Vitrification of oocytes and embryos in cattle and horses: as clear as a glass?

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

6-DMAP	6 Dimethyl aminopurine
Ab	Antibody
ANOVA	Analysis of variance
AQP	Aquaporin
ART	Assisted Reproductive Technology
BSA	Bovine Serum Albumin
COCs	Cumulus-Oocyte-Complexes
CR	Corona Radiata
CPA(s)	Cryoprotectant(s)
Cx(s)	Connexin(s)
DABCO	1,4-diazabicyclo(2.2.2)octane
DAPI	4´,6´-Diamino-2phenylindole
DF	Divalent Free
DMEM/F12	Dulbecco`s Modified Eagle Medium: Nutrient mixture F-12
DMSO	Dimethylsulfoxide
PBS	Phosphated- Buffered Saline
DO(s)	Denuded oocyte(s)
DOscocs	Denuded oocyte fertilized in presence of COCs
EGF	Epidermal Growth Factor
EG	Ethylene Glycol
ES	Equilibration Solution
FITC	Fluorescein Isothiocyanate
FBS	Fetal Bovine Serum
GJ(s)	Gap junction(s)
GV	Germinal vesicle
HC(s)	Hemichannel(s)
HBSS	Hanks Balanced Salt Solution

HEPES	4-(2-Hydroxyethyl)-1-Piperazine-ethanesulfonic acid
HS	Handling Solution
ICSI	Intracytoplasmic Sperm Injection
IVC	In vitro culture
IVC	In vitro fertilization
IVM	In vitro maturation
IVP	In vitro embryo production
kDa	Kilodalton
LN_2	Liquid Nitrogen
MI	Metaphase I
MII	Metaphase II
MW	Molecular Weight
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PI	Propidium Iodide
RT	Room Temperature
SLDT	Scrape Loading Dye Transfer
SOF	Synthetic Oviductal Fluid
TALP	Tyrode´s Albumin Lactate Pyruvate
TCM199	Tissue Culture Medium 199
VS	Vitrification Solution
WT	Wild Type

CHAPTER 1

GENERAL INTRODUCTION

1.1 Cryopreservation of oocytes and embryos in assisted reproduction

Cryopreservation literally means preservation by cold. Typically, cryopreservation refers to storage of live cells or tissue, at temperatures below 0°C, more specifically below −80°C, while maintaining functional intactness. Cryopreservation is applied to various types of somatic cells, tissues, and organs, but also to germplasm, e.g. semen, oocytes, ovaries, embryos, etc. Germplasm may be preserved for gene-banking purposes or for application in human or animal assisted reproductive technologies (ART), because it allows preserving the genetic material for a longer period of time, for later use when it is required.

In humans, cryopreservation of embryos is routinely used to preserve spare embryos produced during in vitro fertilization, allowing patients to make use of these embryos in case the first transfer fails to produce a child. On the other hand, oocyte cryopreservation is beneficial for all female patients because of numerous reasons: (1) it permits women to cryopreserve their fertility in case that they have to go through a chemotherapy treatment or an ovariectomy, (2) it eliminates donor-recipient synchronization problems, (3) it avoids ethical, moral and legal concerns about unused embryos and embryo ownership and (4) it allows women to electively delay childbearing.

In domestic animals, cryopreservation of embryos has become an important part in breeding programs. It permits temporary storage of embryos, which is important when embryos are meant for international trade. In this way, embryos can also be thawed at the desired moment to be transferred into recipient animals, which are at day 6-8 of the cycle after exhibiting natural oestrus. On the other hand, cryopreservation of oocytes and banking would provide a repeatable, accessible supply of oocytes for research. This is particularly the case for the horse: in some countries like the USA, slaughtering of horses is forbidden, and hence there is no supply of slaughterhouse ovaries from mares for research. Moreover, it would provide a means to preserve the genetic material of a genetically valuable female donor animal, something which has been done for over fifty years for male animals by semen freezing.

Furthermore, cryopreservation of embryos and oocytes has become an important tool for the conservation of endangered wild species, providing a safeguard against disease, genetic drift and catastrophic and unexpected losses. Also for domestic species, this application becomes more important for ancient breeds, because during the last decades, farm animal genetic diversity has rapidly declined due to changing market demands and intensification of agriculture. Approximately

20% of the world's breeds of cattle, goats, pigs, horses and poultry are currently at risk of extinction (Prentice and Anzar 2010).

1.2 History of cryopreservation of gametes and embryos

The basis of cryopreservation was established at the end of 1940´s with the discovery of the protective effect of glycerol for semen freezing (Polge *et al.* 1949).This discovery marked the beginning of an era in which practical methods for freezing and banking of blood, cells, semen, various tissues and organs were developed. However, more than two decades went by before the successful cryopreservation of a mammalian embryo was reported (Whittingham 1972). After this report many other offspring obtained from cryopreserved embryos were born in different species, as in cow (Wilmut and Rowson 1973), rabbit (Bank and Maurer 1974), sheep (Willadsen *et al.* 1976), goat (Bilton and Moore 1976), horse (Yamamoto *et al.* 1982), human (Zeilmaker *et al.* 1984), cat (Dresser *et al.* 1988), and pig (Hayashi *et al.* 1989).

The first attempts to cryopreserve oocytes were published in 1958 (Sherman and Lin 1958), but the first live offspring from cryopreserved mouse oocytes was only reported in 1976 (Parkening *et al.* 1976). A major breakthrough occurred in 1996, when Martino and coworkers reported that oocytes are extremely sensitive to cooling, making their cryopreservation difficult. Further studies focused on understanding the oocyte sensitivity to cryopreservation, and tried to minimize the damage in order to maintain oocyte competence. However, the overall success is still limited, and just a few offspring have been reported using oocyte cryopreservation, including rabbit (Al-Hasani *et al.* 1989), cow (Fuku *et al.* 1992), human (Chen 1986), and horse (Hochi *et al.* 1994).

1.3 Basics in cryobiology

Cryopreservation is the use of very low temperatures to preserve intact cells and tissues. At -196°C, cells can be stored without a lethal effect due to the fact that there is no aqueous diffusion, and thermal energy is insufficient for chemical reactions to occur. However, when cells are cooled to subzero temperatures, ice crystals can be formed. At first, water freezes extracellularly as pure ice and an unfrozen fraction remains, which contains all the solutes. The high concentration of the remaining unfrozen solution establishes an osmotic gradient across the cell membrane, causing an efflux of water from the cell. Slow cooling allows keeping the cytoplasm in a near equilibrium state with the extracellular solution. However, a high cooling rate does not allow enough time for the water to leave the cell and maintain a near equilibrium state with the extracellular solution, leading to intracellular ice formation that normally is fatal for the cells (Figure 1).

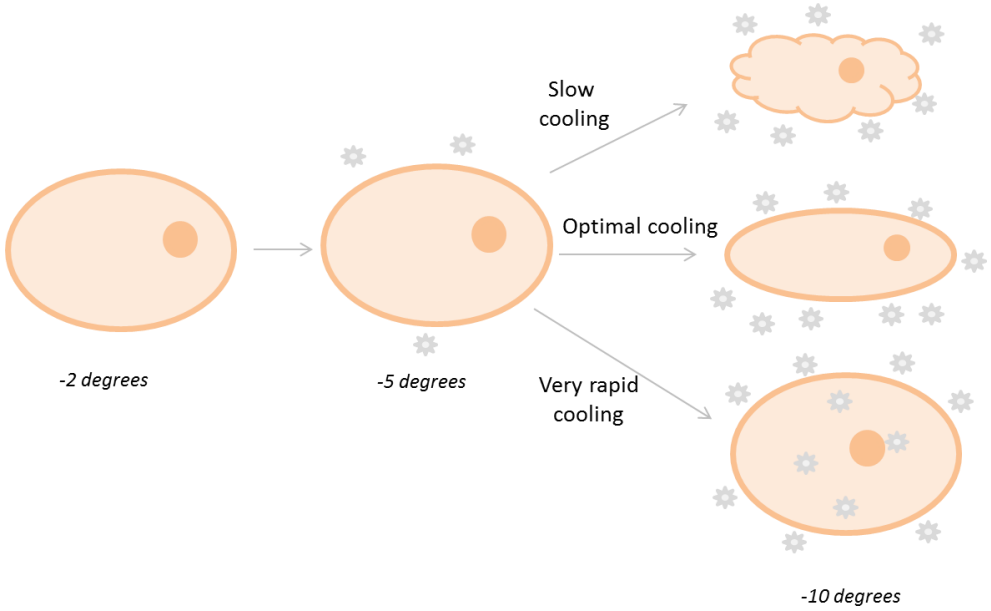

Figure 1. Schematic diagram representing the physical events that occur when cells are preserved at different cooling rates. Slow cooling produces an intense dehydration in the cell and a strong concentration of intracellular solutes, which may lead to cell damage. On the other hand, when very rapid cooling rates are applied, cells are not correctly dehydrated and the remaining water forms intracellular ice crystals. Modified from Mazur 1985.

Moreover, lowering the temperature exerts significant effects on the cell, even though there is no ice formation, such as the thermotropic behavior suffered by the membranes. Membranes are composed of proteins and lipids, such as phospholipids and cholesterol that form a lipid bilayer with the hydrophilic ends of the lipids externally and the hydrophobic fatty acyl chains internally. Phospholipids are randomly arranged in a lamellar structure, and move free laterally within one leaflet of the membrane bilayer. Under this condition, membranes are permeable to water that can diffuse across cellular membrane and impermeable for solutes. However, a drop in the temperature produces a shift from a liquid crystal phase to a gel phase (Figure 2), which will result in clustering of specific lipids and membranes proteins concentrated in a specific area. The aggregation of proteins can result in a decrease of the membrane permeability and decreased metabolic function (De Leeuw *et al.* 1990).

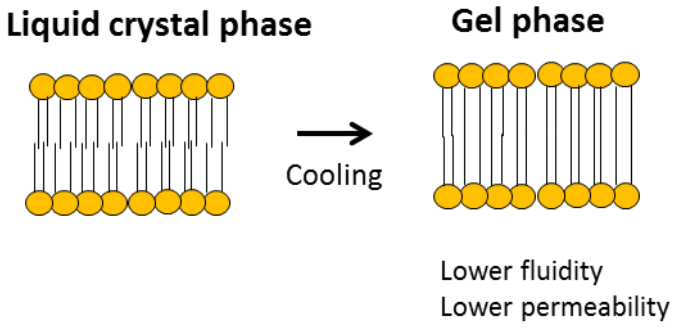

Figure 2. Effect of temperature on the phospholipids bilayer permeability. When membranes are cooled, phase transition takes place from the liquid crystal phase to the gel phase resulting in a lower fluidity and permeability of the membrane.

