MASTER INTERUNIVERSITARIO EN MEJORA GENÉTICA ANIMAL Y BIOTECNOLOGÍA DE LA REPRODUCCIÓN

Effects of cryopreservation on the meiotic spindle, cortical granule distribution and development of rabbit oocytes

Tesis de Master
Valencia, Julio 2011

D.ª Estrella Jiménez Trigos

Director:
D. Francisco Marco Jiménez

INDEX

INTRODUCTION

1. Introduction

1.1. Application 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, minimizing the impact of the genetic drift and to facilitate the diffusion of the lines to different countries avoiding animal transportation and its sanitary risks (García and Baselga 2002; Lavara et al. 2011). Moreover, these techniques allow us to conserve and widespread animal genetics biodiversity and to preserve endangered species to maintain biodiversity (Woelders et al. 2006; Andrabi and Maxwell 2007; Pereira and Marques 2008; Prentice and Anzar 2011). Despite all the advantage however, gamete cryopreservation presents disadvantage that, only the haploid genotype is conserved. Nevertheless, oocyte banks allows female genetic material to be stored unfertilized until an appropriate male germplasm is selected, moreover it would also preserve the genetic material from unexpectedly dead animals and facilitate many assisted reproductive technologies (Ledda et al. 2001; Checura and Seidel 2007; Pereira and Marques 2008). In human, oocyte 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 loosing ovarian function (Ledda et al. 2001; Nottola et al. 2008; Porcu et al. 2008).

1.2 Methods for gamete and embryos cryopreservation: slow freezing and vitrification

Currently, there are two methods for gamete and embryo cryopreservation according with presence or absence of ice formation: slow freezing and vitrification.

1.2.1 Slow freezing

Slow freezing uses low concentrations of permeating cryoprotectants, as propyleneglycol, ethyleneglycol, dimethylsulphoxide and glycerol (Otoi et al., 1993;

Lim et al. 1999). The final concentration of permeating cryoprotectants is from 10 to 15%. This has the advantage of producing less chemical toxicity and osmotic shock, but their ability to prevent ice-crystal formation is limited. Non permeating cryoprotectants are used in combination with a permeating cryoprotectant, to increase the net concentration of the permeating cryoprotectant inside the cell and also preventing ice-crystal formation. The most commonly used nonpermeating cryoprotectants are sucrose, galactose and threalose.

In slow freezing method oocytes are mounted between two air bubbles in 0.25 mL sterile French mini straws sealed by a sterile plug (Figure 1). The straws are then placed into a programmable freezer where temperature is lowered from 20º C to about -5ºC to -7º C where they are kept for several minutes to equilibrate. After equilibration manual seeding is performed and temperature is then lowered to about -30ºC to -35ºC at a rate of about 0.3ºC-0.5ºC/min. Finally, straws are directly plunged into LN2 and stored for later use (Figure 2).

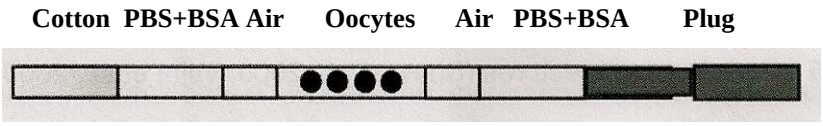

Figure 1. Diagram of packed in straws

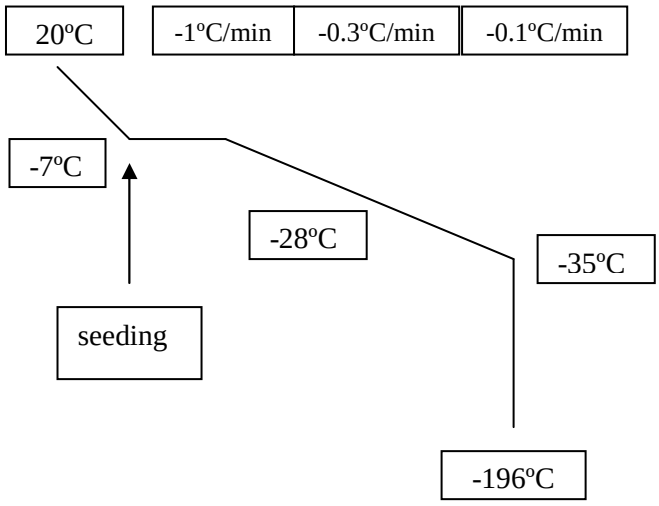

Figure 2. Temperature curve of slow freezing method

The induction of seeding (ice formation in the external solution) by touching the wall of the container with an object cooled at -196 ºC, prevents supercooling and starts the dehydration process (Shaw 1993). The seeding induces the concentration of the solutes in the non-frozen fraction that gradually increases because water is incorporated into the extracellular ice crystals causing a progressive dehydration of the cell thanks to the osmotic gradient, that has been generated across the cell membrane.

2.1.2. Vitrification

Vitrification is the process by which the solution containing sample is cooled so rapidly that the water molecules do not have time enough to form ice crystals and instantly solidify into a “glass-like” structure. The concept is based upon the idea that if the cell is dehydrated to a certain degree and then cooled fast enough, everything will “freeze” in place and damage will not have time to occur, crystals will not be able to organize themselves and a vitrified amorphous glass, like solid, will be formed instead of ice.

To achieve vitrification state three factors should be considered: cooling rate, medium viscosity and volume.

Cooling rate. High cooling rate is achieved plunging the sample into liquid nitrogen. The cooling rate depends on the container, the volume, the termal conductivity, the solution, etc.

Viscosity. The viscosity of the medium depends on the concentration and behaviour of cryoprotectants and other additives in the vitrification solution. The combination of different cryoprotectants is used to increase the viscosity and to reduce the level of toxicity. Cryoprotectants are additional chemicals used in cryopreservation to avoid ice formation and shocking effects. They are classified in two groups: permeating and non-permeating group.

The permeating group includes small molecules that readily penetrate the membranes of cells, form hydrogen bonds with intracellular water molecules and lower the freezing

temperature of the resulting mixture, preventing ice crystallization. Some permeating cryoprotectants are Glycerol, Ethylene glycol (EG), propane-1,2-diol (Pr-OH), Dimethylsulphoxide…

No permeating agents (glucose, sucrose, trehalose, Ficoll, etc.) are large molecules that do not permeate the cell membrane, but they increase the concentration of extracellular solutes and generating an osmotic gradient across the cell membrane, which draws water out of the cell causing the cell dehydratation before the freezing procedure. When they are combined with permeating cryoprotectants, they contribute to the overall vitrification properties.

The concentration of cryoprotectants in the freezing solution (equilibration solution) is really very important as it determines the dehydration rate of the oocyte: the higher the concentration is, the quickies the oocyte dehydrates and the faster the water leaves cytoplasm to dilute the high concentration of extracellular solutes (Shaw 1993).

Volume. The smaller the volume is, the higher probability of vitrification is. Smaller volumes allow better heat transfer, which facilities higher cooling rates. Different carrier tools have been applied to minimise the volume, including surface and tubing techniques. Surface techniques include electron microscope grids (Martino et al. 1996), cryoloops (Fuchinoue et al. 2004), cryotops (Kuwayama et al. 2005), Hemi-straw (Vanderzwalmen et al. 2000), solid surface (Dinnyes et al. 2000), nylon mesh (Matsumoto et al. 2001), Cryoleaf (Chian et al. 2005), direct cover vitrification (Chen et al. 2006), fiber plug (Muthukumar et al. 2008), vitrification spatula (Tsang & Chow 2009), Cryo-E (Petyim et al. 2009), plastic blade (Sugiyama et al. 2010), and Vitri-Inga (Almodin et al. 2010). Tubing techniques include the plastic straw (Rall & Fahy 1985), open pulled straw (OPS; Vajta et al. 1997, 1998), closed pulled straw(CPS; Chen et al. 2001), flexipet-denuding pipette (Liebermann et al. 2002), superfine OPS (Isachenko et al. 2003), CryoTip (Kuwayama et al. 2005), pipette tip (Sun et al. 2008), high-security vitrification device (Camus et al. 2006), sealed pulled straw (Yavin et al. 2009), Cryopette (Portmann et al. 2010), Rapid-i (Larman & Gardner 2010), and JY Straw (R C Chian, personal communication). An examples of the different devices are shown in Figure 3 (adapted from Saragusty and Arav 2010).

