*., 2005, Salvetti *et al*., 2010) and chromosome aberration (Diedrich *et al*., 1988, Salvetti *et al*., 2010). Consequently, blastocyst production after warming is very low (Diedrich *et al*., 1988, Al-Hasani *et al*, 1989, Siebzehnruebl *et al*., 1989, Vincent *et al*., 1989, Cai *et al*., 2005, Salvetti *et al*., 2010, Wang *et al*., 2010). For this reason, in an attempt to improve post-warming survival and subsequent development of cryopreserved rabbit oocytes, the modification of cryopreservation procedures, for example by varying types of cryoprotectants, using additives, or modifying the oocytes themselves to make them more cryopreservable, was proposed in this thesis as a possible way to improve the cryotolerance. Nevertheless, our results demonstrated that stabilising the cytoskeleton system during vitrification using Taxol or Cytochalasin B (CB) did not improve the normal spindle and chromosome configuration or the development to blastocyst stage after parthenogenetic activation. Additionally, we tried to increase cholesterol membrane content of rabbit oocytes by incubation with cholesterol-loaded-cyclodextrin (CLC) prior to cryopreservation in order to make the cell membrane more fluid at temperatures above the phase transition and resistant to cold stress, and increase cleavage and blastocyst rates that way. However, results showed that the addition of cholesterol had no effect on embryo development after cryopreservation. This could be because we were unable to observe cholesterol incorporation into oocyte membrane and therefore any change at the plasma membrane. We observed that this cholesterol diffused through the zona pellucida and penetrated into oocyte cytoplasm. However, previous studies assumed that cholesterol was added into plasma membrane using CLC

as a vehicle (Horvath and Seidel 2006; Sprícigo *et al*., 2012). Additionally, we observed that part of this cytoplasmic cholesterol was lost after cryopreservation. It has been previously reported that embryo quality, developmental potential and the success rates of embryo cryopreservation appear to be highly correlated with cytoplasmic lipid content (Dobrinsky *et al*., 2000; Horvath and Seidel 2006; Park *et al*., 2005). Species with large amount of lipids in the cytoplasm exhibit reduced tolerance to cooling (Polge *et al*., 1974; Didion *et al.,* 1990; Dobrinsky 1997). Moreover, lesser phospholipid content after freezing and thawing has been proposed as the main reason for the low survival and development rates (Lim 1992; Parks and Ruffing 1992; Van Blerkom and Davis 1994). In this sense, our study showed an increase in cytoplasmic cholesterol content after CLC pre-treatment and a loss of this cholesterol content after cryopreservation, without any improvement in developmental competence of cryopreserved oocytes.

Currently, there are two basic techniques that rule the field of oocyte cryopreservation: slow-freezing and vitrification. When we compared both procedures, no differences were observed between vitrified and slow-frozen oocytes in terms of spindle integrity. This may be attributed to the use in our study of VM3 solution for vitrification, previously designed to present low toxicity (Fahy *et al.,* 2004), following the minimum essential volume method, using Cryotop as device, which allowed a high cooling rate, minimising the toxic and osmotic effects (Vajta and Kuwayama 2006; Yavin *et al.,* 2009). However, Cortical Granules (CGs) distribution generally appeared to be altered after cryopreservation, especially after vitrification, which confirms that rabbit oocytes are particularly sensitive to cryoprotectants and low temperatures,

leading to depolymerisation of microtubules and disrupting the network of the meiotic spindle and CGs regardless of the cryopreservation procedure. These structural alterations, more evident in vitrified than in slow-frozen oocytes, would be responsible for the reduced developmental competence after parthenogenetic activation observed in our study, as abnormal spindle and dispersed chromosomes have been related with poor rates of fertilisation and development (Chen *et al.,* 2003; Magli *et al.,* 2010). Nevertheless, considering our results, both methods compromised developmental competence in the same way after warming (around 20% of cryopreserved oocytes presented intact meiotic spindle configuration, which could be related to the developmental decrease after the process).