1.3.1 Cryopreservation techniques

Two techniques are traditionally used to cryopreserve oocytes and embryos: Slow freezing and vitrification. Slow freezing routinely involves equilibration of oocytes or embryos in freezing medium, containing low concentrations of cryoprotectants (CPAs, which are organic compounds used to protect cells during freezing) for periods up to 10 min before loading (in a volume of ~200 µl) into plastic straws, which are sealed at both ends. Next, straws are placed in the chamber of a programmable freezing machine, which slowly reduces the temperature (~0.3°C/min) to ~−30°C. During this cooling phase, ice nucleation (seeding) is induced manually at a temperature between −5 and −8°C. On reaching −30°C, the temperature is then reduced rapidly (at ~−50°C/min) to −150°C before storage in liquid nitrogen. Rapid thawing is accompanied by re-swelling of the cells to regain approximately their original volume. After that, the CPAs can be removed by incubation of the embryos or oocytes in successive media with decreasing concentrations of permeating CPAs (Edgar and Gook 2012). This technique has been successfully used in mouse embryos (Shaw and Jones 2003, Kader *et al.* 2009), but poor results have been reported in more sensitive species, such as pig, sheep or horse. Many studies focused on finding an alternative to slow freezing, because the process takes long time, and requires expensive equipment (Table 1).

In 1985, Rall and Fahy reported the cryopreservation of mouse morula by vitrification. Since then, vitrification became a promising alternative technique to preserve oocytes and embryos. Vitrification is defined as the solidification of a solution at a low temperature without ice crystal formation. This can be achieved by using very high concentrations of CPAs (Rall and Fahy 1985). The CPA concentrations should then be so high that the tendency of the water molecules to form ice has

become zero, and vitrification can be achieved regardless of cooling rate. This is so-called *thermodynamically stable vitrification*. Alternatively, vitrification can also be achieved at less extreme CPA concentrations, provided that the rates of both cooling and rewarming are very high (so-called *meta-stable vitrification*).

Several studies comparing conventional slow freezing and vitrification have reported better survival and development after vitrification of oocytes and embryos (Nedambale *et al.* 2004, Stehlik *et al.* 2005, Mucci *et al.* 2006, Huang *et al.* 2007, Cao *et al.* 2009, Chen and Yang 2009, Martínez-Burgos *et al.* 2011), suggesting that with time, conventional slow freezing may be replaced entirely by vitrification (Vajta and Kuwayama 2006). Nowadays, slow freezing is still the technique of choice for cryopreservation of bovine and equine embryos in the breeding industry. On the other hand, most laboratories working in human ART have completely replaced slow freezing by vitrification.

Table 1. Differences between slow freezing and (metastable) vitrification with current minimal volume approaches. Adapted from Pereira and Marquez 2008.

	Slow Freezing	**Vitrification**
Device	Standard straw, Cryovial	OPS, Cryoloop, Cryotop, Cryoleaf, Electron microscopic grids, etc.
Volume	Large (0.2-2ml)	Very small (<1µL)
Cryoprotectants	Low concentration CPAs (*1.5M)*	High concentration CPAs *(5-7M)*
Cooling rate	Progressive *(0.1 to 0.3°C/min).*	Immediate (*-2500°C/min to 20000°C/ min*).
Equipment	Programmable freezer	No special equipment needed
Procedure	Long time required, complicated, mostly depending on equipment	Rapid, depending on the operator skills

1.4 Vitrification of oocytes and embryos

Successful vitrification of oocytes and embryos is influenced by different variables that have been studied for years. These can be classified into technical and biological variables.

1.4.1 Technical variables

Different protocols have been used for oocyte and embryo vitrification in order to minimize effects caused by CPAs. These protocols make use of different types and concentrations of cryoprotectants (Wani *et al.* 2004) and also several cryodevices are available (Liu *et al.* 2008).

1.4.1.1 Type of cryoprotectants

Cryoprotectants are organic compounds that reduce the freezing point of aqueous solution, increase the viscosity of aqueous solution and lower the ice nucleation temperatures of cells or solutions (Rall *et al.* 1983). They can be classified as permeable and non-permeable.

Permeable CPAs, including dimethyl sulfoxide (DMSO), glycerol, propylene glycol (PG), ethylene glycol (EG) and methanol are capable of passing oocyte and embryo membranes and provide protection both within and around the cells (Lovelock 1954). Prior to cooling, the addition of CPAs makes the medium hyperosmotic, resulting in a shrinking of cells due to the efflux of water (Figure 3). But because of the difference in concentration of the CPA between the extra and intracellular solutions, the CPA begins to permeate the cell by simple diffusion. Simultaneously water begins to re-enter the cell to maintain osmotic equilibrium between the extra and intracellular solutions (Figure 3).

Upon removal of the CPA (warming, Figure 4), cells can also be subjected to osmotic shock. Osmotic shock occurs because water enters the cell more rapidly than an intracellular CPA can leave it. As a result, cell volume may increase to a critical volume and the cell may burst (Oda *et al.* 1992).

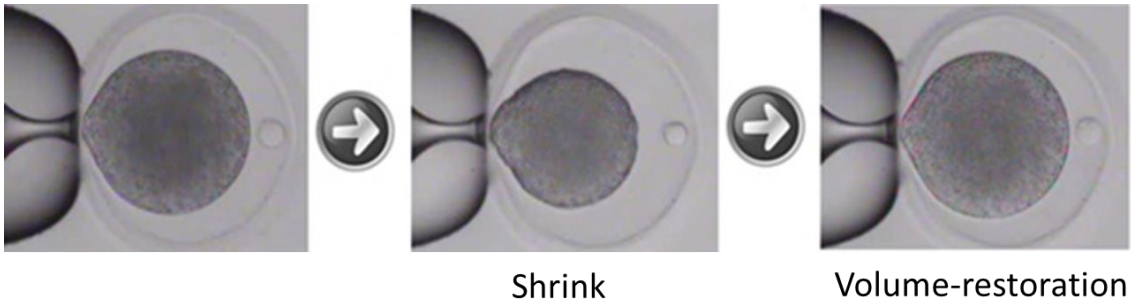

Figure 3. Osmotic volume changes of an oocyte after exposition to cryoprotectants.

Moreover permeable CPAs inducing direct or indirect effects are known to affect cells and cell constituents. More specifically CPAs can cause; (1) depolymerization and disorganisation of microtubules and microfilaments, resulting in chromosomal scattering, the development of aneuploidy or abnormal cytokinesis (Van der Elst *et al.* 1988, Vincent and Johnson 1992); (2) alterations in membrane integrity, metabolism and developmental potential of embryos (Damien *et al.* 1989); (3) hardening of the zona pellucida of oocytes (Vincent *et al.* 1990); (4) destabilisation of proteins (Arakawa *et al.* 1990) and (5) disturbance of intracellular calcium homeostasis in oocytes (Litkouhi *et al.* 1999).

Non permeable CPAS include monosaccharides (galactose), disaccharides (sucrose and threhalose), polysaccharides (dextrans) and polymers (polyvinylalcohol) (Ashwood-Smith 1986). Non permeable CPAs protect through dehydration, stabilisation of lipid bilayers and proteins, or they can change the water properties in the vicinity of membranes (Franks *et al.* 1977, Crowe *et al.* 1990). When oocytes or embryos are exposed to mono or disaccharides, cells respond osmotically by losing water. Since these cryoprotectants do not pass membranes, cells remain contracted when equilibrium is reached.

1.4.1.2 Concentration and time of exposure to CPAs

In cryopreservation, equilibrium refers to the relative amount of water inside the cell and outside the cell being the same (or nearly so). If sufficiently high concentration of CPAs could be added at the beginning of freezing, the system would vitrify with no supercooling no matter how slowly it was cooled. However, the concentration of CPA necessary to achieve thermodynamically stable vitrification is extremely high (80% w/v) leading to osmotic and toxic effects (Allahbadia *et al*. 2015). Two approaches are followed in order to minimize these effects of CPAs. The first approach is the reduction of the temperature and the time of exposure to CPAs following two step-vitrification. In the first step, equilibration (Figure 4), cells are exposed to lower concentration of CPAs for a considerable time of exposure (~10-15 min). This allows the entry of CPAs producing cells to re-swell and thus avoiding osmotic damage. In the second step, vitrification (Figure 4), higher concentration of CPAs are used, with the aim to osmotically reduce the water content of the cells and rapidly increase intracellular CPA concentrations, without having to wait for the slower further influx of CPAs.

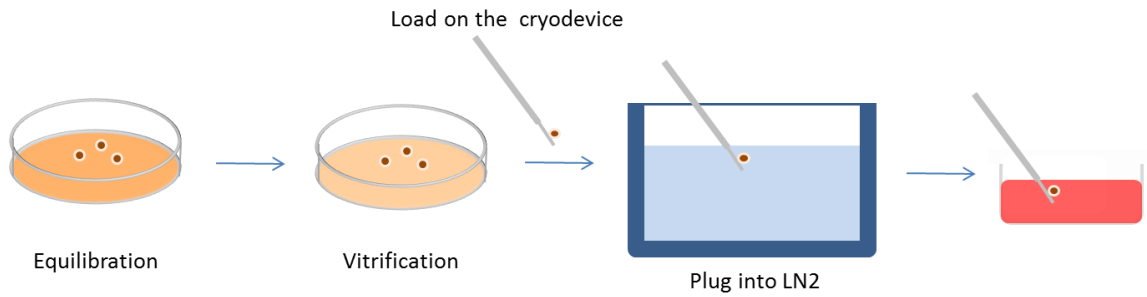

Figure 4. Schematic overview of vitrification. (1) Cells are exposed to low concentration of cryoprotectants (equilibration). (2) Cells are moved to a medium containing higher concentration of cryoprotectants (vitrification). (3) Cells are loaded on the cryo-device, which is directly plugged into liquid nitrogen (LN_2) (4). For warming, cryodevice is directly introduced in the medium and oocytes are recovered.

The second approach is the reduction of the concentration of CPAs by using metastable vitrification protocols, in which cooling rates and warming rates are strongly increased by using minimum volume cryo-devices (described below). Minimizing the volume of the sample decreases the amount of liquid which has to be cooled, thereby increasing cooling and warming rates. High cooling rates reduce the likelihood of ice nucleation, while high warming rates prevent the lethal growth of small crystals, if they were formed during cooling.

1.4.1.3 Cooling and warming rates

As previously described, the concentration of CPAs can be also reduced when higher cooling and warming rates are achieved. While straws of 0.25 ml or 0.5 ml are normally used for slow freezing (Table 1), a large number of cryodevices have been developed for vitrification in order to increase the cooling rate: The Minimum Drop Size (Arav 1992), Electron Microscope Copper Grids (Steponkus and Caldwell 1990), Open Pulled Straws (Vajta *et al.* 1998), Cryoloops (Lane *et al.* 1999), Superfine-pulled Open Pulled Straw (Isachenko *et al.* 2000), Micro-drops (Papis *et al.* 2000), Hemi-straw (Vanderzwalmen 2000), Solid-surface (Dinnyes *et al.* 2000), Nylon-mesh (Matsumoto *et al.* 2001), Closed Open Pulled Straw (Chen *et al.* 2001), Flexipet-Denuding Pipettes (Liebermann and Tucker 2002), Cryotop (Hamawaki *et al.* 1999, Kuwayama and Kato 2000), Cryoleaf (Chian *et al.* 2005), Cryotip (Kuwayama *et al.* 2005a), Direct Cover Vitrification (Chen *et al.* 2006), High Security Vitrification (Camus *et al.* 2006), Fiber Plug (Muthukumar *et al.* 2008), Vitrification Spatula (Tsang and

Chow 2009), Cryo-E (Petyim *et al.* 2009), Cryopette (Portmann *et al.* 2010), Plastic Blade (Sugiyama *et al.* 2010), Vitri-Inga (Almodin *et al.* 2010) and Rapid-I (Balaban *et al.* 2010).