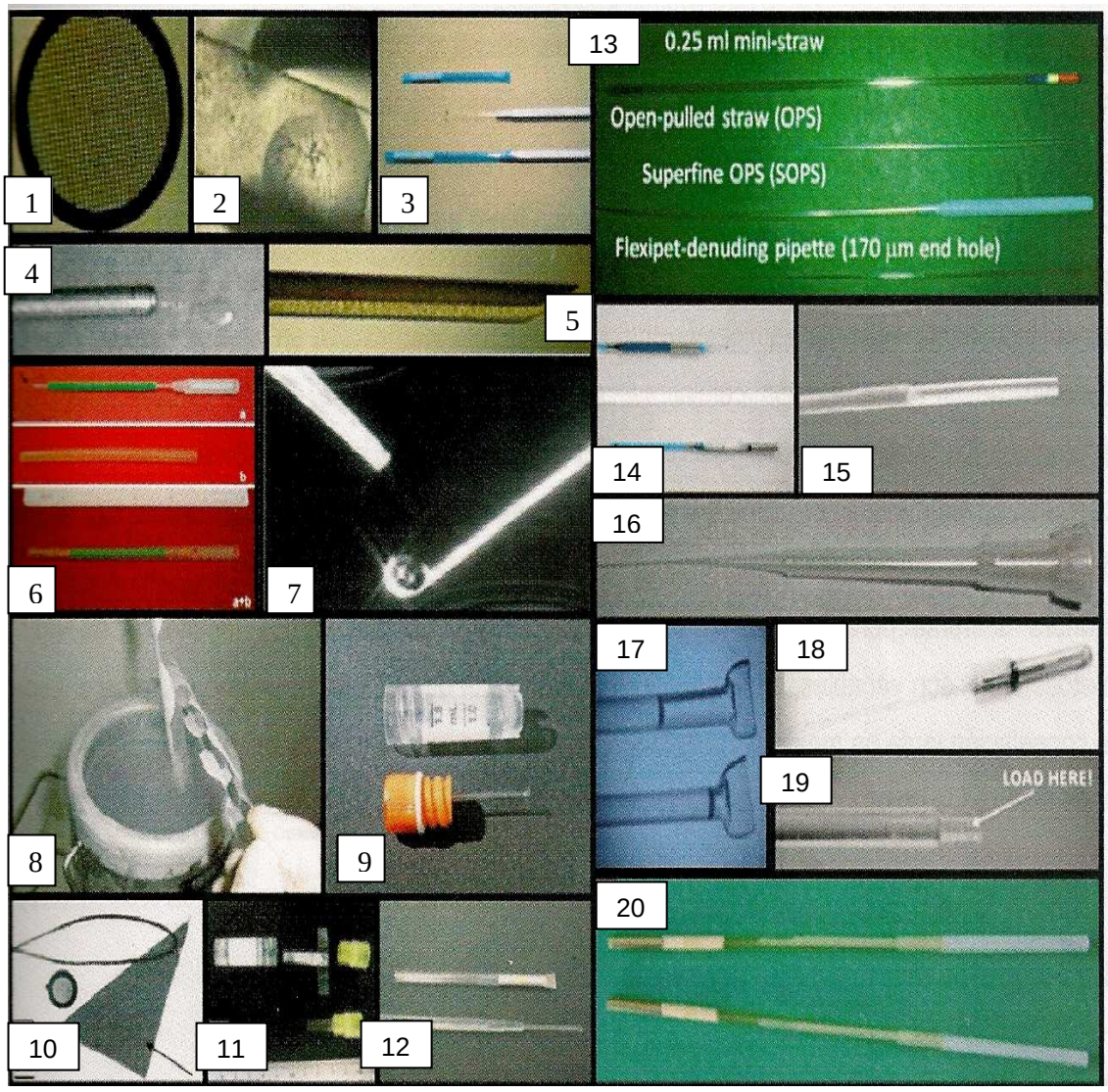

Figure 3. Decivers for vitrification. Electron microscope grids (1), minim volume drop (2), Cryotop (3), Cryoloop (4), Hemi straw (5), CryoleafTM (6), Fibre plug (7), Direct cover vitrification (8), vitrification spatula (9), nylon maya (10), Cryotube (11), Vitri-Inga (12), Straw, open-pulled straw, super open-pulled straw (13), Cryotip® (14), flexipet-denuding pipette (15), Pipette tip (16), Sealed pulled straw (17), Cryopette® (18), Rapid-iTM (19), JY Straw (20). (Adapted from Saragusty and Arav 2010).

Decreasing the volume of vitrification and increasing the cooling rate allow a moderate decrease in cryoprotectants concentration to minimize its toxic and osmotic hazardous effects. Combination of these three factors can result in the following general equation for the probability of vitrification:

$$\text{Probability of vitrification} = \frac{\text{Cooling rate} \times \text{Viscosity}}{\text{Volume}}$$

1.3. Slow freezing vs. vitrification

Cryopreservation by slow freezing is a process where extracellular water crystallizes, resulting an osmotic gradient that draws water from the intracellular compartment till intracellular vitrification occurs. In cryopreservation by vitrification, both intra and extracellular compartments apparently vitrify after cellular dehydration has already occurred. Owing to these differences, the terms freezing and thawing are relevant to the slow freezing process while cooling and warming are relevant to vitrification.

Unlike the controlled rate freezing method, which requires sophisticated equipment to manage the cooling rate, vitrification can be done relatively cheaply and even under field conditions with no need for special equipment, making it a good alternative for the its use in various settings often encountered with wildlife species, such as zoos, poorly equipped locations, and field work in remote sites. However, performing vitrification, and in particular loading the sample properly into or onto the container, does require much experience to be done properly.

Despite 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 (Berthelot et al. 2000; Vajta et al. 1998; Martinez et al. 2003; Lavara et al. 2011). Table 1 shows the differences between both methods.

Table 1. Differences between slow freezing and vitrification. (Adapted form Pereira and Marques 2008).

Freezing procedures	
Slow-freezing	**Vitrification**
Standard 0.25 ml straws	Several devices for loading embryos and oocytes (standard 0.25 ml straws, OPS, cryoloop, cryoleaf…)
Sophisticated equipment	No special equipment
Low cryoprotectant concentrations	High cryoprotectant concentrations /reduced volume and time with vitrification solution
Seeding at -5 to -7ºC, controlled slow cooling (0.1 to 0.3ºC/min)	Ultrarapid cooling rates (-2500ºC/min or 20000ºC/min using OPS or cryoloop)
Plunging at -30 to -70ºC and storage in liquid nitrogen (-196ºC)	Plunging into liquid nitrogen (-196ºC)
Extracellular water crystallizes and intracellular compartment vitrify	Both intra and extracellular compartments vitrify

1.4. Difficulties to oocyte cryopreservation

Although many progresses have been done in oocytes cryopreservation, general protocol has not been established yet (Nottola et al. 2008; Pereira and Marques 2008; Noyes et al. 2010). Moreover, procedures developed for one specie are difficult to adapt to an other specie (Paynter et al. 1999:2001).

Oocytes are particularly difficult to cryopreserved successfully resulting in low rates of blastocyst production after thawing, fertilization and culture. In general, the low efficiency might be due to the complex structure of the oocyte and to the differences in

membrane permeability and physiology between embryos and oocytes (Gardner *et al.*, 2007). Most of the components present in the oocyte are particularly sensitive to temperature and osmotic pressure (Figure 4).

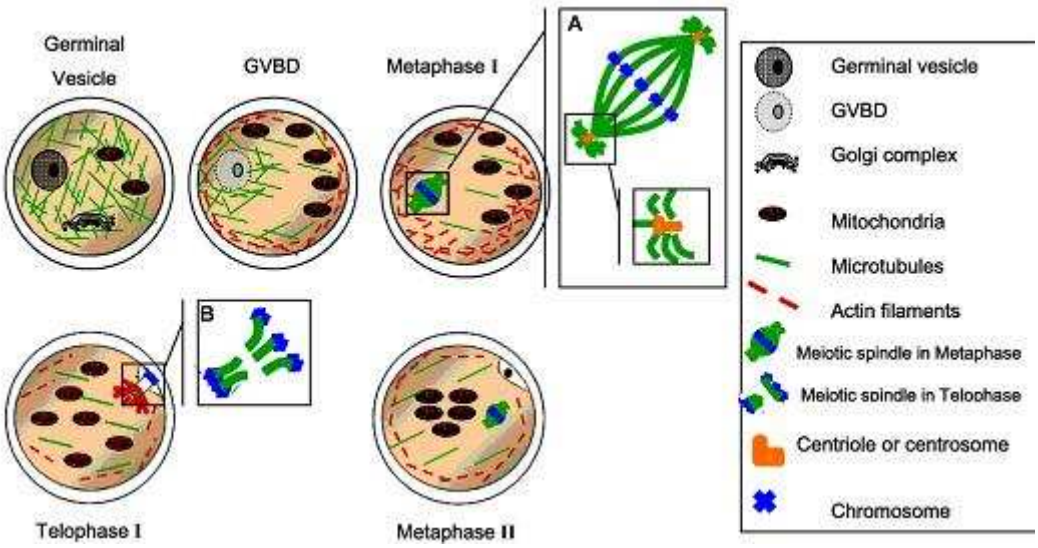

Figure 4. Diagram of an Oocyte. (Adpated from Ferreira et al. 2009)

The large size of oocytes and the low surface to volume ratio, make it more difficult for water and cryoprotectants to move across the cell. Moreover, the plasma membrane of oocytes at the second metaphase stage has a low permeability coefficient, making the movement of cryoprotectants and water slower. They are also surrounded by zona pellucida, which acts as an additional barrier for the movement of water and cryoprotectants into and out of the oocyte. Additionally, oocytes have less submembranous actin microtubules making their membrane less robust.

1.5. Cellular components damaged during cryopreservation

Cryopreservation can cause cytoskeleton disorganization and chromosome and DNA abnormalities. Oocytes cryopreserved after maturation 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. Oocytes also have high cytoplasmic lipid content that increases chilling sensitive.

Chilling and freezing are associated with an altered distribution of cortical granules and an increasing polispermy or, on the contrary, with zona pellucida hardening by premature cortical granule exocytosis impairing fertilization. Frequently oocytes present zona pellucida or cytoplasmic membrane fracture. These problems and their effects are summarized in Table 2.

Table 2. Problems and effects associated with chilling and freezing of oocytes.

Alteration	Effect
Meiotic spindle depolymerisation Chromosome abnormality	Increase polyploidy and aneploidy
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)

1.6. Current status of oocyte cryopreservation

Up to now, oocytes have been cryopreserved by both methods in some species and although several breakthroughs have been made in oocyte cryopreservation since 1971, live offspring have only been obtained in a few species, such as mouse (Withingham, 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).

Results remain low, and pregnancy rates remain higher using cryopreserved embryos. The literature reports a great variability between both methods (Table 3) and the results obtained are different depending on the species. In human, vitrification

shows better results than slow-freezing (Fadini et al. 2009) but in rabbit, slow-freezing shows higher results than vitrification (Salvetti et al. 2010).

Table 3. Pregnancy rate per oocyte cryopreserved in some species.

Specie	Author	Year	Cryopreservation method	Pregnancy rate/oocyte cryopreserved (%)
Human	Virant-Klun et al.	2011	Slow-freezing	2.41
	Kuwayama	2007	Vitrification	9.90
	Fadini	2009	Slow freezing	0.89
			Vitrification	1.05
Horse	Maclellan	2002	Vitrification	7.69
Bovine	Suzuki et al.	1996	Slow-freezing	0.83
	Kubota et al.	1998	Slow-freezing	0.64-0.73
	Vieira et al.	2002	Vitrification	0.41
Rabbit	Al-Hasani et al.	1989	Slow-freezing	0.84
Mouse	Bos-Mikich et al.	1995	Vitification	0.83
	Aono et al.	2004	Vitification	2.89
	Lee et al.	2010	Vitrification	0.08

Although many advances have been made, continued optimization of oocyte cryopreservation techniques is challenging due to the scarcity of material for experimentation. Rabbit has been used as a model organism for studying mammalian reproduction for decades (Heape 1891; Pincus 1939; Chang et al. 1970). Nevertheless, while numerous reports of studies designed to investigate oocyte cryopreservation in some species have been published (Mullen 2007) a 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 a recent work compare slow-freeze and vitrification methods (Salvetti et al. 2010), moreover, live offspring has been only obtained using slow-freezing method (Al-Hasani et al. 1989).

Rabbit embryos matured *in vitro* have no mucin coat. When cultured blastocysts are transferred to recipients, the lack of mucin coat might in part account for subsequent failure of pregnancy These embryos must be transferred to recipients after 24-48 h for mucin coat deposition during passage through the oviduct in order to obtain higher rates of implantation of the in vitro-cultured blastocysts (Joung et al. 2004). The thickness of the mucin layer appears to be an important factor for successful implantation of rabbit embryos (Murakami et al. 1996).