Although much progress been made since the first successful report of oocyte cryopreservation (Whittingham 1977), no general protocol has yet been established. A possible explanation for the few works done in rabbit and the scant progress in this field may be the lack of successful development of different ART in this species (Keefer 1989, Curry *et al*., 2000, Zheng *et al*., 2004). Cryopreserved oocytes at germinal vesicle state (GV) might overcome the problem of damage in meiotic spindle followed by associated microtubule depolymerisation in matured cryopreserved oocytes (MII) (Aman and Parks 1994; Shaw *et al*., 2000; Rojas *et al*., 2004; Prentice *et al.,* 2011) because the meiotic spindle is not organised yet (Díez *et al.,* 2012). However, although rabbit oocytes are widely used as an *in vitro* model in experimental procedures on mammalian oocytes and embryos, to date there is little information about production of oocytes able to undergo successful maturation (Arias-Alvarez *et al.*, 2010; Sugimoto *et al.,* 2012). Regarding IVF, early works done in rabbit

provided a background to facilitate the spread of IVF technology to other mammalian species (Chang 1959; Bedford and Chang 1962; Brackett and Williams 1968). Nevertheless, this technique has not been set up yet, possibly due to the lack of an efficient *in vitro* capacitation system for rabbit spermatozoa, linked to the poor permeability of sperm plasma membrane (Curry *et al*., 2000; Viudes-de-Castro *et al.,* 2005). Several reports pointed out that ejaculated rabbit spermatozoa capacitated *in vitro* were not entirely equivalent to *in vivo* capacitated spermatozoa in fertilising ability (Bedford 1969; Viriyapanich and Bedford 1981). Viudes-de-Castro *et al.,* (2005) showed that fertilising ability *in vitro* ranged between 8.7% to 26.7%. Similarly, the success of ICSI is still very limited (less than 30% of the injected oocytes developed to blastocyst stage) (Deng and Yang 2001, Li *et al*., 2001; Li *et al.,* 2010), mainly because of the difficulty in performing it due to the presence of rough, dark granules in the plasma that easily lyse and die after the process (Cai *et al.,* 2005). Moreover, Fernández-González *et al.,* (2008) demonstrated that ICSI is capable of producing alterations in the early embryo and long-term consequences as well as genetic and epigenetic changes during preimplantation and, as a consequence, mouse offspring with aberrant growth, behaviour, early aging, and tumours. On the other hand, to date, *in vitro* conditions have been unable to mimic the dynamic changes of oviduct and uterus secretion that respond to the varying metabolism of a developing embryo (Rizos *et al.,* 2002, Saenz-de-Juano *et al.,* 2013). For this reason, our efforts were focused on developing a precise and reliable method for *in vivo* fertilisation as the best option to obtain live offspring from cryopreserved oocytes. Additionally, this environment could be more beneficial when oocyte quality is not optimal.

In all our assays, we performed intraoviductal oocyte transfer technique because rabbit has the advantage of belonging to a species in which ovulation is induced by mating, and this allowed us to manage oocyte transfer according to the timing of gamete biological events. When recipient does are induced to ovulate, sperm transport, fertilisation and embryo development can be exactly synchronised with the donor. In a first attempt to develop an accurate technique for *in vivo* fertilisation, the effect of two recipient models, ovariectomised and oviduct ligated immediately after transfer, were compared. Results showed that both models are able to fertilise rabbit oocytes. Nevertheless, they affected the reproductive tract functionality as a consequence of the surgery in ovariectomised females and a tubal fluid accumulation induced by oviduct ligation. Moreover, a low recovery rate was observed after transfer into both models. This low recovery rate during the first day after transfer has been observed previously (Ryan and Moore 1988; Cortell *et al.,* 2010). Nevertheless, in our case this is not related with the transfer procedure, as high recovery rates were obtained in our control-transferred and control groups. Nor is it due to an altered tubal migration, as all uterine horns were perfused separately and no oocytes or embryos were collected. Moreover, oviduct ligation ensures that no loss of oocytes occurs in the peritoneal cavity. Several hypotheses could be the cause of this low recovery rate in all transferred groups. One of them could be the absence of the follicular fluid that could induce oocyte retention in the oviduct. Another hypothesis could be the absence of cumulus cells. In all experiments, oocytes were transferred without cumulus cells in order to identify morphologically normal oocytes and allow their passage through the transfer needle. This removal of cumulus cells could induce an inadequate oocyte adhesion and

transport into the oviduct and could be also related with reduced fertilisation and cleavage rate through the loss of several factors secreted by these cells.