Differences in cooling and warming rates have been observed when diverse cryodevices are being used, for example, 16,700- 13,900°C/min achieved with Open Pulled Straw (OPS) and 23,000-42,000°C/min obtained by Cryotop (reviewed in Zhang et al. 2011). This is due to the fact that higher cooling rates are achieved when lower volumes are used, 1.5µL for OPS compared to< 0.1 µL for CryoTop. Therefore, better results have been reported after vitrification using CryoTop compared to OPS (Liu *et al.* 2008, Morato *et al.* 2008).

In our laboratory, we have developed a custom-adapted device consisting of a 0.25 ml straw with a cut in one end to allow loading of the oocytes in a minimal volume (<1 µL). At the opposite end, a wire is added to prevent floating in LN_2 (Figure 5). Oocytes are loaded using a 130 µm pipette in order to minimize the volume surrounding the oocytes, and excess medium is removed with the pipette by capillarity in order to increase cooling and warming rates.

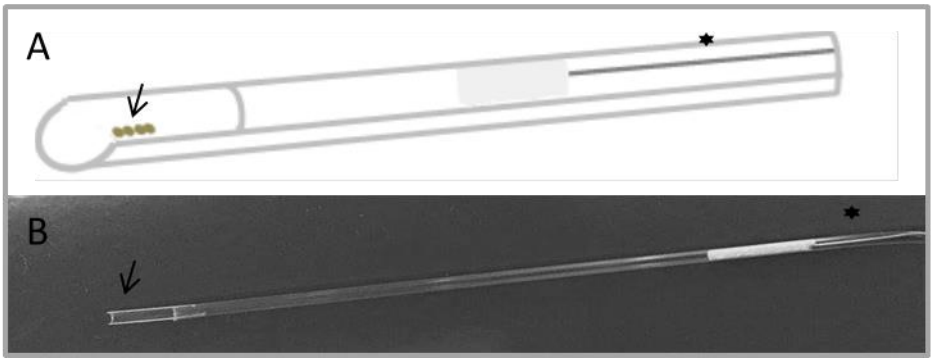

Figure 5. Drawing (A) and representative image (B) of the custom-adapted device used in the present study. The arrows denote where the oocytes are loaded and the asterisks denote the wire added.

Higher cooling rates can be also achieved when slush nitrogen (SN_2) is used (135,000°C/min). Slush nitrogen is liquid nitrogen mixed with nitrogen ice, i.e. nitrogen at its melting point (−210°C), rather than at its boiling point (−196°C). Obviously, the temperature of melting N_2 is lower than that of boiling N_2, but more importantly, N_2 at its melting point will not boil of the heat it receives from a specimen to be cooled. In conventionally used LN_2, (boiling point), an insulating sheath of N_2 gas is generally formed around an object that needs to be cooled (Leidenfrost effect), which can slow down heat transfer from that object. The high cooling rate that can be achieved in N_2 slush may allow the

use of lower CPA concentrations for metastable vitrification, or may help prevent to 'outrun' damaging cellular changes that may occur during cooling (Huang *et al.* 2005, Lee *et al.* 2007, Yoon *et al.* 2007, Cha *et al.* 2011). However, SN_2 is difficult to produce since it is necessary to reduce the pressure above a Dewar of LN_2 using a vacuum pump in a sealed system until conversion occurs.

Although a lot of efforts have been made to achieve a very high cooling rate, Seki et al. 2009 reported that warming has a higher influence in cell survival. This occurs because intracellular water can freeze or vitrify during cooling. But, the outcome would be mainly influenced during warming under the three situations described below.

Firstly, if large intracellular ice crystals have formed during cooling, survival was near zero independently of warming rate. Secondly, if small intracellular crystals are formed during cooling, slow warming would result in (1) growth of large and small crystals, (2) recrystallization (small crystals that are thermodynamically unstable tend to melt with larger ice crystals), and (3) de novo ice nucleation. These three phenomena could have lethal effect on the cells. However, if warming rate is sufficiently rapid, recrystallization may be blocked (Seki and Mazur 2009). This explains why cells can survive even when small crystals are formed during cooling.

1.4.2 Biological variables

1.4.2.1 Variables that influence oocytes vitrification

In general, oocytes are difficult to preserve due to the fact that they have certain features that make them very sensitive to cooling. Firstly, the oocyte is the largest cell in the body, resulting in a low surface-volume ratio. This lower surface volume ratio, compared to other cells makes the movement of water and cryoprotectants slowly, because it needs to be accommodated through a relatively small surface area.

As a single cell, the oocyte needs to maintain its integrity of several unique structures to undergo maturation, fertilization and subsequently embryo development. Those structures include the surrounding cumulus cells, the zona pellucida, the oolemma, the cortical granules and the metaphase II spindle (Figure 6). All of these are really susceptible to suffer cryoinjuries during cryopreservation, affecting vitrification.

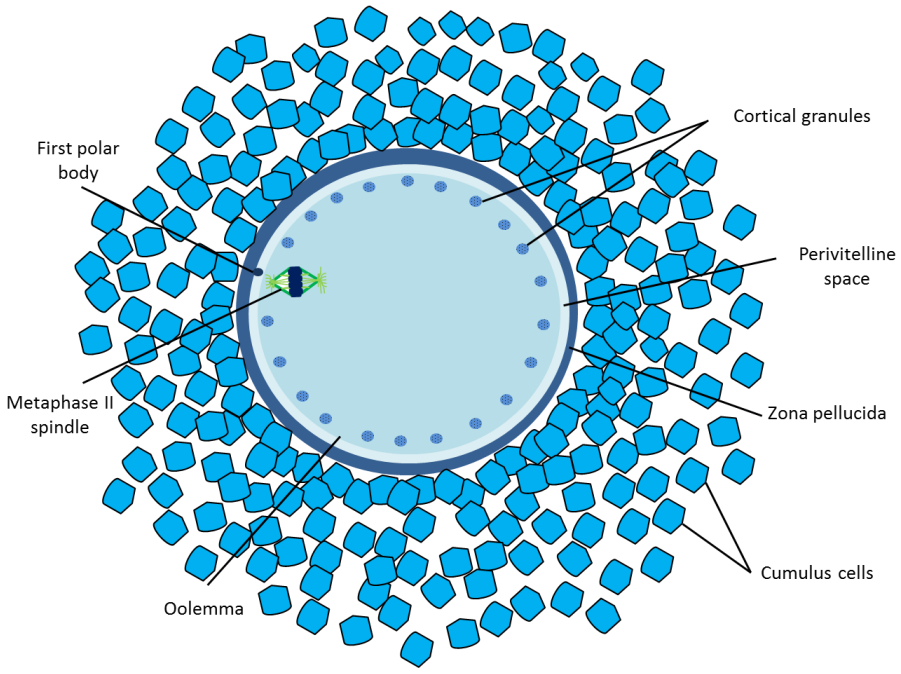

Figure 6. Schematic diagram of a mature oocyte with its investments and internal structures.

On the outside, oocytes are surrounded by different layers of cumulus cells and communicating with them through cellular projections. Such communication is necessary to prepare the oocyte for normal maturation and fertilization (Bloor *et al.* 2004). Moreover, cumulus cells are responsible to trap and select the spermatozoa, and induce sperm capacitation, acrosome reaction and penetration during the fertilization (Van Soom *et al.* 2002, Tanghe *et al.* 2003). While it is clear that cumulus cells play a fundamental role during in vitro culture, their role during vitrification is still not clear. It seems that cumulus cells protect oocytes from the adverse effects of chilling injuries (Tharasanit *et al.* 2009), and may enhance their chance for fertilization by preventing zona hardening (Vincent *et al.* 1990), but it also has been proposed that cumulus cells may hinder the movement of water and cryoprotectants leading to an undesired shield, preventing the transport of these molecules (Papis *et al.* 2013). Different authors have tried to clarify this subject (reviewed in Table 2), but apparently the effect is species-specific and it also depends on the oocyte stage (Fujihira *et al.* 2005). The use of corona radiata (CR) oocytes has been proposed as an alternative to the vitrification of denuded or cumulus oocyte complexes (Figure 7). Corona radiata oocytes are surrounded by two or three layers of cumulus cells, which less affects the movement of CPAs and water contribute to a normal oocyte development (Papis *et al.* 2013).

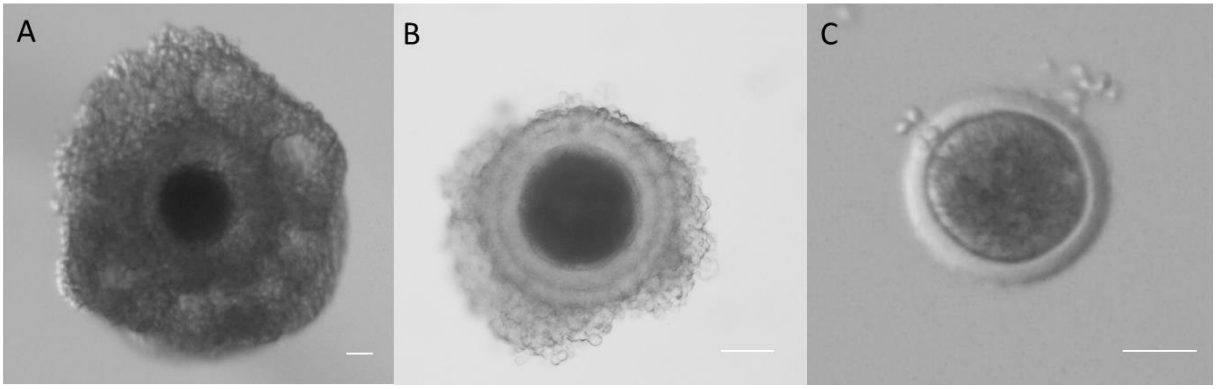

Figure 7. Microscopic images of a cumulus oocyte complex (A), corona radiata oocyte (B) and denuded oocyte (C).Scale bar: 50 µm.

Table 2. Comparative survival, spindle quality and competence of denuded or partially denuded oocytes versus cumulus-oocyte- complexes after vitrification at immature (GV) and mature (MII) stage in different species.

Species	Survival	Meiotic compet.	Spindle quality	Fertili-zation	Embryo develop.	Reference
			Germinal vesicle			
Sheep	Higher	Higher	ND	-	-	*Bogliolo et al. 2007*
Goat	Lower	Lower	-	-	-	*Purohit et al. 2012*
Cattle	Lower	-	-	-	Lower	*Zhou et al. 2010*
Cattle	Higher	-	-	-	Higher	*Papis et al. 2013*
Horse	-	Lower	Lower		-	*Tharasanit et al. 2009*
			Mature			
Mouse	ND	-	ND	Lower	ND	*Park et al. 2001*
Mouse	Lower	-	-	-	Lower	*Zhou et al. 2016*
Sheep	ND	-	-	-	ND	*Zhang et al. 2009*
Goat	-	-	-	ND	-	*Purohit et al. 2012*
Cattle	Higher	-	-	-	ND	*Chian et al. 2004*
Cattle	ND	-	-	-	ND	*Zhou et al. 2010*
Buffalo	Lower	-	-	-	Lower	*Gasparrini et al. 2007*
Horse	-	-	Lower	-	-	*Tharasanit et al. 2009*

ND: No differences

Below the cumulus cells, we encounter the zona pellucida and the oolemma (Figure 6). The oolemma is really sensitive to chilling injuries, and when it is cooled, it exhibits a thermotropic behavior changing the usual lipids arrangement, and resulting in significant deleterious effects on its function, as previously described. One level deeper we find the cytoplasm, which may or may not contain structures susceptible to cooling depending upon the stage of maturation (germinal vesicle or mature).