Rabbit is a standard laboratory animal in biomedical research, and transgenic rabbits are used as animal models for a variety of human diseases both genetic and acquired. The rabbit (Oryctolagus cuniculus) is phylogenetically closer to primates than rodents (Graur et al. 1996) and is large enough to permit non-lethal monitoring of physiological changes. For these reasons, several research groups have chosen transgenic rabbits as animal models for the study of lipoprotein metabolism, atherosclerosis, cardiovascular research and hypertrophic cardiomyopathy (Bosze and Houdebine 2006). The rabbit may be a better model organism for experimental investigation of oocyte cryopreservation due to their smaller size, ease of handling, relatively short gestation time, and economy when it comes to applying embryo transfer procedures compared to cattle and pigs. Furhtermore, experimental evidences suggest that the extreme sensitivity of cattle and pig oocytes higher than human oocytes may make them relatively poor models (Hunter et al. 1991; Martino et al. 1996; Liu et al. 2003).

1.7. References

Al-Hasani S, Kirsch J, Diedrich K, Blanke S, van der Ven H, Krebs D, 1989: Successful embryo transfer of cryopreserved and in-vitro fertilized rabbit oocytes. Hum Reprod *4* 77–79.

Almodin CG, Minguetti-Camara VC, Paixao CL, Pereira PC, 2010: Embryo development and gestation using fresh and vitrified oocytes. Hum Reprod. *25* 1192-1198.

Aono N, Abe Y, Hara K, Sasada H, Sato E, Yoshida H, 2005: Production of live offspring from mouse germinal vesicle-stage oocytes vitrified by a modified stepwise method, SWEID. Fertil Steril. *84 2* 1078-1082.

Andrabi SMH, Maxwell WMC, 2007: A review on reproductive biotechnologies for conservation of endangered mammalian species. Anim Reprod Sci *99* 223-243.

Berthelot F, Marinat-Botte F, Locatelli A, Perreau C, Terqui M, 2000: Piglets born after vitrification of embryos using the open pulled straw method. Cryobiology 41 116–124.

Bos-Mikich A, Wood MJ, Candy CJ, Whittingham DG, 1995: Cytogenetical Analysis and Developmental Potential of Vitrified Mouse Oocytes. Biol Reprod. *53* 780-785.

Cai XY, Chen GA, Lian Y, Zheng XY, Peng HM, 2005: Cryoloop vitrification of rabbit oocytes. Hum Reprod. *20* 1969-1974.

Camus A, Clairaz P, Ersham A, Van Kappel AL, Savic G, Staub C, 2006: The comparison of the process of five different vitrification devices. Gynecol Obstet Fertil. *34* 737-745.

Chang MC, Casas JH, Hunt DM, 1970: Prevention of pregnancy in the rabbit by subcutaneous implantation of silastic tube containing oestrogen, Nature *226* 1262–1263.

Checura CM, Seidel GE Jr, 2007: Effect of macromolecules in solutions for vitrification of mature bovine oocytes. Theriogenology *67* 919-930.

Chen C, 1986: Pregnancy after human oocyte cryopreservation. Lancet. *19* 884-886.

Chen SU, Lien YR, Cheng YY, Chen HF, Ho HN, Yang YS, 2001: Vitrification of mouse oocytes using closed pulled straws (CPS) achieves a high survival and preserves good patterns of meiotic spindles, compared with conventional straws, open pulled straws (OPS) and grids. Hum Reprod *16* 2350–2356.

Chen SU, Chien CL, Wu MY, Chen TH, Lai SM, Lin CW, Yang YS, 2006: Novel direct cover vitrification for cryopreservation of ovarian tissues increases follicle viability and pregnancy capability in mice. Hum Reprod. *21* 2794–2800.

Chian RC, Son WY, Huang JY, Cui SJ, Buckett WM, Tan SL, 2005: High survival rates and pregnancies of human oocytes following vitrification. J Reprod Dev. 2004 *50* 685-696.

Diedrich K, al-hasani S, van der Ven H, Krebs D,1988: Successful in Vitro fertilization of frozen-thawed Rabbit and human oocytes. Ann N Y Acad Sci. *541* 562-570.

Dinnyes A, Dai Y, Jiang S, Yang X, 2000: High developmental rates of vitrified bovine oocytes following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer. Biol Reprod. *63* 513–518.

Fadini R, Brambillasca F, Renzini MM, Merola M, Comi R, De Ponti E, Dal Canto MB, 2009: Human oocyte cryopreservation: comparison between slow and ultrarapid methods. Reprod Biomed Online. *19* 171-180.

Ferreira EM, Vireque AA, Adona PR, Meirelles FV, Ferriani RA, Navarro PA, 2009: Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. Theriogenology. *71* 836-848.

Fuchinoue K, Fukunaga N, Chiba S, Nakajo Y, Yagi A, Kyono K, 2004: Freezing of human immature oocytes using cryoloops with Taxol in the vitrification solution. J Assist Reprod Genet. 2004 *21* 307-309.

Fuku E, Kojima T, Shioya Y, Marcus GJ, Downey BR, 1992: In vitro fertilization and development of frozen–thawed bovine oocytes. Cryobiology *29* 485–492.

García ML, Baselga M, 2002: Estimation of genetic response to selection in litter size of rabbits using a cryopreserved control population. Livest Prod Sci *99* 565-571.

Gardner DK, Sheehan CB, Rienzi L, Katz-Jaffe M, Larman MG, 2007: Analysis of oocyte physiology to improve cryopreservation procedures. Theriogenology. *67* 64-72.

Gómez MC, Kagawa N, Pope CE, Kuwayama M, Leibo SP, Dresser BL, 2008: In vivo survival of domestic cat oocytes after vitrification, intracytoplasmic sperm injection, and transfer to recipients. Reproduction, Fertility and Development 20:118.

Heape W, 1891: Preliminary note on the transplantation and growth of mammalian ova within a uterine foster-mother, Proc. R. Soc. *48* 457–458.

Hiripi L, Negre D, Cosset FL, Kvell K, Czömpöly T, Baranyi M, Gócza E, Hoffmann O, Bender B, Bosze Z, 2010: Transgenic rabbit production with simian immunodeficiency virus-derived lentiviral vector. Transgenic Res. *19* 799-808.

Hochi S, Fujimoto T, Braun J, Oguri N, 1994: Pregnancies following transfer of equine embryos cryopreserved by vitrification. Theriogenology *42* 483–488.

Hunter JE, Bernard A, Fuller B, Amso N, ShawRW, 1991: Fertilization and development of the human oocyte following exposure to cryoprotectants, low temperatures and cryopreservation: a comparison of two techniques, Hum. Reprod. 6 1460–1465.

Isachenko V, Folch J, Isachenko E, Nawroth F, Krivokharchenko A, Vajta G, DattenaM, Alabart JL, 2003: Double vitrification of rat embryos at different developmental stages using an identical protocol. Theriogenology *60* 445–452.

Joung SY, Kim HJ, Choi WS, Im KS, Lee SH, Park CS, Jin DI, 2004: Effects of transferring in vitro-cultured rabbit embryos to recipient oviducts on mucin coat deposition, implantation and development. Zygote. *12* 215-219.

Kubota C, Yang X, Dinnyes A, Todoroki J, Yamakuchi H, Mizoshita K, Inohae S, Tabara N, 1998: In vitro survival frozen-thawed bovine oocytes after IVF, nuclear transfer, and parthenogenetic activation, Mol Reprod Dev. *51* 281-286.

Kuwayama M, Vajta G, Ieda S & Kato O 2005 Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. Reprod Biomed Online. *11* 608-14.

Kuwayama M, Vajta G, Kato O, Leibo SP, 2005: Highly efficient vitrification method for cryopreservation of human oocytes. Reprod Biomed Online *11* 300–308.

Kuwayama M, 2007: Highly efficient vitrification for cryopreservation of human oocytes and embryos: The Cryotop method. Theriogenology. *67* 73-80.

Larman MG, Gardner DK, 2010: Vitrifying mouse oocytes and embryos with super-cooled air. Fertil Steril. 95 1462-1466.

Lavara R, Baselga M, Vicente JS, 2011: Does storage time in LN2 influence survival and pregnancy outcome of vitrified rabbit embryos? Theriogenology doi:10.1016/j.theriogenology.2011.03.018.

Ledda S, Leoni G, Bogliolo L, Naitana S, 2001: Oocyte cryopreservation and ovarian tissue banking. Theriogenology. *55* 1359-1371.

Lee HJ, Elmoazzen H, Wright D, Biggers J, Rueda BR, Heo YS, Toner M, Toth TL, 2010: Ultra-rapid vitrification of mouse oocytes in low cryoprotectant concentrations. Reprod Biomed Online. *20* 201-208.

Liebermann J, Tucker M, Graham J, Han T, Davis A, Levy M, 2002: Blastocyst development after vitrification of multipronuclear zygotes using the felxipet denuding pipette. Reprod Biomed Online. *4* 146-150.

Lim JM, Ko JJ, Hwang WS, Chung HM, Niwa K, 1999: Development of in vitro matured bovine oocytes after cryopreservation with different cryoprotectants. Theriogenology. 51 1303-1310.

Liu RH, Sun QY, Li YH, Jiao LH, Wang WH, 2003: Effects of cooling on meiotic spindle structure and chromosome alignment within in vitro matured porcine oocytes, Mol. Reprod. Dev. *65* 212-218.

Maclellan LJ, Carnevale EM, Coutinho da Silva MA, Scoggin CF, Bruemmer JE, Squires EL, 2002: Pregnancies from vitrified equine oocytes collected from super-stimulated and non-stimulated mares. Theriogenology. *58* 911-919.

Martínez AG, Valca´cel A, Furnus CC, de Matos DG, Iorio G, de las Heras MA, 2006: Cryopreservation of in Vitro produced bovine embryos. Small Rum Res *63* 288-296.

Martino A, Songsasen N, Leibo SP, 1996: Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling, Biol. Reprod. 54 1059-1069.

Matsumoto H, Jiang JY, Tanaka T, Sasada H, Sato E, 2001: Vitrification of large quantities of immature bovine oocytes using nylon mesh. Cryobiology *42* 139-144.

Mullen SF, 2007: Advances in the Fundamental Cryobiology of Mammalian Oocytes, Veterinary Pathobiology, University of Missouri, Columbia. p. 350.

Murakami H, Imai H, 1996: Successful implantation of in vitro cultured rabbit embryos after uterine transfer: a role for mucin. Mol Reprod Dev. *43* 167-170.

Muthukumar K, Mangalaraj AM, Kamath MS, George K, 2008: Blastocyst cryopreservation: vitrification or slow freeze. Fertil and Steril *90* S426-S427.