According to the results obtained with fresh oocytes, unilateral oviduct ligated females were employed as a model to generate live offspring from slow-frozen oocytes. Offspring rates obtained were similar to those reported in other species such as human, (Fadini *et al.,* 2009; Virant-Klun *et al.,* 2011; Siano *et al.,* 2013), bovine, (Suzuki *et al.,* 1996; Kubota *et al.,* 1998; Vieira *et al.,* 2002; Morató *et al.,* 2008) and mouse (Aono *et al*, 2005; Lee *et al.,* 2010). However, intraoviductal oocyte transfer and oviduct manipulation to prevent the entrance of the recipient's own oocytes into the oviduct generated a tubal disorder in this kind of animal models that reduced the likelihood of fertilisation. This is why we proposed an alternative method to generate less oviductal damage using cyanoacrylate tissue adhesive to block the oviducts. In a first attempt, the ability of this tissue adhesive to block the entrance of the oocytes into the oviducts after ovulation and the effect on *in vivo* fertilising ability were evaluated. Then, *in vivo* fertilisation and live birth rate after cryopreserved oocyte transfer were assessed. Results obtained suggested that this kind of animal model for *in vivo* fertilisation showed better results than ovariectomised and unilateral oviduct ligation models. Nevertheless, the lower *in vivo* fertilisation after fresh oocyte transfer confirms that oviduct manipulation or oocyte handling is hazardous to oocyte competence and successful fertilisation. It has been previously observed that oviduct manipulation could trigger an inflammatory reaction that may affect the normal oviduct function and even generate a direct deleterious effect on oocyte (David *et al.,* 1969, Wainer *et al.,* 1997, Tuffrey *et al*., 1990). On the other hand, oocyte

manipulation itself may retard the developmental rate of transferred oocytes (Tarkowski 1959, Adams 1973).

To our knowledge, this is the first study to obtain live offspring from vitrified rabbit oocytes. The use of cyanoacrylate tissue adhesive to block the oviduct and fertilised cryopreserved oocytes reported higher live birth rates after transfer than those obtained in 1989 by Al-Hasani *et al.,* (1989) using slow-freezing method. However, based on the results obtained with fresh oocytes, further experiments to improve the efficiency of both fresh and cryopreserved oocyte transfer and *in vivo* fertilisation are still needed, especially the establishment of successful fertilisation conditions to achieve higher success rates for future application. Based on our results, around 18-33% of cryopreserved oocytes had intact nuclei after warming. When we transferred these oocytes into recipient oviducts, around 3.3-13.2% were fertilised. When ICSI was used on fresh oocytes, the birth rate ranged between 0.4-6.0% (Deng and Yang 2001; Li *et al*., 2001; Li *et al*., 2010), whereas when using *in vivo* fertilisation technique we reported offspring between 19.2-37.5%. Therefore, improvements of this latter technique (*in vivo* fertilisation) could obtain better results. Additionally, the technique provides optimal environmental conditions for fertilisation and embryo development to generate live offspring from cryopreserved oocytes, minimising the disorders derived from *in vitro* embryo production, as described previously.

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10. CONCLUSIONS

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The conclusions of this thesis are:

Cryopreservation of rabbit matured oocytes (MII) led to structural damage which had been related with reduced developmental competence after cryopreservation, regardless of the procedure. However, these structural alterations are more evident in vitrified than in slow-frozen oocytes, probably due to the high sensitivity of rabbit oocytes to high concentrations of cryoprotectants.

Modifying oocytes to make them more cryotolerant by stabilising the cytoskeleton system during cryopreservation with Taxol or Cytochalasin B, as well as the addition of cholesterol to the plasma membrane to increase its fluidity and stability during the process, showed that these modifications had no effect on the structure and development capacity of cryopreserved rabbit oocytes after the procedure.

Live offspring from cryopreserved, slow-frozen and vitrified rabbit oocytes have been obtained after *in vivo* fertilisation using intraoviductal oocyte transfer assisted by laparoscopy. This technique has arisen as a reliable and reproducible method to induce pregnancies in this species and has allowed live kits to be obtained from vitrified rabbit oocytes for the first time.