Mature oocytes have a number of secretory organelles named cortical granules in the cytoplasm (Figure 8). Cortical granules release secretions into the perivitelline space, as a reaction on the oscillations of calcium induced by the entrance of one spermatozoa to the oocyte. The released substances change the composition of the zona pellucida, and induce the so-called zona-block, avoiding the further entrance of more spermatozoa. However, during cryopreservation, the use of some CPAs can also induce calcium oscillations, resulting in premature release of cortical granules, and subsequently zona pellucida hardening, which prevents normal fertilization (Carroll *et al.* 1990, Mavrides and Morroll 2005). Zona hardening effect can be minimized in cryopreserved oocytes by adding 20% of fetal bovine serum to the medium (George *et al.* 1992) or by using a calcium-free medium (Larman *et al.* 2006).

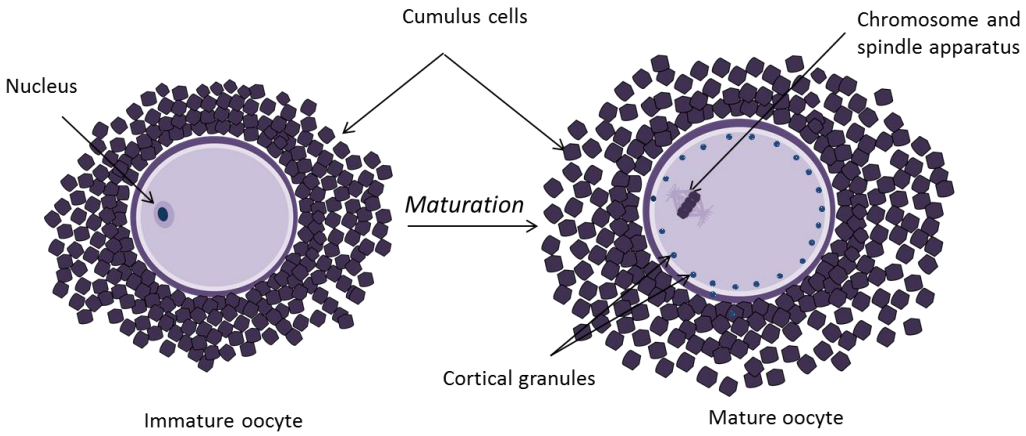

Figure 8. Diagram of an immature oocyte surrounded by compact cumulus cells and with the genetic material contained within the nucleus. During maturation cortical granules are relocated and the metaphase plate that is composed of the chromosomes and the spindle apparatus is organized in the cytoplasm.

Moreover, mature oocytes are in the middle of the meiotic division, thus genetic material is condensed forming chromosomes, which are organized by the spindle apparatus (Figure 9). The spindle apparatus is formed by microtubules, which organize centers at opposite poles and keep the

chromosomes aligned at the equatorial plane of the meiotic spindle (Figure 9). A drop in temperature causes depolymerization of microtubules, resulting in spindle disorganization (Aman and Parks 1994). Meiotic spindle can be also altered by the use of CPAs (Vincent *et al.* 1990, Vincent and Johnson 1992). For example, DMSO and PG change the polymerization pattern of nearly all the microtubules in the MII spindle of mouse oocytes (Johnson and Pickering 1987) , while a similar effect is observed in cattle when oocytes are exposed to EG (Saunders and Parks 1999).

Even though microtubules repolarization occurs after cryopreservation, a wrong alignment of chromosomes increases the chance for aneuploidic embryos, which is undesirable (Wang and Sun 2006, Bromfield *et al.* 2009). Additionally, alterations in microtubules lead to an abnormal distribution of the mitochondria and a multiple aster formation. In cattle, a single sperm aster is the result of the microtubule organizer center, which is formed by polymerization of microtubules and produces the migration and fusion of the male and female pronucleus. However, vitrification adversely affects the recruitment of the centrosomal proteins by the sperm centrosome due to the cytoskeleton disorganization, resulting in an erroneous fertilization and subsequently embryo development (Hara *et al.* 2012).

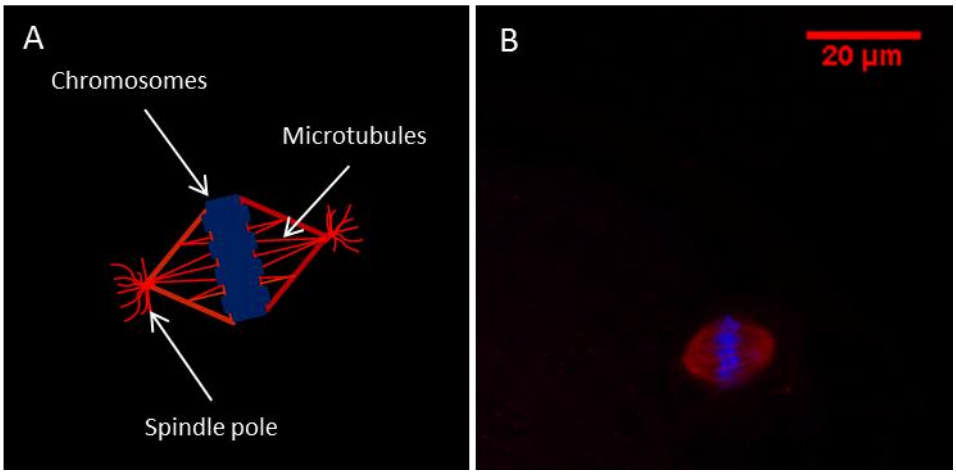

Figure 9. Schematic diagram (A) and confocal picture (B) of a normal MII oocyte with its typical barrel-shaped metaphase II spindle configuration and the chromosomes perfectly aligned in blue.

On the other hand, germinal vesicle (GV) oocytes do not contain any of these structures (Figure 8), and therefore, they have been proposed as a sound alternative to mature oocytes for vitrification. However, immature oocytes also present some difficulties. They have a less permeable membrane, which may hamper the movement of water and CPAs (Agca et al. 1998).

Different stages have been reported as most favorable for vitrified bovine oocytes, GV (Zhou *et al.* 2010) and MII (Otoi *et al.* 1995, Men *et al.* 2002, Diez *et al.* 2005). In porcine, GV oocytes seem to be more sensitive to cooling than MII oocytes (Rojas *et al.* 2004), while the opposite was reported for equine oocytes (Tharasanit *et al.* 2006).

1.4.2.2 Variables that influence embryo vitrification

An embryo is an early stage of development of a multicellular diploid eukaryotic organism. The fact that embryos are diploid may be considered advantageous, as such cells show higher resistance to mutagenic and stress factors (Cherfas and Zoy 1984). However, it is worth noticing that embryos are susceptible to cooling, because they contain actively dividing cells.

An embryo is formed when an oocyte is successfully fertilized by a spermatozoon. After one or two days, cells start to divide and become a spherical structure denominated morula around day five or six post fertilization. At day six to seven, a cavity is formed, the blastocoel, and the morula is transformed into a blastocyst, consisting of two type of cells, which form the inner cell mass and the trophoblast (Figure 10).

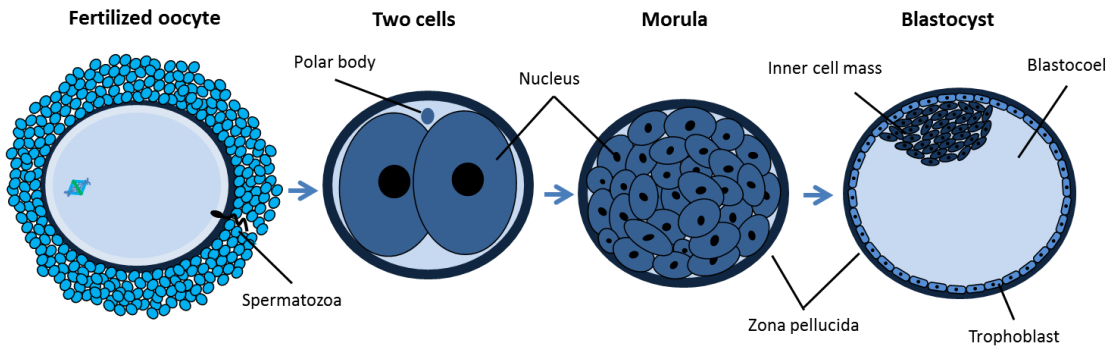

Figure 10. Bovine embryo development after fertilization.

Like oocytes, embryos are surrounded by the zona pellucida. Fractures in the zona pellucida and lysis of the cellular membranes of the embryonic cells are frequently observed after cryopreservation (Cuello *et al.* 2007). In addition, ultrastructural investigations revealed cryoinjuries in mitochondria and rough endoplasmic reticulum alterations. Similarly, poorly developed desmosomes, disintegration of cell adhesions and communication between adjacent trophoblastic cells have been reported after embryo cryopreservation (Dalcin *et al.* 2013).

The communication of cell-cell is necessary for successful development and appropriate implantation in mammalian embryos (Bloor *et al.* 2004). The most direct form of cell-cell communication is provided by gap junctions, which are formed by two assembled hemichannels. Hemichannels (HCs) consist of six connexin proteins and each connexin has four transmembrane segments, two extracellular loops, one intracellular loop and N- and C- terminal tails projecting into the cytoplasm (Figure 11). While the function of gap junctions is the communication of cell to cell, hemichannels are responsible for connecting the cytoplasm of the cell with the exterior.

Unapposed hemichannels, which are normally closed, open under certain osmotic and chemical conditions (Wang *et al.* 2013) allowing the movement out of the cell of small metabolites and molecules necessary for normal cell functioning (Decrock *et al.* 2009).

Cryopreservation is a dynamic process during which a number of physical and chemical factors, such as osmotic and hydrostatic pressure, ionic intracellular content, pH and temperature, fluctuate over a wide non-physiological range. In the case of cryopreservation of human blood vessels, it was found that cryopreservation can lead to opening of HCs in endothelial cells and gap junctions in smooth muscle cells, which can cause extensive cell death (Bol *et al.* 2013). Interestingly, it has been described that the open HCs can be blocked with connexin-targeting peptides. The connexin peptide binds to extracellular domains of connexins during the processes of cryopreservation of human vascular grafts, reducing cell death of endothelial and smooth muscle cells (Bol *et al.* 2013).