Nakagata N, 1992: Cryopreservation of unfertilized rat oocytes by ultrarapid freezing. Jikken Dobutsu. *41* 443-7.

Nottola SA, Coticchio G, De Santis L, MAcchiarelli G, Maione M, Bianchi S, Laccarino M, Flamigni C, Borini A, 2008: Ultrastructure of human mature oocytes after slow cooling cryopreservation with ethylene glycol. Reprod Biomed online *17* 368-377.

Noyes N, Boldt J, Nagy ZP, 2010: Oocyte cryopreservation. Is it time to remove its experimental label? J Assist Reprod Genet. *27* 69-74.

Otoi T, Tachikawa S, Kondo S, Suzuki T, 1993: Developmental capacity of bovine oocytes frozen in different cryoprotectants. Theriogenology. *40* 801-807.

Paynter SJ, Cooper A, Gregory L, Fuller BJ, Shaw RW, 1999: Permeability characteristics of human oocytes in the presence of the cryoprotectant dimethylsulphoxide. Hum Reprod. *14* 2338-2342.

Paynter SJ, O'Neil L, Fuller BJ, Shaw RW, 2001: Membrane permeability of human oocytes in the presence of the cryoprotectant propane-1,2-diol. Fertil Steril. *75* 532-538.

Pereira RM, Marques CC, 2008: Animal oocyte and embryo cryopreservation. Cell Tissue Bank. *9* 267-277.

Petyim S, Makemahar O, Kunathikom S, Choavaratana R, Laokirkkiat P, Penparkkul K, 2009: The successful pregnancy and birth of a healthy baby after human blastocyst vitrification using Cryo-E, first case in Siriraj Hospital. J Med Assoc Thai. *92* 1116-1121.

Pincus G, 1939: The development of fertilized and artificially activated eggs, J. Exp. Zool. *82* 85–130.

Porcu E, Bazzocchi A, Notarangelo L, Paradisi R, Landolfo C, Venturoli S, 2008: Human oocyte cryopreservation in infertility and oncology. Curr Opin Endocrinol Diabetes Obes. *15* 529-535.

Portmann M, Nagy ZP, Behr B, 2010: Evaluation of blastocyst survival following vitrification/warming using two different closed carrier systems. Hum Reprod 25 i261 (abstract).

Prentice JR, Anzar M, 2010: Cryopreservation of Mammalian oocyte for conservation of animal genetics. Vet Med Int. doi.

Rall WF, Fahy GM, 1985: Ice-free cryopreservation of mouse embryo at -196 degrees C by vitrification. Nature. *313* 573–575.

Salvetti P, Buff S, Afanassieff M, Daniel N, Guérin P, Joly T, 2010: Structural, metabolic and developmental evaluation of ovulated rabbit oocytes before and after cryopreservation by vitrification and slow freezing. Theriogenology. *74* 847-*855*.

Saragusty J, Arav A, 2011: Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. Reproduction. *141* 1-19.

Shaw J.M, 1993: In: Trounson A. and Gardner D. (eds), In Vitro Fertilization. Chap. 11. CRC Press, Boca Raton, Florida.

Siebzehnruebl ER, Todorow S, van Uem J, Koch R, Wildt L, Lang N, 1989: Cryopreservation of human and rabbit oocytes and one-cell embryos: a comparison of DMSO and propanediol. Hum Reprod 4 312-317.

Sugiyama R, Nakagawa K, Shirai A, Sugiyama R, Nishi Y, Kuribayashi Y, Inoue M, 2010: Clinical outcomes resulting from the transfer of vitrified human embryos using a new device for cryopreservation (plastic blade). J Assist Reprod Genet. *27* 161–167.

Sun X, Li Z, Yi Y, Chen J, Leno GH, Engelhardt JF, 2008: Efficient term development of vitrified ferret embryos using a novel pipette chamber technique. Biol Reprod. *79* 832–840.

Suzuki T, Boediono A, Takagi M, Saha S, Sumantri C, 1996: Fertilization and development of frozen-thawed germinal vesicle bovine oocytes by a one-step dilution method in vitro, Cryobiology. *33* 515-524.

Tsang WH, Chow KL, 2009: Mouse embryo cryopreservation utilizing a novel high-capacity vitrification spatula. BioTechniques *46* 550–552.

Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H, 1998: Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol Reprod Dev. *51* 53–58.

Vajta G, Holm P, Greve T, Callesen H, 1997: Vitrification of porcine embryos using the open pulled straw (OPS) method. Acta Veterinária Scandinavica 38 349–352.

Vanderzwalmen P, Bertin G, Debauche Ch, Standaert V, Bollen N, van Roosendaal E, Vandervorst M, Schoysman R, Zech N, 2003: Vitrification of human blastocysts with the Hemi-Straw carrier: application of assisted hatching after thawing. Hum Reprod. *18* 1504-1511.

Vincent C, Garnier V, Heyman Y, Renard JP, 1989: Solvent effects on cytoskeletal organization and in vivo survival after freezing of rabbit oocytes. J Reprod Fertil *87* 809-820.

Vieira AD, Mezzalira A, Barbieri DP, Lehmkuhl RC, Rubin MI, Vajta G, 2002: Calves born after open pulled straw vitrification of immature bovine oocytes. Cryobiology *45* 91–94.

Virant-Klun I, Bacer-Kermavner L, Tomazevic T, Vrtacnik-Bokal E, 2011: Slow oocyte freezing and thawing in couples with no sperm or an insufficient number of sperm on the day of in vitro fertilization. Reprod Biol Endocrinol. *2* 9:19.

Wang J, Cong L, Zhang ZG, Cao YX, Wei ZL, Zhou P, Zhao JH, He XJ, 2010: Double activation improves rabbit freeze-thawed oocytes developmental potential. Zygote. 18 27-32.

Whittingham DG, 1977: Fertilization in vitro and development to term of unfertilized mouse oocytes previously stored at -196ºC. J Reprod Fertil. *49* 89-94.

Woelders H, Zuidberg CA, Hiemstra SJ, 2006: Animal genetic resources conservation in the Netherlands and Europe: poultry perspective. Poult Sci. *85* 216-222.

Yavin S, Aroyo A, Roth Z, Arav A, 2009: Embryo cryopreservation in the presence of low concentration of vitrification solution with sealed pulled straws in liquid nitrogen slush. Hum Reprod. *24* 797–804.

ARTICLE

2. Article

Effects of cryopreservation on the meiotic spindle, cortical granule distribution and development of rabbit oocytes[1]

E. Jiménez-Trigos, C. Naturil, J.S. Vicente, F. Marco-Jiménez*

Institute of Science and Animal Technology, Laboratorio de Biotecnología de la Reproducción, Universidad Politécnica de Valencia, Valencia, 46022

Corresponding author:
*Prof. PhD Francisco Marco Jiménez, Laboratory of biotechnology of reproduction. Institute of Science and Animal Technology (ICTA) at the Polytechnic University of Valencia, C/Camino de Vera s/n, 46022 Valencia, Spain.
Email address: fmarco@dca.upv.es.

Tel.: +34 96 3879435; fax: +34 96 3877439.

[1]. *This manuscript has been submitted to the Reproduction in Domestic Animals*

2.1. 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, only the slow-freezing method has resulted in live offspring with a total of 0.8%. The aim of our study was to evaluate the effect of vitrification and slow-freezing on the meiotic spindle, cortical granule distribution and their developmental competence. Oocytes were vitrified with 16.84% EG, 12.86% formamide, 22.3% DMSO, 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 cortical granule distribution were assessed with a confocal laser-scanning microscope. To determine oocyte developmental competence, the in vitro development of oocytes from each cryopreservation procedure was assessed with parthenogenesis activation. Our data showed that oocyte cryopreservation was significantly affected by both procedures. In particular, meiotic spindle organisation was dramatically altered after cryopreservation. Oocytes with peripheral cortical granule migration after slow-freezing procedures were better preserved compared to vitrified oocytes. In addition, slow-frozen oocytes led to higher cleavage and blastocyst rates compared to vitrified oocytes. 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. Moreover, the slow-freezing method is currently the recommended option for rabbit oocyte cryopreservation.

Keywords: Vitrification; slow-freezing; cryotop; parthenogenesis; confocal microscopy

2.2. Introdution

Cryopreservation of embryos and oocytes in animal species is considered an important tool in reproduction biotechnology. 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. Although several breakthroughs have been made in oocyte cryopreservation since 1971, live offspring have only been obtained in a few species, such as mouse (Withingham, 1977), human (Chen, 1986), rabbit (Al-Hasani et al. 1989), cattle (Fuku et al. 1992), rat (Nakagata 1192), 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 and 2001; Nottola et al. 2008; Pereira and Marques 2008; Noyes et al. 2010).

In general, the low efficiency 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 exocytosis leading to zona pellucida hardening (Mavrides and Morrol 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 (Loutradi et al. 2008; Keskintepe et al. 2009; Vutyavanich et al. 2009; Martínez-Burgos et al. 2010) and the results are different depending on the species. In human, vitrification shows better results than slow-freezing (Fadini et al. 2009) but in rabbit, slow-freezing shows higher results than vitrification (Salvetti et al. 2010). In human, Fadini et al. (2009) drew a comparison of the outcomes obtained with both methods in several studies and the births per oocyte cryopreserved showed that this rate ranged between 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).

The aim of this study was to evaluate the effects of vitrification and slow freezing for the cryopreservation of rabbit oocytes in terms of meiotic spindle configuration, cortical granule distribution and viability by their parthenogenetic activation.

2.3. Materials and methods

All chemicals in this study 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).

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 hours after induction by flushing each oviduct with Dulbecco's Phosphate Buffered Saline without calcium chloride (DPBS) and supplemented with 0.1% of bovine serum albumin (BSA). Finally, oocytes were treated for 15 min at room temperature with 0.1% hyaluronidase in DPBS and cumulus cells were removed by mechanical pipetting.

Cryopreservation procedures

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). Later, the oocytes were transferred and 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 then transferred to vitrification solution consisting of 16.8% w/v EG, 12.9% 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 into cryotop devices and directly plunged into liquid nitrogen (LN2) 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 sec before being equilibrated for 10 min in TCM-199 containing 20% FBS at 38 °C. After warming, the oocytes were incubated for 2 hours in medium TCM-199 containing 20% FBS at 38.5ºC, 5% CO_2 in humidified atmosphere.

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) sealed by a sterile plug. The straws were then placed into a programmable freezer (Cryologic, CL-8800) for the freezing process. Temperature was lowered from 20º C to -7º C at 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 LN2 and stored for later use. For thawing, the straws were taken out from the LN2 into ambient temperature for 10-15 sec 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. As with the vitrification group, after warming the oocytes were incubated 2 hours in medium TCM-199 containing 20% FBS at 38.5ºC, 5% CO_2 in humidified atmosphere.

Meiotic spindle status

Structural evaluation 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 4º C and permeabilised 30 min at 37º C using 0.1% Triton X-100 in DPBS. Mouse anti-α-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% BSA). Then, oocytes were labelled with fluorescein isothiocyanate-conjugated Donkey anti-mouse antibody (Jackson ImmunoResearch) diluted by a ratio of 1:200 for 45 min at 37ºC in darkness. After extensive washing, DNA of samples was counterstained with propidium iodide (PI). Finally, samples were mounted between a coverslip and a glass slide and stored at 4 ºC and protected from the light until they were examined. The localisations of meiotic spindle and chromosomes were assessed with a confocal microscope (TCS SL, Leica) provided with an argon-krypton laser. When FICT fluorescence was monitored, the excitation light wavelength was 488 nm and emission light wavelength was 515-535nm. When PI fluorescence was monitored, the excitation light wavelength was 543 nm and emission light wavelength was 590-630nm.

The meiotic spindle was classified as normal when the classic symmetrical barrel shape was observed, with organised 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 disorganised, 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 5.

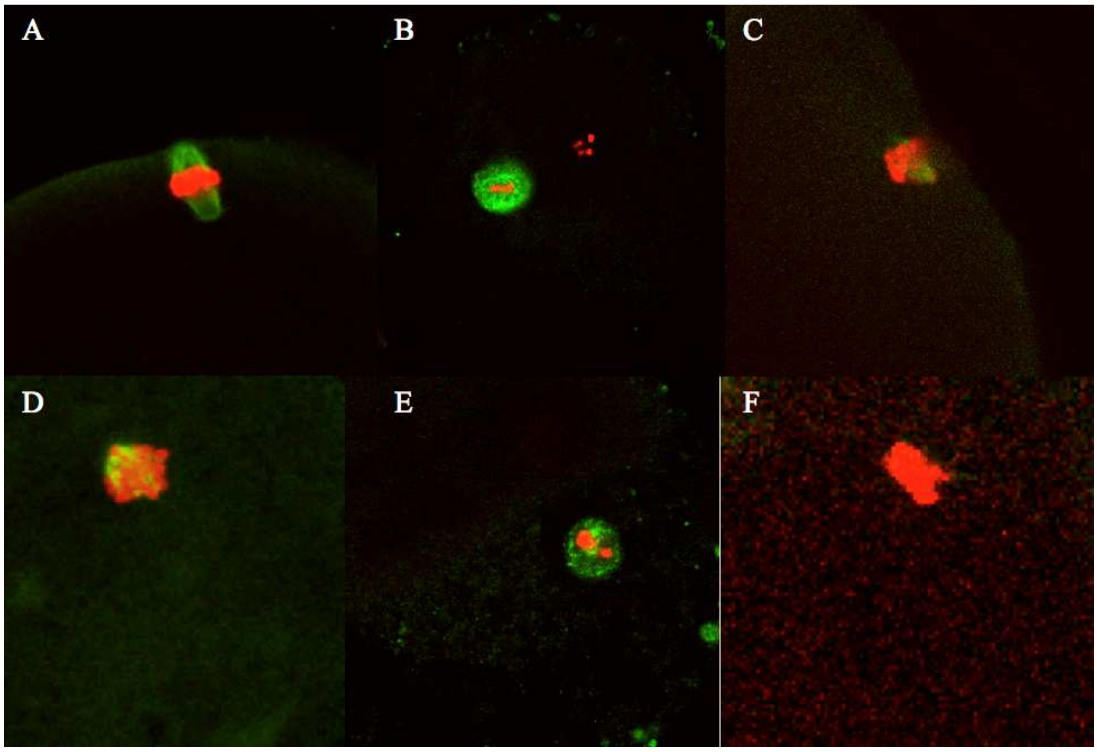

Figure 5. (A) Normal meiotic spindle of rabbit oocyte with chromosomes arrayed at the metaphase plate, evaluated using confocal microscope. (B-E) Abnormal meiotic spindle configuration. (F) Absence of meiotic spindle**.**

Cortical granule distribution

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 4 ºC. Then, oocytes were incubated 30 min at 37 ºC with permeabilisation solution (0.02 % vol/vol Triton X-100). Next, samples were incubated 15 min at 37 ºC in the dark with 100 µg/mL lens culinaris fluorescein isothiocyanate (FITC-LCA) for cortical granule (CG) staining. The oocytes were then washed with blocking solution (7.5% w/v BSA), mounted between a coverslip and a glass slide and examined under a confocal laser-scanning microscope (TCS SL, Leica). Cortical granule distribution was classified as (A) peripheral: CGs were adjacent to the plasma membrane, showing they were cytoplasmically matured; (B) abnormal: this group included samples where most of the CGs were spread throughout the cortical area in a non-homogeneous, anomalous distribution of CGs compatible with a poor quality or degenerated oocytes (Figure 6).

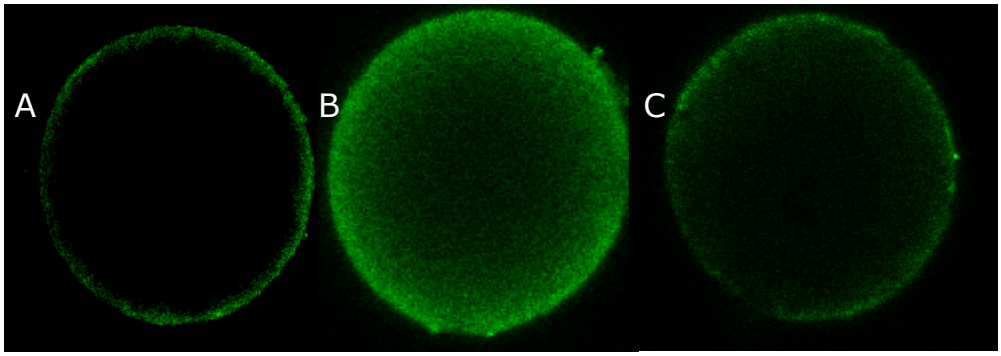

Figure 6. (A) Normal cortical granule distribution of rabbit oocyte evaluated using confocal microscope. (B) Abnormal cortical granule distribution with CGs spread through the cortical area (C) Loose of part of CGs.

Parthenogenetic activation

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 sec apart in an activation medium (0.3 M mannitol supplemented with 100 µM $MgSO_4$ and 100 µM $CaCl_2$), followed by 1h exposure in TCM-199 medium supplemented with 5 µg/µL of cycloheximide and 2 mM of 6-DMAP. Parthenotes were cultured in 500 µL of TMC-199 supplemented with 20% FBS and layered under paraffin oil at 38.5ºC in 5% CO_2 and saturated humidity. Activation rate was recorded at 24 hours after in vitro activation and the blastocyst development rate was assessed at 102 hours after oocyte activation (Figure 7).

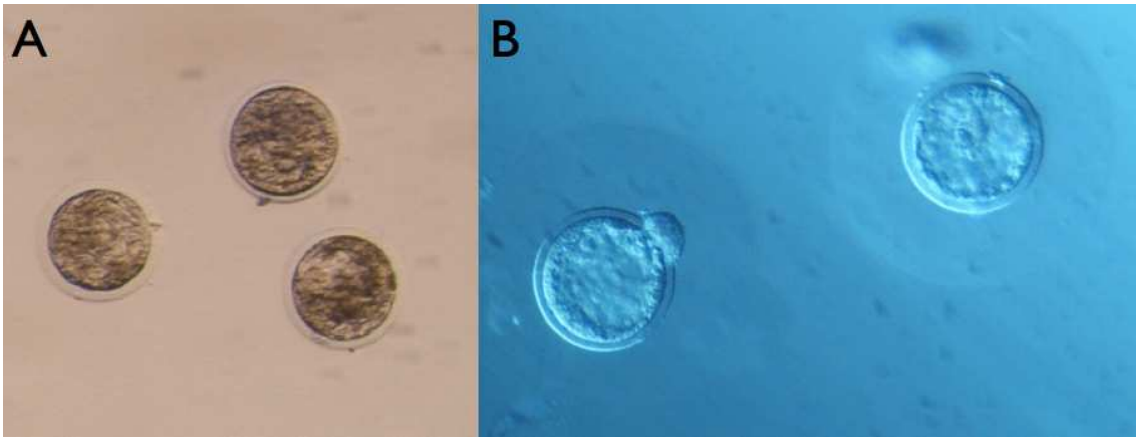

Figure 7. (A) Blastocyst at 102 hours after oocyte activation. (B) Blastocyst at 102 hours after *in vivo* fertilization.

Statistical Analysis

The Chi-square test was used to determine the differences between cryopreservation procedure on oocyte meiotic spindle status, cortical granule distribution and parthenogenetic activation. Statistical analysis were performed using the statistical software program Statgraphics Plus (Version 5.1, STSC Inc., Rockville, MD, USA) and a probability of $P \leq 0.05$ was considered to be the minimum level of significance.

2.4. Results

Effect of cryopreservation method on the meiotic spindle

The spindle morphology was assessed in a total of 258 oocytes. The proportion of meiotic spindle with a normal shape decreased from 89.7% for fresh oocytes to 21.8% after slow freezing and 18.2% after vitrification (Table 4). Differences between the two cryopreservation methods were not significant.

Table 4. Proportion of fresh, vitrified and frozen rabbit metaphase II oocytes with normal meiotic spindle organization.

Procedure	n	Meiotic spindle (%)
Fresh	29	89.7[a]
Frozen	119	21.8[b]
Vitrified	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 cortical granule distribution analysis was assessed in a total of 149 oocytes. Table 5 shows the peripheral cortical granule migration rates of fresh, slow-frozen and vitrified oocytes. Some 95.2% of fresh oocytes presented normal peripheral cortical granule migration. Cryopreservation had a major influence on the normal cortical

granule distribution decreasing to 34.9% after slow-freezing and 14.5% after vitrification. The difference between both cryopreservation methods was significant.

Table 5. Percentage of fresh, vitrified and frozen metaphase II rabbit oocytes with peripheral cortical granules migration.

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

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

Effect of cryopreservation method on parthenogenetic oocyte activation

Parthenogenetic activation was assessed in a total of 346 oocytes. Table 6 shows the developmental rates of fresh, slow-frozen and vitrified oocytes at 24 h and 102 h after parthenogenetic activation. Twenty four hours after parthenogenetic activation, 79.3% of fresh oocytes cleaved. Cryopreservation had influence on the cleavage rates decreasing 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 6.Parthenogenetic development rate at 24 hours and 102 hours after activation of fresh, vitrified and frozen oocytes.