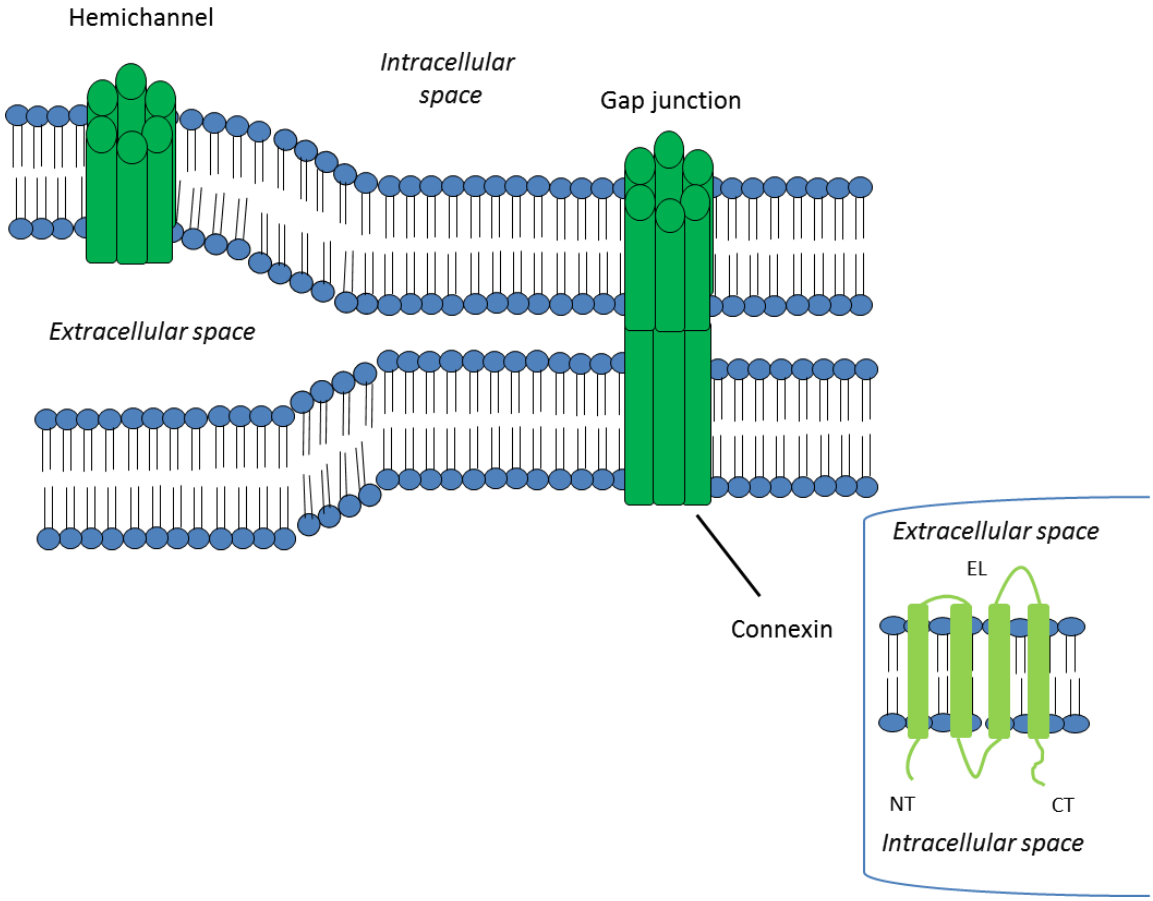

Figure 11. Diagram of the gap junctions and hemichannels. EL; extracellular loop, NT; N-terminal tail and CT; C-terminal tail.

1.4.3 Biological variables influenced by technical variables

Although oocytes and embryos of farm animals, such as cattle and pigs, are rich in cytoplasmic lipids in comparison with human or murine oocytes (McEvoy *et al.* 2000), this variable can also be altered by the environment in which oocytes and embryos are cultured.

Oocytes and embryos are routinely cultured in a medium with serum, because it contains components such as hormones, vitamins, lipids, proteins and growth factors which are important for embryo development. However, the success of cryopreservation is highly correlated with cytoplasmic lipid content, the specific mechanism is unknown and may be indirectly related to cytoplasmic lipids. It is known that serum induces the neosynthesis of triacyglycerides (Razek *et al.* 2000), changes in the membrane composition, and changes in the function of beta oxidation in the mitochondria (Abe *et al.* 2002), which may compromise the survival and further embryonic development of vitrified oocytes and embryos (Abe *et al.* 2002, Gómez *et al.* 2008, Shirazi *et al.* 2012).

In this chapter we have discussed the importance of cryopreservation in assisted reproductive technology, as well as the basics of cryobiology that allow us to understand how technical and biological variables have an effect on a successful vitrification. In the next chapter we will describe the aims that have guided our work in this dissertation.

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CHAPTER 2

AIMS

Despite the major progress that has been made, vitrification of oocytes and embryos remains a challenge because after warming, their survival and development are compromised. As previously described in the introduction, technical and oocyte/embryo variables influence successful vitrification. These variables need to be addressed to find the most adequate protocol to minimize the damage suffered during the vitrification and to achieve higher survival rates and developmental competence. The aim of this work is to develop and optimize a vitrification strategy for equine and bovine oocytes and for bovine embryos. In order to realize this general aim, specific objectives were formulated as follows:

I. To determine the effect of cumulus cells during the vitrification of mature bovine oocytes (Chapter 3).

II. To study the effect of the level of cumulus cells surrounding the equine immature oocyte at vitrification time (Chapter 4).

III. To compare two protocols for vitrification of immature equine COCs and CR, one with a short exposure to a high concentration of cryoprotectants, and one with a longer exposure to a lower concentration of cryoprotectants (Chapter 5).

IV. To study the effect of maturation in the presence of serum on the vitrification of oocytes (Chapter 5.1) and blastocysts (Chapter 5.2).

V. To investigate whether blocking Cx channels with Gap26, which mimics a sequence of the first extracellular Cx loop, could improve the outcome of vitrified bovine blastocysts matured as oocytes in serum containing or serum-free media (Chapter 5.2).

CHAPTER 3

EFFECT OF CUMULUS CELLS DURING THE VITRIFICATION OF MATURE BOVINE OOCYTES

Modified from:

Role of Cumulus Cells During Vitrification and Fertilization of Mature Bovine Oocytes: Effect on Survival, Fertilization and Blastocyst Development.

Ortiz-Escribano N, Smits K, Piepers S, Van den Abbeel E, Woelders H, Van Soom A. Theriogenology 2016; 86: 635-641.

Summary

This study was designed to determine the role of cumulus cells during vitrification of bovine oocytes. Mature cumulus oocytes complexes (COCs) surrounded by many layers of cumulus cells, corona radiata (CR) oocytes, with a few layers of cumulus cells and denuded oocytes (DOs) without cumulus cells were vitrified in 15% ethylene glycol (EG), 15% dimethylsulfoxide (DMSO) and 0.5M sucrose. Oocytes that survived the vitrification process were fertilized. Denuded oocytes were fertilized with or without supplementation of intact COCs (DOscocs). First, survival and embryo development rates were studied. Higher survival rates were obtained for DOs and DOscocs (94% and 95% respectively) compared to COCs (82.7%, $P<0.05$). Corona radiata oocytes showed similar survival rates when compared to denuded oocytes. The cleavage and blastocyst rates of vitrified DOs were compromised, since cumulus cells were not present during the fertilization (34% and 2.7% respectively). However, the situation could be reverted when DOs were supplemented with intact COCs (DOscocs) (62.7% and 12.7% respectively, $p<0.05$). Vitrified CR oocytes showed similar cleavage and blastocyst rate (49.3% and 7.7% respectively) compared to COCs (54.8% and 4.9% respectively). In the second experiment, the penetration rate was analyzed. Removing cumulus cells before fertilization reduced the fertilization of vitrified DOs compared to COCs (24.3% vs.52.8%, $p<0.05$). The supplementation of DOs with intact COCs (DOscocs) improved the fertilization rate though (49.6%, $p<0.05$). No differences in the fertilization rate were found between CR oocytes and COCs. In the third experiment, parthenogenetic activation was examined. Interestingly, the CR oocytes showed higher cleavage and blastocyst rates (76.8% and 29.6% respectively) than the COCs (39.1% and 7.5 % respectively, $p<0.05$). Furthermore, oocytes from vitrified CR oocytes had the same odds to become a blastocyst as fresh oocytes (1.1 vs. 1.5, respectively). In conclusion, our data demonstrated that cumulus cells reduce survival after the vitrification of mature bovine oocytes. Since cumulus cells are required for fertilization, the use of partially denuded (CR) oocytes or the addition of intact COCs (DOscocs) during fertilization can result in higher survival and embryo development after vitrification.

INTRODUCTION

The vitrification of oocytes provides many benefits for assisted reproductive technology (ART). In humans, it allows to reduce the number of embryos produced at any given time, it permits synchronization in donor-recipient programs and it preserves the fertility of young women receiving cancer treatment; in animals, it allows preserving genetic diversity and it increases the material available for research and animal breeding programs (Ledda *et al.* 2001, Pereira and Marques 2008). Oocytes are very sensitive to vitrification because of their high lipid content and low surface-to-volume ratio. Furthermore, their complex structure (zona pellucida, oolemma, cortical granules, metaphase plate or germinal vesicle) can be severely damaged during cooling and warming (Chen *et al.* 2003). Oocyte vitrification can induce rupture of the oolemma, distortion of the metaphase plate in the mature oocyte, and premature extrusion of the cortical granules leading zona hardening, all of which prevent normal fertilization of the vitrified-warmed oocytes (Mavrides and Morroll 2005). Human ART has circumvented these problems and laboratories have reported obtaining blastocyst formation and live births from vitrified oocytes at rates equivalent to those from fresh oocytes (Cobo *et al.* 2008) . However, the development rates of vitrified-warmed bovine oocytes remain low in comparison with their fresh counterparts, since bovine oocytes are more sensitive to chilling because they contain high amounts of lipids (Martino *et al.* 1996).

The success of oocyte vitrification depends on many variables that have been studied for several years (Saragusty and Arav 2011). These variables can be divided in two groups: biological variables, referring to the presence of cumulus cells or the developmental stage of the oocyte (mature or germinal vesicle) and technical variables, referring to different protocols, cryoprotectants (CPAs) and cyo-devices used. Although most of these variables have been studied in the last decade, some of them, such as the effect of cumulus cells during vitrification still remain unclear. In cattle, it is known that the presence of cumulus cells is necessary for a correct maturation, fertilization and subsequently embryo development (Zhang *et al.* 1995, Tanghe *et al.* 2003). However, it is still controversial if the presence of cumulus cells is beneficial during vitrification of bovine mature oocytes (Dinnyes *et al.* 2000, Chian *et al.* 2004, Zhou *et al.* 2010). It has been suggested that cumulus cells may protect against cryo-injury during vitrification by minimizing the release of cortical granules, thus preventing premature zona hardening (Vincent *et al.* 1990). On the other hand, the presence of cumulus cells during cryopreservation could limit the exchange of water and CPAs, which could cause inadequate dehydration and/or CPA entry and consecutive ice crystal formation, which leads to an inappropriate oocyte protection (Gook *et al.* 1993) . In cattle, Zhou et al. showed that vitrified partially denuded immature oocytes develop at significantly lower rates than vitrified

cumulus enclosed oocytes. However, no significant differences were detected when mature oocytes were partially denuded before vitrification (Zhou *et al.* 2010). These authors suggested that cumulus enclosed and partially denuded mature oocytes displayed the same survival, cleavage and blastocyst rates after vitrification, probably due to the fact that cumulus cells were detrimental during vitrification, compromising the benefits of cumulus cells during in vitro fertilization (IVF). The aim of our study was to further determine the possible detrimental or beneficial effect of cumulus cells during the vitrification of mature bovine oocytes. In particular, we analyzed whether an intact, partially removed or completely removed cumulus, or the mere presence of cumulus cells, affects survival, fertilization and subsequent embryo development of vitrified and fresh mature bovine oocytes. To disentangle effects on fertilization and development respectively, we also studied embryo development after parthenogenetic activation.