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

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

2.5. Discussion

Many studies of oocyte cryopreservation have been carried out in recent years, but the methods are still inefficient (Gardner et al. 2007; Nottola et al. 2008) and to date, live offspring has been obtained only in a few species, such as mouse (Withingham, 1977), human (Chen, 1986), cattle (Fuku et al., 1992), rat (Nakagata 1992), horse (Hochi et al. 1994) and cat (Gómez et al. 2008). In rabbit, slow-freezing and vitrification has been used for oocyte cryopreservation, but only using slow-freezing method has obtained live offspring (Al-Hasani et al. 1989). Until now, few studies have been performed and, to our knowledge, only a recent report compared both cryopreservation methods (Salvetti et al., 2010).

The impaired meiotic spindle and peripheral cortical granule competence observed in our study for both cryopreserved methods could result from the exposure of oocytes to low temperatures and high concentrations of cryoprotectants, as well as from a drastic reduction in the development up to the blastocyst stage. The spindle is very 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 it 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). So, the high concentration of cryoprotectants (from 5 to 7 M of cryoprotectants) required to achieve vitreous state should produce a higher detrimental effect on spindle configuration. However, our results did not confirm this hypothesis, as we observed that structural alterations were similar between slow-freezing and vitrification procedures. These discrepancies may be related to differences in our vitrification protocol. In our study, the VM3 solution previously designed to present low toxicity (Fahy et al. 2004) was used, following the minimum essential volume method, using cryotop as device, which allowed high cooling rate, minimising the toxic and osmotic effects (Vatja and Kuwayama 2006; Yavin et al. 2009). However, inappropriate condition of exposure to cryoprotectants and cooling induced exocytosis and disorder of cortical granules after vitrification of the oocytes (Bernard and Fuller 1996). In our

study, the cortical granule distribution generally appeared to be altered after cryopreservation, especially after vitrification. To our knowledge, no previous studies of cortical granule distribution after cryopreservation have been reported in rabbit. In other species, it has been reported that cryopreservation has an effect on cortical granule exocytosis as a consequence of disruption of the cytoskeleton that might lead to premature release of cortical granules and zona hardening (Vicent et al. 1990; Ghetler et al. 2006; Morató et al. 2008; Notolla et al. 2009; Tan et al. 2009; Coticchio et al. 2010). This hardening decrease in sperm permeability has been observed in several species (Mavrides and Morrol 2005; Tian et al. 2007) and in vitro fertilisation can be compromised (Coticchio et al. 2001). Therefore, our vitrification protocol seems to induce damage on cytoskeleton filaments involved in exocytosis of cortical granules but not leading to meiotic spindle alteration. Although ICSI can overcome that problem, survival rate in rabbit ICSI is low (Deng and Yang 2001). Parthenogenesis activation therefore seems to be an appropriate tool to assess in vitro developmental rates into blastocysts of cryopreserved rabbit oocytes.

Thus, the cryoprotectants and low temperatures lead to depolymerisation of microtubules and disrupt the network of the meiotic spindle and cortical granules in rabbit oocytes regardless of the cryopreservation procedure. Abnormal spindle and dispersed chromosomes have been related with poor rates of fertilisation and development (Chen et al. 2003; Magli et al. 2010). The cleaved and blastocyst rates of fresh oocytes 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. Parthenogenesis appears to be 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). Our developmental rate to blastocyst status 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 fertilisation (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 levels of cryoprotectants. Considering our results, slow-freezing method seems to be a valuable option for rabbit oocyte cryopreservation, althought both methods need more studies to clarify cellular mechanisms associated with cryoinjury and assure better outcomes.

2.6. Acknowledgements

This work was supported by funds from Generalitat Valenciana research programme (Prometeo 2009/125). English text version revised by N. Macowan English Language Service. Estrella Jiménez was supported by a research grant from Conselleria de Educación of Comunidad Valenciana (programme VALi+d. ACIF/2010/262).

2.7. References

Al-Hasani S, Kirsch J, Diedrich K, Blanke S, van der Ven H, Krebs D, 1989: Successful embryo transfer of cryopreserved and in-vitro fertilized rabbit oocytes. Hum Reprod *4* 77–79.

Andrabi SMH, Maxwell WMC, 2007: A review on reproductive biotechnologies for conservation of endangered mammalian species. Anim Reprod Sci *99* 223-243

Aono N, Abe Y, Hara K, Sasada H, Sato E, Yoshida H, 2005: Production of live offspring from mouse germinal vesicle-stage oocytes vitrified by a modified stepwise method, SWEID. Fertil Steril. *84 2* 1078-1082.

Bernard A, Fuller BJ, 1996: Cryopreservation of human oocytes: a review of current problems and perspectives. Hum Reprod Update. *2* 193-207.

Bos-Mikich A, Wood MJ, Candy CJ, Whittingham DG, 1995: Cytogenetical Analysis and Developmental Potential of Vitrified Mouse Oocytes. Biol Reprod. *53* 780-785.

Cai XY, Chen GA, Lian Y, Zheng XY, Peng HM, 2005: Cryoloop vitrification of rabbit oocytes. Hum Reprod. *20* 1969-1974.

Checura CM, Seidel GE Jr, 2007: Effect of macromolecules in solutions for vitrification of mature bovine oocytes. Theriogenology *67* 919-930.

Chen C, 1986: Pregnancy after human oocyte cryopreservation. Lancet. *19* 884-886.

Chen SU, Lien YR, Chao KH, Ho HN, Yang YS, Lee TY, 2003 : Effects of cryopreservation on meiotic spindles of oocytes and its dynamics after thawing: clinical implications in oocyte freezing a review article. Mol Cell Endocrinol. *202* 101-107.

Ciotti PM, Porcu E, Notarangelo L, Magrini O, Bazzocchi A, Venturoli S, 2009: Meiotic spindle recovery is faster in vitrification of human oocytes compared to slow freezing. Fertil Steril. *91* 2399-2407.

Coticchio G, Bonu MA, Borini A, Flamigni C, 2004: Oocyte cryopreservation: a biological perspective. Eur J Obstet Gynecol Reprod Biol. *115 1* S2-7

Coticchio G, Borini A, Distratis V, Maione M, Scaravelli G, Bianchi V, Macchiarelli G, Nottola SA, 2010 : Qualitative and morphometric analysis of the ultrastructure of human oocytes cryopreserved by two alternative slow cooling protocols. J Assist Reprod Genet. *27* 131-140.

Coticchio G, Garetti S, Bonu MA, Borini A, 2001: Cryopreservation of human oocytes. Hum Fertil *4* 152-157.

Deng MQ, Yang XZ, 2001: Full term development of rabbit oocytes fertilized by intracytoplasmic sperm injection. Mol Reprod Dev. *59* 38–43.

Diedrich K, al-hasani S, van der Ven H, Krebs D,1988: Successful in Vitro fertilization of frozen-thawed Rabbit and human oocytes. Ann N Y Acad Sci. *541* 562-570.

Fadini R, Brambillasca F, Renzini MM, Merola M, Comi R, De Ponti E, Dal Canto MB, 2009: Human oocyte cryopreservation: comparison between slow and ultrarapid methods. Reprod Biomed Online. *19* 171-180.

Fahy GM, Wowk B, Wu J, Phan J, Rasch C, Chang A, Zendejas E, 2004: Cryopreservation of organs by vitrification: perspectives and recent advances. Cryobiology. *48* 157-178

Fuku E, Kojima T, Shioya Y, Marcus GJ, Downey BR, 1992: In vitro fertilization and development of frozen–thawed bovine oocytes. Cryobiology *29* 485–492.

Fuller B, Paynter S, 2004: Fundamentals of cryobiology in reproductive medicine. Reprod Biomed Online. *9* 680-691.

Gardner DK, Sheehan CB, Rienzi L, Katz-Jaffe M, Larman MG, 2007: Analysis of oocyte physiology to improve cryopreservation procedures. Theriogenology. *67* 64-72.

Ghetler Y, Skutelsky E, Ben Nun I, Ben Dor L, Amihai D, Shalgi R, 2006: Human oocyte cryopreservation and the fate of cortical granules. Fertil Steril. *86* 210-6.

Gómez MC, Kagawa N, Pope CE, Kuwayama M, Leibo SP, Dresser BL, 2008: In vivo survival of domestic cat oocytes after vitrification, intracytoplasmic sperm injection, and transfer to recipients. Reproduction, Fertility and Development 20:118.

Hochi S, Fujimoto T, Braun J, Oguri N, 1994: Pregnancies following transfer of equine embryos cryopreserved by vitrification. Theriogenology *42* 483–488.

Jain JK, Paulson RJ, 2006: Oocyte cryopreservation. Fertil Steril. *86* 1037–1046.

Johnson MH, Pickering SJ, 1987: The effect of dimethylsulphoxide on the microtubular system of the mouse oocyte, Development. *100* 313-324.

Keefer CL, 1989: Fertilization by sperm injection in the rabbit. Gamete Res. *22* 59-69.

Keskintepe L, Sher G, Machnicka A, Tortoriello D, Bayrak A, Fish J, Agca Y, 2009: Vitrification of human embryos subjected to blastomere biopsy for pre-implantation genetic screening produces higher survival and pregnancy rates than slow freezing. J Assist Reprod Genet. *26* 629-635.

Kubota C, Yang X, Dinnyes A, Todoroki J, Yamakuchi H, Mizoshita K, Inohae S, Tabara N, 1998: In vitro survival frozen-thawed bovine oocytes after IVF, nuclear transfer, and parthenogenetic activation, Mol Reprod Dev. *51* 281-286.

Kuwayama M, Vajta G, Kato O, Leibo SP, 2005: Highly efficient vitrification method for cryopreservation of human oocytes. Reprod Biomed Online *11* 300–308.

Lee HJ, Elmoazzen H, Wright D, Biggers J, Rueda BR, Heo YS, Toner M, Toth TL, 2010: Ultra-rapid vitrification of mouse oocytes in low cryoprotectant concentrations. Reprod Biomed Online. *20* 201-208.

Li XH, Chen SU, Zhang X, 2005: Cryopreserved oocytes of infertile couples undergoing assisted reproductive technology could be an important source of oocyte donation: a clinical report of successful pregnancies. Hum Reprod *20* 3390-3394.

Loudrati KE, Kolibianakis EM, Venetis CA, Papanikolaou EG, Pados G, Bontis I, Tarlatzis BC, 2008: Cryopreservation of human embryos by vitrification or slow freezing: a systematic review and meta-analysis. Fertil Steril. *90* 186-193.

Magli MC, Lappi M, Ferraretti AP, Capoti A, Ruberti A, Gianaroli L, 2010: Impact of oocyte cryopreservation on embryo development. Fertil Steril. *93* 510-516.

Mandelbaum J, Anastasiou O, Lévy R, Guérin JF, de Larouzière V, Antoine JM, 2004: Effects of cryopreservation on the meiotic spindle of human oocytes. Eur J Obstet Gynecol Reprod Biol. *113 1* 17-23.

Martinez-Burgos M, Herrero L, Megías D, Salvanes R, Montoya MC, Cobo AC, García-Velasco JA, 2011: Vitrification versus slow freezing of oocytes: effects on morphologic appearance, meiotic spindle configuration, and DNA damage. Fertil Steril. *95* 374-377.

Mavrides A, Morroll D, 2005: Bypassing the effect of zona pellucida changes on embryo formation following cryopreservation of bovine oocytes. Eur J Obstet Gynecol Reprod Biol. *118* 66-70.

Morato R, Izquierdo D, Paramio MT, Mogas T, 2008: Cryotops versus open-pulled straws (OPS) as carriers for the xryopreservation of bovine oocytes: effects on spindle and chromosomes configuration and embryo development. Cryobiology. 57 137-141.

Nakagata N, 1992: Cryopreservation of unfertilized rat oocytes by ultrarapid freezing. Jikken Dobutsu. *41* 443-7.

Nottola SA, Coticchio G, De Santis L, MAcchiarelli G, Maione M, Bianchi S, Laccarino M, Flamigni C, Borini A, 2008: Ultrastructure of human mature oocytes after slow cooling cryopreservation with ethylene glycol. Reprod Biomed online *17* 368-377.

Nottola SA, Coticchio G, Sciajno R, Gambardella A, Maione M, Scaravelli G, Bianchi S, Macchiarelli G, Borini A, 2009: Ultrastructural markers of quality in human mature oocytes vitrified using cryoleaf and cryoloop. Reprod Biomed Online. *19 3* 17-27.

Noyes N, Boldt J, Nagy ZP, 2010: Oocyte cryopreservation. Is it time to remove its experimental label? J Assist Reprod Genet. *27* 69-74.

Paynter SJ, Cooper A, Gregory L, Fuller BJ, Shaw RW, 1999: Permeability characteristics of human oocytes in the presence of the cryoprotectant dimethylsulphoxide. Hum Reprod. *14* 2338-2342.

Paynter SJ, O'Neil L, Fuller BJ, Shaw RW, 2001: Membrane permeability of human oocytes in the presence of the cryoprotectant propane-1,2-diol. Fertil Steril. *75* 532-538.

Pereira RM, Marques CC, 2008: Animal oocyte and embryo cryopreservation. Cell Tissue Bank. *9* 267-277.

Pickering SJ, Johnson MH, 1987: The influence of cooling on the organization of the meiotic spindle of the mouse oocyte. Hum Reprod. *2* 207-216.

Rojas C, PAlomo MJ, Albarracin JL, Mogas T, 2004: Vitrification of inmatureand in vitro matured pig oocytes: study of distribution of chromosomes, microtubules, and actin microfilaments. Cryobiology *49* 211-220.

Salvetti P, Buff S, Afanassieff M, Daniel N, Guérin P, Joly T, 2010: Structural, metabolic and developmental evaluation of ovulated rabbit oocytes before and after cryopreservation by vitrification and slow freezing. Theriogenology. *74* 847-*855*.

Saragusty J, Arav A, 2011: Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. Reproduction. *141* 1-19.

Shamonki MI, oktay K, 2005: Oocyte and ovarian tissue cryopreservation: indications, techniques, and applications. Semin Reprod Med. *23* 266-276.

Siebzehnruebl ER, Todorow S, van Uem J, Koch R, Wildt L, Lang N, 1989: Cryopreservation of human and rabbit oocytes and one-cell embryos: a comparison of DMSO and propanediol. Hum Reprod 4 312-317.

Succu S, Leoni GG, Berlinguer F, Madeddu M, Bebbere D, Mossa F, Bogliolo L, Ledda S, Naitana S, 2007: Effect of vitrification solutions and cooling upon in vitro matured prepubertal ovine oocytes. Theriogenology. *68* 107-114.

Suzuki T, Boediono A, Takagi M, Saha S, Sumantri C, 1996: Fertilization and development of frozen-thawed germinal vesicle bovine oocytes by a one-step dilution method in vitro, Cryobiology. *33* 515-524.

Tan X, Song E, Liu X, You W, Wan F, 2009: Factors affecting the survival, fertilization, and embryonic development of mouse oocytes after vitrification using glass capillaries. In Vitro Cell Dev Biol Anim. *45* 420-429.

Tian SJ, Yan CL, Yang HX, Zhou GB, Yang ZQ, Zhu SE, 2007: Vitrification solution containing DMSO and EG can induce parthenogenetic activation of in vitro matured ovine oocytes and decrease sperm penetration. Anim Reprod Sci. *101* 365–371.

Vajta G, Kuwayama M, 2006: Improving cryopreservation systems. Theriogenology. *65* 236-244.

Vajta G, 2000: Vitrification of the oocytes and embryos of domestic animals. Anim Reprod Sci. *60 61* 357-364.

Vieira AD, Mezzalira A, Barbieri DP, Lehmkuhl RC, Rubin MI, Vajta G, 2002: Calves born after open pulled straw vitrification of immature bovine oocytes. Cryobiology *45* 91–94.

Vincent C, Garnier V, Heyman Y, Renard JP, 1989: Solvent effects on cytoskeletal organization and in vivo survival after freezing of rabbit oocytes. J Reprod Fertil *87* 809-820.

Vincent C, Pickering SJ, Johnson MH, Quick SJ, 1990: Dimethylsulphoxide affects the organization of microfilaments in the mouse oocyte. Mol Reprod Dev. *26 3* 227–235.

Vutyavanich T, Sreshthaputra O, Piromlertamorn W, Nunta S, 2009: Closed-system solid surface vitrification versus slow programmable freezzin of mouse 2-cell embryos. J Assist Reprod Genet. *26* 285-290.

Wang J, Cong L, Zhang ZG, Cao YX, Wei ZL, Zhou P, Zhao JH, He XJ, 2010: Double activation improves rabbit freeze-thawed oocytes developmental potential. Zygote. 18 27-32.

Whittingham DG, 1971 Survival of mouse embryos after freezing and thawing. Nature. *233* 125-126.

Whittingham DG, Leibo SP, Mazur P, 1972: Survival of mouse embryos frozen to -196º and -269ºC. Science. *178* 411-414.

Whittingham DG, 1977: Fertilization in vitro and development to term of unfertilized mouse oocytes previously stored at -196ºC. J Reprod Fertil. *49* 89-94.

Yang D, Brown SE, Nguyen K, Reddy V, Brubaker C, Winslow KL, 2007: Live birth after the transfer of human embryos developed from cryopreserved oocytes harvested before cancer treatment. Fertil Staril *87* 1469.e1-4.

Yavin S, Aroyo A, Roth Z, Arav A, 2009: Embryo cryopreservation in the presence of low concentration of vitrification solution with sealed pulled straws in liquid nitrogen slush. Hum Reprod. *24* 797-804.

Zheng YL, Jiang MX, Zhang YL, Sun QY, Chen DY, 2004: Effects of oocyte age, cumulus cells and injection methods on in vitro development of intracytoplasmic sperm injection rabbit embryos. Zygote. *12* 75-80.

FUTURE PERSPECTIVES

3. Future perspectives

In most cases, modifying cryopreservation methods to fit the cells to be cryopreserved is likely to be preferable to modifying cells to fit procedures for cryopreservation (Seidel, 2006). Various studies with reproductive and non-reproductive cells provide numerous ideas for further studies. Stabilizing the cytoskeleton system during vitrification could be beneficial for improving the post-thaw survival and subsequent development of vitrified oocytes (Morató et al. 2008). Adding more cholesterol via cyclodextrin may be worth pursuing. Horvath and Seidel (2006) showed that the zona pellucida of oocytes did not prevent transfer of cholesterol from occurring withing this procedure. Another area worth investigated is stabilizing cell membranes with trehalose, a compound that plants use naturally to increase cryotolerance ("Ice blockers"). One issue is how to transfer the compound to the cytoplasm of the cell, where it normally functions. Satpathy et al. (2004) developed a method for loading red blood cells with trehalose for freeze-drying, and a variation of this one might work to move trehalose into oocytes and embryonic cells, or trehalose might be injected into individual oocytes (Eroglu et al. 2002). It is unclear whether trehalose would be harmful after thawing, and if so whether the molecule could be removed from cells readily. Kim et al. (2005) used another approach by modifying red blood cells with phosphoenolpyruvate to decrease membrane fragility. Following, this one, have been described some possible modifications or alternatives to improve oocyte vitrification and the necessary tools to asses *in vivo* viability of embryos. The complexity of these techniques is combined with the low output obtained.

3.1. Alternatives

3.1.1. Cytoskeleton stabilizing agents

The main damage observed during vitrification is an abnormal spindle configuration mainly due to the disorganization or disassembly of meiotic microtubules. One possible way to improve the cryotolerance of oocytes and improve the postthaw survival and subsequent development of vitrified oocytes or embryos may be the use of cytoskeleton stabilizing agents such as cytochalasin B or taxol.

Cytochalasin B is a cytoskeletal relaxant considered to make the cytoskeletal elements less rigid (Fujihira et al. 2004). The Cytochalasin B effects in oocyte vitrification are controversial and may depend on the species and procedures used. In mature oocyte, CCB reduced damage to microtubules and may enhance stabilization of spindle microtubules during vitrification. In the case of GV oocytes, where no organised meiotic spindle is present, this relaxant effect may preserve the functionality of the gap junctions between oocyte and granulosa cells, permitting a faster and more uniform penetration of the cryoprotectants. Studies on the effect of pre-treatment with CCB on the vitrification of pig (Fujihira et al. 2004) and sheep (Silvestre et al. 2006) oocytes have been reported, but more studies are needed in order to increase oocyte or embryos survival after vitrification with these additives.

Taxol TM (paclitaxel) is a diterpenoid taxane used as an antineoplastic agent in patients diagnosed with ovarian cancer, metastatic breast carcinoma and nonsmall cell lung carcinoma. Taxol interacts with microtubules and increases the rate of polymerization by reducing the critical concentration of tubulin needed for polymerization. The addition of Taxol to the vitrification solution improves the post-warming development of immature human (Fuchinoue et al. 2004) and mature porcine (Shi et al. 2006) and bovine (Morato et al. 2008) oocytes.