MATERIALS AND METHODS

Media and reagents

Basic Eagle's Medium, Tissue Culture medium (TCM) 199, Minimal Essential Medium non-essential amino acids, kanamycin, and gentamycin were purchased from Life Technologies Europe and all other components were obtained from Sigma (Bornem, Belgium).

Collection and *in vitro* maturation of oocytes

Bovine ovaries obtained from a local slaughterhouse were rinsed twice in physiological saline supplemented with kanamycin (25 mg/ml). Cumulus-oocyte complexes (COCs) were aspirated from 2-8 mm follicles with an 18 gauge needle attached to 10 ml syringe and matured in groups of 60 oocytes in TCM199 supplemented with 50 mg/ml gentamycin and 20 ng/ml epidermal growth factor for 22 h at 38.5°C in 5% CO_2 in air.

Vitrification and Warming

Matured oocytes were vitrified as described by (Kuwayama *et al.* 2005) with some modifications. The handling solution (HS) used was TCM199/ Hank's/ Hepes supplemented with 20% Fetal Bovine Serum (FBS, Greiner Bio-one). All the vitrification media were prepared using this HS. Vitrification and warming steps were performed at 38.5°C on a heated plate.

Vitrification was performed in two steps: equilibration and vitrification. Oocytes were equilibrated by transferring them sequentially in three drops of 75 µl of equilibration solution (ES) composed of HS with 7.5% ethylene glycol (EG) and 7.5% dimethyl-sulfoxide (DMSO). After oocytes regained their original volume, they were subsequently transferred into four consecutive 50 µl drops of vitrification solution (VS) composed of HS with 15% EG, 15% DMSO and 0.5 M sucrose. Oocytes were exposed to equilibration solution for 10-15 min and to vitrification solution for 45-60 sec. Four oocytes were loaded to a custom adapted device imitating the Cryotop and within 5 sec submerged in liquid nitrogen (LN_2). After one week in LN_2, oocytes were warmed by transferring them to a warming solution composed of HS with 1 M of sucrose. This was followed by a three step wash-out of the hyperosmolar sucrose reduced from 1 M to 0.5 M (washing 1, 3 min), 0.25 M (washing 2, 5 min) and 0 M in HS (washing 3, 5 min). Oocytes were washed in HS three times and then incubated in maturation medium for 2 h to allow them to recover.

Fertilization and culture

Fresh (non-vitrified) and vitrified oocytes were fertilized in the same conditions. Frozen-thawed bull spermatozoa were separated using a Percoll gradient (45% and 90%; Pharmacia, GE Healthcare). The final sperm concentration of $1x10^6$spermatozoa/mL was adjusted in IVF Tyrode's albumin-pyruvate–lactate (TALP), consisting of bicarbonate-buffered Tyrode solution, supplemented with BSA (6 mg/ml) and heparin (25 mg/ml). At 21 h post-insemination, presumptive zygotes were vortexed to remove cumulus cells, washed and cultured in groups of 25 in 50 µl droplets of synthetic oviductal fluid medium (SOF) supplemented with ITS (5 µg/ml Insulin + 5 µg/ml Transferrin + 5 ng/ml Selenium) and 0.4% BSA. Culture occurred at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2. Cleavage rates were determined after 48h post insemination and blastocyst rate after 8 days post insemination.

Parthenogenetic activation

After maturation, oocytes were denuded by repeated pipetting in TCM199. Oocytes were incubated in 5 µM ionomycin for 5 min. Next, they were incubated in SOF medium supplemented with 2 mM 6-dimethyl-aminopurine (6DMAP) for 4 h. After incubation, presumptive parthenotes were washed twice and cultured in SOF medium in the same conditions as fertilized oocytes. Cleavage rates were determined at 48h post activation and blastocyst rate at 8 days post activation.

Assessment of survival and penetration rate

After fertilization, oocyte survival was evaluated morphologically. The criteria used to classify oocytes as surviving or degenerated have been described elsewhere (Chian *et al.* 2004). Briefly, oocytes with intact oolemma, intact zona pellucida and homogenous and dark cytoplasm are considered as surviving oocytes. Only surviving oocytes were used for in vitro culture.

Fertilization rate was determined with the nuclear staining Hoechst 33342 (Molecular Probes, Invitrogen, Merelbeke, Belgium) which selectively binds to double stranded DNA. Presumed zygotes were fixed in 4% formalin and then stained with Hoechst 10 µg/ml for 10 min. Successful fertilization was characterized by the presence of two pronuclei and three or more pronuclei were considered as indicative for polyspermy.

Experimental design

In experiment 1 (n= 1161), mature oocytes were randomly divided into two groups; fresh and vitrified. Fresh oocytes were subdivided into four groups: cumulus complex oocytes (Fresh COCs), corona radiata oocytes (Fresh CRs), denuded oocytes (Fresh DOs) and denuded oocytes supplemented with intact COCs during fertilization (Fresh DOscocs). Oocytes in the vitrified group were vitrified as COCs, CRs or DOs and subdivided into four groups after warming; cumulus complex oocytes (Vitrified COCs), corona radiata oocytes (Vitrified CRs), denuded oocytes (Vitrified DOs), and denuded oocytes supplemented with fresh COCs during fertilization (Vitrified DOscoc). For distribution see figure 1. DOs and CR oocytes were partially or completely denuded by gently pipetting in HS. Intact COCs which were supplemented to vitrified oocytes during fertilization were obtained one day before warming and matured in the same conditions as the rest of the oocytes. These supplemented COCs were removed by pipetting after fertilization and before assessing survival, cleavage and blastocyst rates. In experiment 2 (n= 1073), the experimental design was similar, except that presumptive zygotes were not cultured, but were denuded, fixed and stained to

study the fertilization rate. In experiment 3 (n= 601), mature oocytes were randomly divided in four groups; fresh control, vitrified COCs, vitrified DOs and CR oocytes. Parthenotes were produced by parthenogenetic activation and cleavage and blastocyst rates were assed.

Statistical analysis

Several binary logistic regression models were fit to determine the role of cumulus cells and the impact of the treatment on the likelihood of survival, embryo development, fertilization and polyspermy in experiment 1 and 2, and on the likelihood of parthenote development in experiment 3, using SPSS statistics version 22. The models included the likelihood of survival, embryo development, fertilization, and polyspermy and parthenote development respectively, as binary outcome variables and the oocyte group (COCs, CRs, DOs, and DOscocs), the treatment (fresh and vitrified), and the interaction term between the oocyte group and treatment as categorical independent variables. Six replicates were performed for experiment 1, five for experiment 2 and four for experiment 3. For all outcome variables, the replicate was forced in the model to account for clustering of observations within a replicate. Furthermore, each vitrified oocyte was compared with its respective fresh counterparts. A Bonferroni´s correction was applied to correct for multiple comparisons. Statistical significance was assessed at $p< 0.05$.

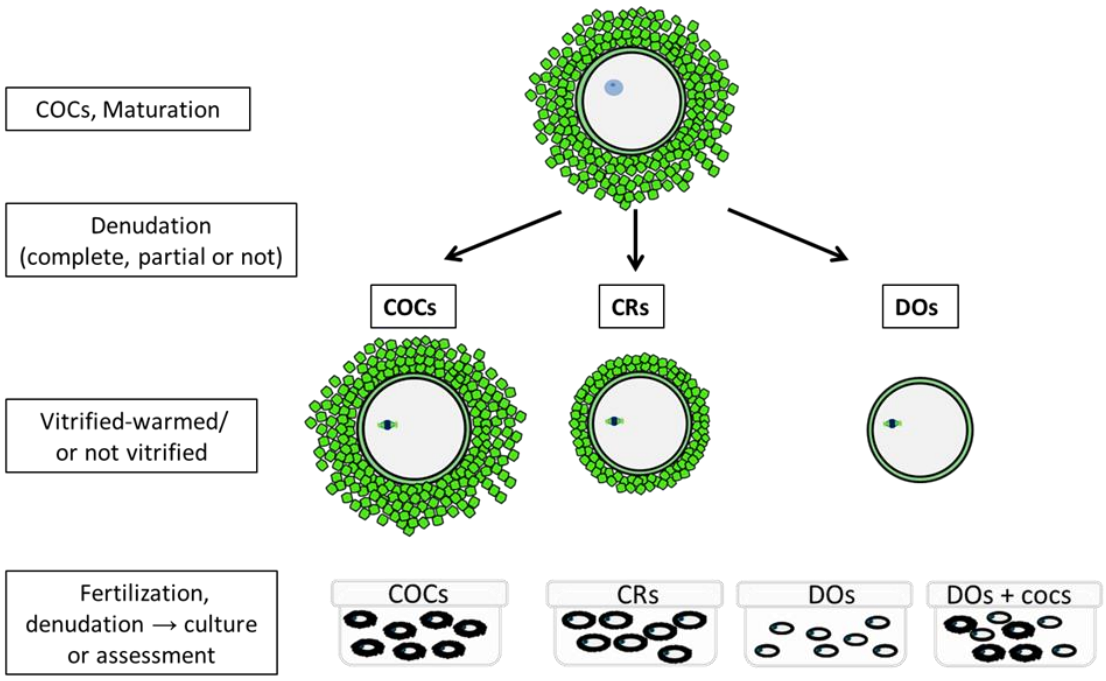

Figure 1. Experimental design of experiment 1 and 2. In vitro matured cumulus oocyte complexes (COCs) were randomly assigned to three groups: COCs, corona radiata oocytes (CRs), and denuded oocytes (DOs). CRs and DOs were created by partial or complete removal of the cumulus cells. Then, oocytes in all three groups were randomly assigned to be either vitrified and warmed, or not vitrified. The vitrified and not-vitrified DOs were further subdivided in DOs and DOs supplemented with not-vitrified COCs (DOs+cocs). The thus obtained four vitrified and four not-vitrified groups were fertilized in vitro followed by embryo culture (experiment 1) or assessment of fertilization (experiment 2).

RESULTS

Experiment 1

Survival rates of fresh groups were all very close to 100%, with no significant differences between groups (Figure 2). However, survival rates of vitrified denuded oocytes in the groups DOs (94%) and DOscocs (95%) were higher than vitrified COCs (82.7%), $p<0.05$. Survival rates of vitrified CR oocytes (86.2%) did not differ significantly from those of vitrified denuded oocytes. Vitrified COCs and CR oocytes had significantly lower survival rates compared to their fresh counterparts (82.7% and 86.2% vs.98 % and 98.6% respectively, $p<0.05$).