3.1.2. Lipid content

Lipid content and membrane lipid composition affects oocytes and embryo's ability to resist chilling injuries during cryopreservation 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. 2000a; Zeron et al. 2001, 2002).

Some factors as oocyte and embryo origin (*in vivo or in vitro*), specie, breed, physiologic state, and nutrition affect lipid content (McEvoy et al. 2000; Zeron et al. 2001, 2002; Genicot et al. 2005). For example, bovine oocytes are more resistant to cryopreservation than porcine ones, probably because porcine oocytes have 2.4 times

more lipid drops than bovine and their structure and composition are different (McEvoy et al. 2000; Isachenko et al. 2001; 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. Mechanical delipidation has been applied to earlier stages of porcine embryo development (Nagashima et al. 1999) and to in vitro produced and cloned bovine embryos (Ushijima et al. 1999; Tominaga et al. 2000; Diez et al. 2001), through polarization of the cytoplasmic lipid droplets by centrifugation and physical removal of excess lipid. In both cases embryo sensitivity to chilling was reduced, increasing their cryopreservation. However, besides being an invasive and extremely laborintensive method, mechanical delipidation alters 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 by Men et al. (2006). This agent promoted the cryosurvival of porcine IVP embryos after partial delipidation through chemical stimulation of intracellular lipolysis. Besides reducing lipid content, Pereira et al. (2008) proposed the possibility of a 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.

3.2. Offspring production

The main objective is to develop a method for oocyte cryopreservation that allow generate offspring. In rabbit, only two studies have shown results of embryo implantation (Vincent et al. 1989; Wang et al. 2010) and only one obtained live offspring using slow-freezing method (Al-Hasani et al. 1989).

It is necessary to asses the results of cryopreservation *in vivo*, because although it seems possible, 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) linked to the poor permeability of sperm plasma membrane (Curry et al. 1995, 2000). Best results are obtained today when semen is capacitated *in vivo*.

On the other hand, ICSI of rabbit oocytes is difficult because they have rough, dark granules in the plasma, and they easily lyse and die after the ICSI process. Only two studies of ICSI success in fresh rabbit oocytes have been reported (Deng and Yang, 2001; Li et al. 2001), and two in vitrified oocytes (Cai et al. 2005; Wang et al. 2009).

An alternative to avoid IVF and ICSI variability is necessary to asses the success of oocyte cryopreservation, for example using *in vivo* fertilization.

3.3. References

Al-Hasani S, Kirsch J, Diedrich K, Blanke S, van der Ven H, Krebs D, 1989: Successful embryo transfer of cryopreserved and in-vitro fertilized rabbit oocytes. Hum Reprod *4* 77–79.

Arav A, Pearl M, Zeron Y, 2000: Does membrane lipid profile explain chilling sensitivity and membrane lipid phase transition of spermatozoa and oocytes? Cryo Lett. *21* 179–186.

Brackett BG, Bousquet D, Dressel MA, 1982 : In vitro sperm capacitation and in vitro fertilization with normal development in the rabbit. J Androl *3* 402–411.

Cai XY, Chen GA, Lian Y, Zheng XY, Peng HM, 2005: Cryoloop vitrification of rabbit oocytes. Hum Reprod. *20* 1969-1974.

Curry MR, Kleinhans FW, Watson PF, 2000: Measurement of the water permeability of the membranes of boar, ram, and rabbit spermatozoa using concentration-dependent self-quenching of an entrapped fluorophore. Cryobiology *41* 167–73.

Curry MR, Redding BJ, Watson PF, 1995: Determination of water permeability coefficient and its activation energy for rabbit spermatozoa. Cryobiology *32* 175– 181.

Deng MQ, Yang XZ, 2001: Full term development of rabbit oocytes fertilized by intracytoplasmic sperm injection. Mol Reprod Dev. *59* 38–43.

Diez C, Heyman Y, Bourhis D, Guyader-Joly C, Degrouard J, Renard JP, 2001: Delipidating in vitro-produced bovine zygotes: effect on further development and consequences for freezability. Theriogenology *55* 923–936.

Eroglu A, Toner M, Toth TL, 2002: Beneficial effect of microinjected trehalose on the cryosurvival of human oocytes, *Fertil Steril* 77 152–158.

Fuchinoue K, Fukunaga N, Chiba S, Nakajo Y, Yagi A, Kyono K, 2004: Freezing of human immature oocytes using cryoloops with Taxol in the vitrification solution. J Assist Reprod Genet *21* 307–309

Fujihira T, Kishida R, Fukui Y, 2004: Developmental capacity of vitrified immature porcine oocytes following ICSI: effects of cytochalasin B and cryoprotectants. Cryobiology *49* 286–290.

Genicot G, Leroy JL, Van Soom A, Donnay I, 2005: The use of a fluorescent dye, Nile red, to evaluate the lipid content of single mammalian oocytes. Theriogenology *63* 1181–1194.

Ghetler Y, Yavin S, Shalgi R, Arav A, 2005: The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. Hum Reprod *20* 3385–3389.

Horvath G, Seidel GE Jr., 2006: Vitrification of bovine oocytes in chemically defined media after treatment with cholesterol-loaded methyl-β-cyclodextrin. *Theriogenology*. *66* 1026–1033.

Isachenko V, Isachenko E, Michelmann HW, Alabart JL, Vazquez I, Bezugly N, Nawroth F, 2001: Lipolysis and ultrastructural changes of intracellular lipid vesicles after cooling of bovine and porcine GV-oocytes. Anat Histol Embryol *30* 333–338.

Kim H, Itamoto K, Une S, Nakaichi M, Taura Y, Sumida S, 2005: Application of phosphoenolpyruvate into canine red blood cell cryopreservation with hydroxyethyl starch, Cryo Lett. *26* 1–6.

Li GP, Chen DY, Lian L, Sun QY, Wang KM and Liu JL, 2001: Viable rabbits derived from reconstructed oocytes by germinal vesicle transfer after intracytoplasmic sperm injection (ICSI). Mol Reprod Dev *58* 180–185.

McEvoy TG, Coull GD, Broadbent PJ, Hutchinson JSM, Speake BK, 2000: Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. J Reprod Fertil *118* 163–170.

Men H, Agca Y, Riley L, Critser JK, 2006: Improved survival of vitrified porcine embryos after partial delipation through chemically stimulated lipolysis and inhibition of apoptosis. Theriogenology *66* 2008–2016.

Morato R, Izquierdo D, Albarracin JL, Anguita B, Palomo MJ, Jimenez-Macedo AR, Paramio MT, Mogas T, 2008:Effects of pre-treating in vitro-matured bovine

oocytes with the cytoskeleton stabilizing agent taxol prior to vitrification. Mol Reprod Dev *75* 191–201.

Morato R, Izquierdo D, Paramio MT, Mogas T, 2008: Cryotops versus open-pulled straws (OPS) as carriers for the cryopreservation of bovine oocytes: effects on spindle and chromosomes configuration and embryo development. Cryobiology. *57* 137-141.

Nagashima H, Cameron RDA, Kuwama M, Young M, Beebe L, Blackshaw AW, Nottle MB, 1999: Survival of porcine delipated oocytes and embryos after cryopreservation by freezing and vitrification. J Reprod Dev *45* 167–176.

Orief Y, Schultze-Mosgau A, Dafopoulos K, Al-Hasani S, 2005: Vitrification: will it replace the conventional gamete cryopreservation techniques? Middle East Fertility Society Journal. *10* 171–184.

Pereira RM, Marques CC, 2008: Animal oocyte and embryo cryopreservation. Cell Tissue Bank. *9* 267-277.

Pereira RM, Carvalhais I, Pimenta J, Baptista MC, Vasques MI, Horta AEM, Santos IC, Marques MR, Reis A, Silva Pereira M, Marques CC, 2008: Biopsied and vitrified bovine embryos viability is improved by trans10, cis12 conjugated linoleic acid supplementation during in vitro embryo culture. Anim Reprod Sci *106* 322–332.

Satpathy GR, Török Z, Bali R, Swyre DM, Little E, Walker NJ, 2004: Loading red blood cells with trehalose: a step towards biostabilization, *Cryobiology 49* 123–136.

Seidel GE Jr., 2006: Modifying oocytes and embryos to improve their cryopreservation. *Theriogenology*. *65* 228–235.

Shi, WQ., Zhu, SE, Zhang, D, Wang, WH, Tang GL, Hou YP, Tian SJ, 2006: Improved development by Taxolpretreatment after vitrification of in vitro matured porcine oocytes. Reproduction *131* 795–804.

Silvestre, M.A., Yaniz, J., Salvador, I., Santolaria, P., Lopez-Gatius, F., 2006. Vitrification of pre-pubertal ovine cumulusoocyte complexes: effect of cytochalasin B pre-treatment. Anim. Reprod. Sci. 93, 176–182.

Tominaga K, Shimizu M, Ooyama S, Izaike Y, 2000: Effect of lipid polarization by centrifugation at different developmental stages on post-thaw survival of bovine in vitro produced 16-cell embryos. Theriogenology *53* 1669–1680.

Ushijima H, Yamakawa H, Nagashima H, 1999: Cryopreservation of bovine pre-morula-stage in vitro matured/ in vitro fertilized embryos after delipidation and before use in nucleus transfer. Biol Reprod *60* 534–539.

Vincent C, Garnier V, Heyman Y, Renard JP, 1989: Solvent effects on cytoskeletal organization and in vivo survival after freezing of rabbit oocytes. J Reprod Fertil *87* 809-820.

Wang J, Cong L, Zhang ZG, Cao YX, Wei ZL, Zhou P, Zhao JH, He XJ, 2010: Double activation improves rabbit freeze-thawed oocytes developmental potential. Zygote. 18 27-32.

Wowk B, Leitl E, Rasch CM, Mesbah-Karimi N, Harris SB, Fahy GM, 2000: Vitrification enhancement by synthetic ice blocking agents. Cryobiology *40* 228–236.

Wowk B, Fahy GM, 2002: Inhibition of bacterial ice nucleation by polyglycerol polymers. Cryobiology *44* 14–23.

Wowk B, 2005: Anomalous high activity of a subfraction of polyvinyl alcohol ice blocker. Cryobiology *50* 325–331.

Zeron Y, Ocheretny A, Kedar O, Borochov A, Sklan D, Arav A, 2001: Seasonal changes in bovine fertility: relation to developmental competence of oocytes, membrane properties and fatty acid composition of follicles. Reprod *121* 447–454.

Zeron Y, Sklan D, Arav A, 2002: Effect of polyunsaturated fatty acid supplementation on biophysical parameters and chilling sensitivity o fewe oocytes. Mol Reprod Dev *61* 271–278.