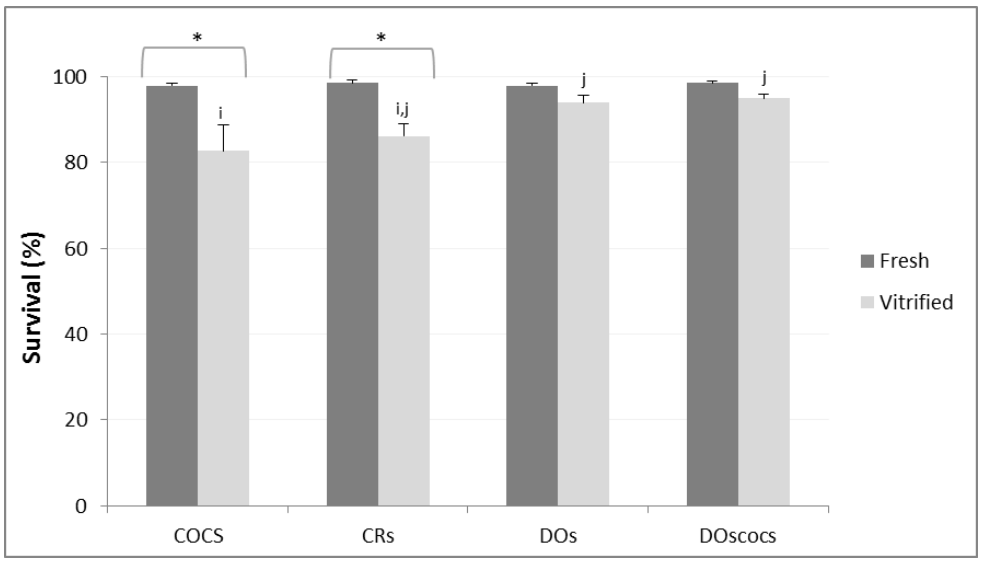

Figure 2. Survival of control and vitrified mature bovine oocytes. COCs: Cumulus complex oocytes, CRs: Corona radiata oocytes, DOs: denuded oocytes, DOscocs: Denuded oocytes supplemented with cumulus complex oocytes. [i,j] Different superscripts indicate significant differences between the oocyte groups for the vitrified oocytes;*Indication of significant differences between each control with its respective vitrified group. Mean ± SEM, n= 1161, $p<0.05$.

Removing cumulus cells after maturation significantly decreased the number of oocytes that cleaved ($p<0.05$; Figure 3). However, an increase of the cleavage rate was found in DOscocs compared to DOs (68.5 % vs. 53.7% respectively, $p<0.05$). The same effect was observed among the vitrified groups ($p<0.05$). Furthermore, the addition of COCs to vitrified DOs (DOscocs) resulted in cleavage rates comparable to those in vitrified COCs (62.8% vs. 54.9%, respectively). Cleavage rates of vitrified COCs (54.9%) and DOs (34%) were significantly lower than fresh COCs (82.7%) and fresh DOs (53.6%), $p<0.05$.

The chances to become a blastocyst were higher in fresh COCs (42%) and DOscocs (26.7%) compared to vitrified COCs (4.9%) and DOscocs (12.7%), $p<0.05$. Removing cumulus cells among fresh groups, decreased the blastocyst development for CR oocytes (20%) and DOs (9.3%) compared to COCs (42%), $p<0.05$. However, the supplementation of the intact COCs to DOs (DOscocs) increased the blastocyst development to rates comparable to those of COCs (26.7% vs. 42% respectively). Among vitrified groups, we observed that removing the cumulus cells did not have a significant effect on the blastocyst development of CR oocytes (7.7%) and DOs (2.7%) compared to COCs (4.9%), but the blastocyst rate of DOscocs was significantly higher compared to DOs (12.7% vs. 2.7% respectively, $p<0.05$).

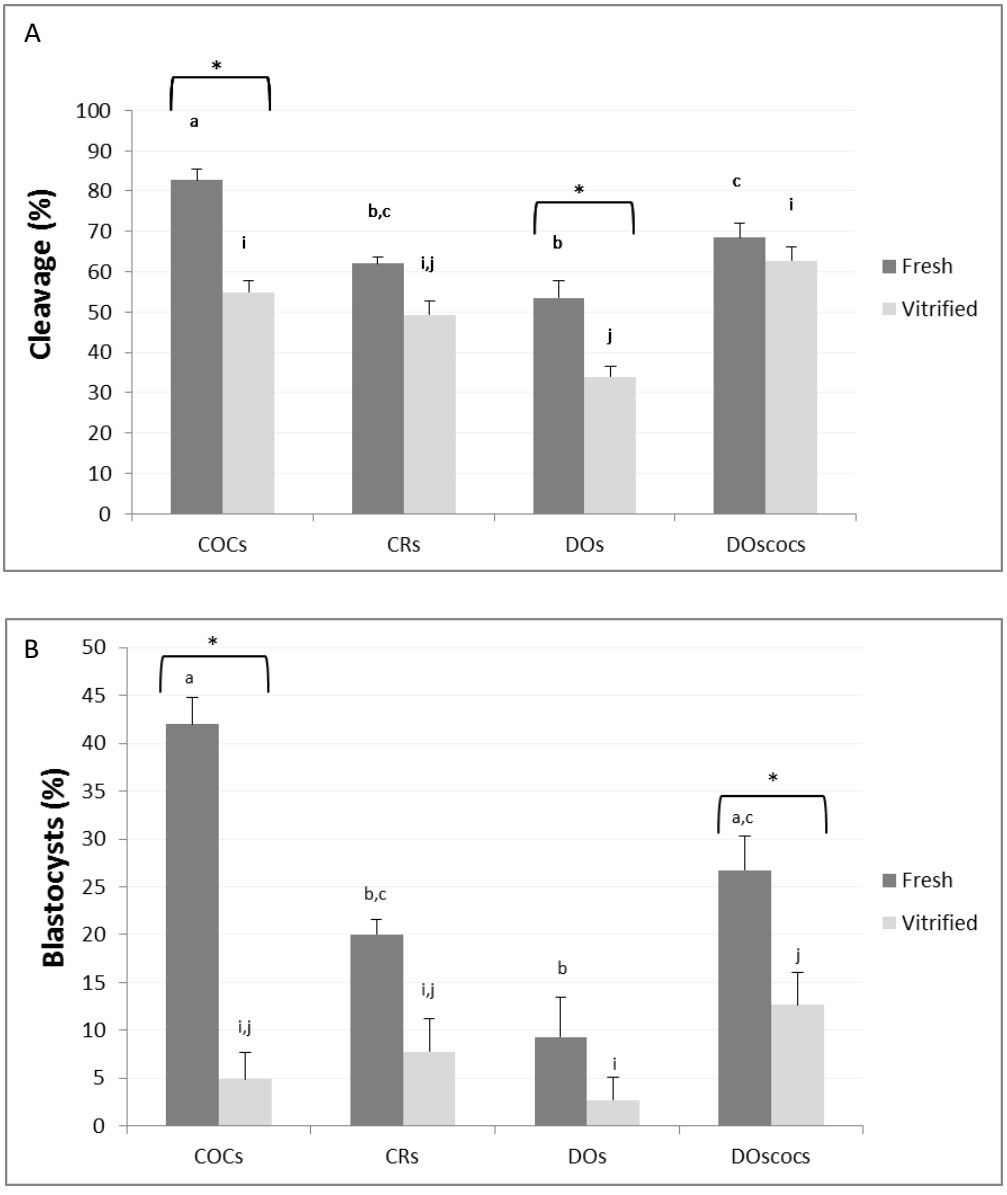

Figure 3. Cleavage (A) and blastocyst (B) development of control and vitrified mature bovine oocytes after in vitro production. COCs: Cumulus complex oocytes, CRs: Corona radiata oocytes, DOs:

denuded oocytes, DOscocs: Denuded oocytes supplemented with cumulus complex oocytes. [a,b,c] Different superscripts indicate significant differences between the oocyte groups for the control group; [i,j,] Different superscripts indicate significant differences between the oocyte groups for the vitrified group;*Indication of significant differences between each control with its respective vitrified group. Data are given as mean + SEM, n=1161, p<0.05.

Experiment 2

Removing cumulus cells before fertilization appeared to have a negative effect on fertilization rates (Figure 4). For fresh oocytes, fertilization rates of CR oocytes (39.5%) and DOs (24.7%) were significantly lower compared to COCs (71.6%), p<0.05. The supplementation of intact COCs to DOs could revert the fertilization rate, being not significantly different from COCs (59.3% vs. 71.6% respectively). We observed the same effect in vitrified oocytes. Fertilization rate was significantly higher for COCs compared to DOs (52.8% vs. 24.3% respectively, p<0.05), and the situation could be reverted when DOs were supplemented with intact COCs (DOscocs, 49.6%). Only vitrified COCs showed a fertilization rate which was significantly different from that in their fresh counterparts (71.6% vs. 52.8%, p<0.05). Polyspermy rates (Figure 4) were also numerically lower in DOs and CR oocytes compared with COCs and DOscocs, but none of the differences between groups were significant.

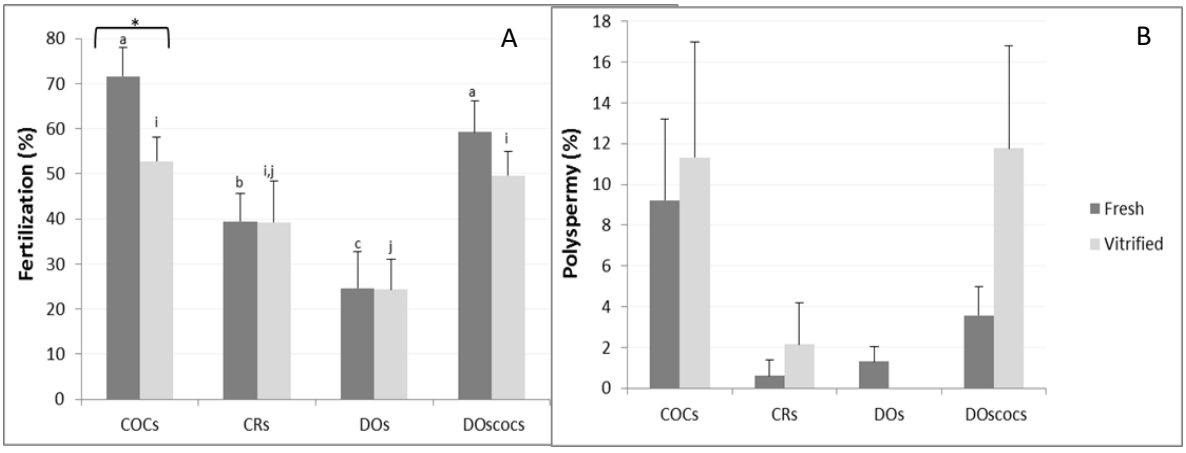

Figure 4. Fertilization (A) and polyspermy (B) rates of control and vitrified mature oocytes. COCs: Cumulus oocyte complexes, CRs: Corona radiata oocytes, DOs: denuded oocytes, DOscocs: Denuded oocytes supplemented with cumulus complex oocytes. Data are given as mean + SEM, n= 1073, five replicates. [a,b,c] Different superscripts indicate significant differences between the oocyte groups for the control group; [i,j,] Different superscripts indicate significant differences between the oocyte groups for the vitrified group;*Indication of significant differences between each control with its respective vitrified group. Significance was assessed at P<0.05.

Experiment 3

In figure 5, we observed that fresh groups had higher chances to cleave after parthenogenetic activation when compared to vitrified groups ($p<0.05$). Among the vitrified groups, cleavage rates of CR oocytes were significantly higher compared to those of COCs (76.8% vs.39.1% respectively, $p<0.05$). Blastocyst development of vitrified CR oocytes (29.6%) was comparable to that of fresh oocytes (41.9%), and significantly higher than that of COCs (7.5%, $p<0.05$).

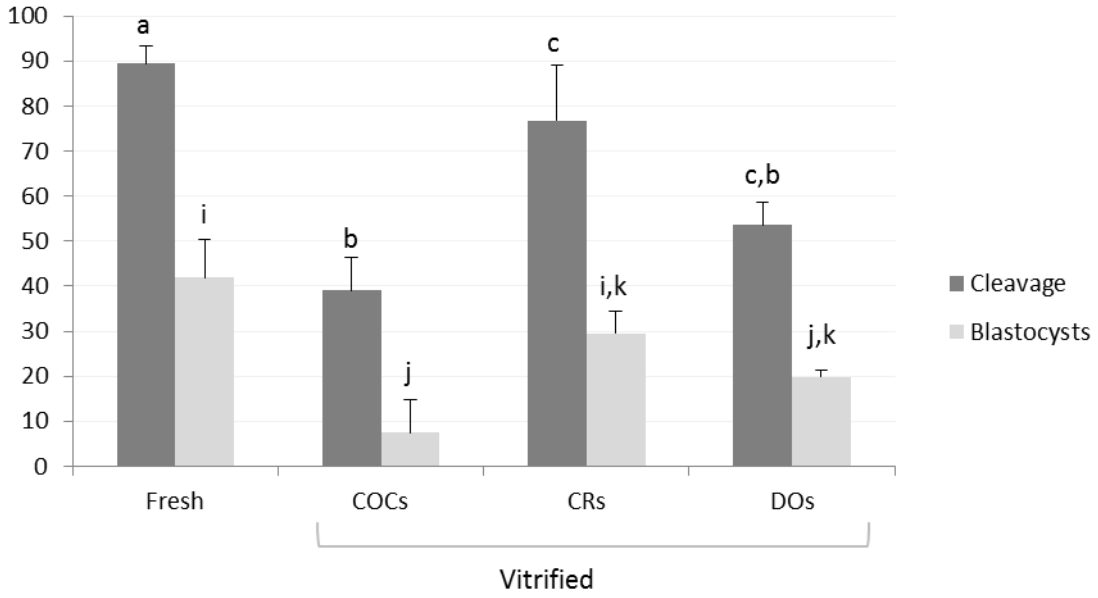

Figure 5. Effect of cumulus cells on embryo development of control and vitrified oocytes after parthenogenetic activation. COCs: Cumulus oocyte complexes CRs: Corona radiata oocytes, DOs: denuded oocytes. Data are given as mean percentages + SEM (four replicates, n=601). Cleavage rates with different superscripts (a-c) and blastocyst rates with different superscripts (i-k) differ significantly ($p<0.05$).

DISCUSSION

The effect of cumulus cells during vitrification has been studied for many years, but discrepant results have been found in several species. In human, a beneficial effect of cumulus cells in the survival of mature oocytes was observed at first (Imoedemhe and Sigue 1992, Kuwayama *et al.* 2005), however (Minasi *et al.* 2012) reported no differences in the survival of COCs compared to denuded oocytes. In equine, the presence of cumulus cells was found to be beneficial during the vitrification of mature oocytes (Tharasanit *et al.* 2009), whilst they were reported to play a detrimental role during the vitrification of buffalo oocytes (Gasparrini *et al.* 2007). On the other

hand, no differences were found in the survival rate between cumulus complex oocytes and denuded ovine oocytes (Zhang *et al.* 2009). In cattle, discrepant results have been reported. (Dinnyes *et al.* 2000) did not observe any effect when cumulus cells were present during vitrification of bovine oocytes. Similarly, (Zhou *et al.* 2010) did not observe any effect in the survival of mature bovine oocytes when they were vitrified with cumulus cells. Nevertheless, (Chian *et al.* 2004) observed that cumulus cells have a detrimental effect on the survival rate of vitrified mature oocytes. Our observations agree with the latter study. We observed that oocytes surrounded by cumulus cells such as oocytes in the COCs or CRs groups have less chance to survive compared to their fresh counterparts, whilst denuded oocytes presented the same survival rate as their fresh counterparts. According to the results observed in our study, we think discrepancies with previous reports were due to the fact that we analyzed the survival of COCs, CRs and DOs, whereas (Zhou *et al.* 2010) studied the survival of COCs and partially denuded oocytes. Those partially denuded oocytes could be considered as a different group than we used in the current study, since they had a different amount of cumulus cells with respect to DOs and CR oocytes. We hypothesize that cumulus cells can hinder the diffusion of water and CPAs, resulting in an inadequate cell protection. It is also remarkable that denuded or corona radiata oocytes were easier to handle when a manufactured cryotop was used during vitrification. Moreover, the amount of liquid surrounding these oocytes was lower compared to COCs. A lower amount of liquid surrounding the oocyte leads to a higher cooling and warming rate (Arav 2014), which could be positive for survival of the DOs and CR oocytes compared with COCs.

It is known that removal of cumulus cells shortly before IVF strongly decreases fertilization rates in cattle (Zhang *et al.* 1995, Tanghe *et al.* 2003). During fertilization, cumulus cells attract, trap and select the spermatozoa (Chian *et al.* 1996, Cox *et al.1993*). They are also important to induce sperm capacitation, acrosome reaction and penetration (Chian *et al.* 1996, Cox *et al. 1993*, Fukui 1990, Younis and Brackett 1991) and to prevent zona pellucida hardening (Katska *et al.* , Downs *et al.* 1986). In our study, removal of cumulus cells after maturation decreased the fertilization, cleavage and blastocyst rates, as expected. To solve this problem, we supplemented DOs with intact COCs during the fertilization. In this way, DOs can restore their developmental capability (Luciano *et al.* 2005). Attanasio *et al.*, showed that the cleavage rate of vitrified buffalo oocytes increases when DOs are supplemented with intact COCs, but not the blastocyst rate. The data of the present study showed that cleavage and blastocyst rates can be improved in denuded vitrified bovine oocytes when they are supplemented with intact COCs.

Another way to solve the negative effect of cumulus cells during vitrification and to allow a positive influence during fertilization involves a partial reduction of cumulus cells before vitrification (Papis *et al.* 2013). Therefore, in the present study, we included a CRs group, which represents the corona radiata consisting of a few layers of cumulus cells. We observed that the survival rate of CR oocytes after vitrification was comparable to that in DOs, while the fertilization rate tended to be higher. Furthermore, vitrified CR oocytes showed similar embryo development than COCs. On the other hand, fresh COCs displayed higher fertilization and subsequently embryo development than CR oocytes.

It has been published that the exposition to low temperatures and cryoprotectants can lead to the release of cortical granules in oocytes, which can cause zona hardening (Fuku *et al.* 1995b). In our study, this effect may have been small, as the differences in the fertilization rate between fresh and vitrified DOs supplemented with fresh COCs (DOscocs) were very similar. On the other hand, polyspermic penetration can be related to abnormalities in release and dispersal of cortical granules (Fuku *et al.* 1995a). A proper cortical reaction was probably present, since we could not find significant differences in polyspermic penetration between vitrified and fresh oocytes.

To investigate the effect of cumulus cells during the vitrification of mature oocytes on embryo development, avoiding its confounding effect during fertilization, we performed parthenogenetic activation of vitrified mature oocytes after warming. We observed a lower developmental capacity of the vitrified COCs compared with the CR oocytes. Interestingly, the CR oocytes showed similar blastocyst rates as fresh oocytes. This suggests that partial removal of an excess of cumulus cells supports developmental competence of mature bovine oocytes after vitrification. This result disagrees with the data published by (Dinnyes *et al.* 2000), who found that the development following parthenogenetic activation was reduced in vitrified oocytes compared to fresh oocytes. Although the reduction of cumulus cells layers compromises the fertilization and subsequently embryo development, our parthenogenetic data proved that this was merely due to a reduced fertilization rate since parthenogenetic embryos could reach the blastocyst stage at high rates. Hence, the detrimental effect of cumulus cell removal can be mended by adding COCs during fertilization of vitrified warmed oocytes, as we showed here, or eventually by intracytoplasmic sperm injection (Mavrides and Morroll 2005).

In conclusion, our data indicate that it is advisable to remove at least a part of the cumulus cells before the vitrification of mature bovine oocytes. Denuded oocytes survive vitrification at higher rates than COCs, although their fertilization and subsequent embryo development is compromised

by the absence of cumulus cells. The supplementation of intact COCs can restore the situation, providing DOs with similar fertilization and embryo development rates as in vitrified COCs. On the other hand, although vitrified COCs have a higher fertilization rate, their survival and embryo development is compromised by the presence of cumulus cells. The use of corona radiata oocyte can overcome these two factors, improving the efficiency of vitrified mature bovine oocytes. However, if future research could show why COCs vitrify more poorly than DOs, perhaps the vitrification medium recipe or the procedures could be optimized for COCs to yield best survival combined with best fertilizing and developmental capacity.

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CHAPTER 4

INFLUENCE OF CUMULUS CELLS AND VITRIFICATION PROTOCOL ON THE MATURATION AND BLASTOCYST DEVELOPMENT OF IMMATURE EQUINE OOCYTES

Modified from:

Optimization of a vitrification protocol for equine immature oocytes, resulting in a first live foal.

Ortiz-Escribano N, Bogado Pascottini O, Woelders H, Vandenberghe L, De Schauwer C, Govaere J, Van den Abbeel E, Vullers T, Ververs C, Roels K, Van de Velde M, Van Soom A, Smits K. Equine Veterinary Journal. Under revision.

Summary

The success rate for vitrification of immature equine oocytes is low. Although vitrified-warmed oocytes are capable of growing to maturity, further embryonic development appears to be compromised. The aim of this study was to compare two vitrification protocols, and to examine the effect of the number of layers of cumulus cells surrounding the oocyte during vitrification of immature equine oocytes. Immature equine oocytes were vitrified after a short exposure to high concentrations of cryoprotectants (CPAs) or a long exposure to lower concentrations of CPAs. In Experiment 1, the maturation of oocytes surrounded by multiple layers of cumulus cells (CC oocytes) and oocytes surrounded by only corona radiata cells (CR oocytes) was investigated. In Experiment 2, spindle configuration was determined for CR oocytes vitrified using the two vitrification protocols. Finally, in Experiment 3, further embryonic development was studied after fertilization and culture. Similar nuclear maturation rates were observed for CR oocytes vitrified using the long exposure compared to the non-vitrified controls. Furthermore, a lower maturation rate was obtained for CC oocytes vitrified with the short exposure compared to control CR oocytes ($p < 0.05$). Both vitrification protocols resulted in significantly higher rates of aberrant spindle configuration than the control groups ($p < 0.05$). Blastocyst development only occurred in CR oocytes vitrified using the short exposure, and even though blastocyst rates were significantly lower than in the control group ($p < 0.05$), embryo transfer resulted in a healthy foal. These results indicate that for vitrification of immature equine oocytes, the use of (1) CR oocytes, (2) a high concentration of CPAs and (3) a short exposure time may be key factors for achieving improved blastocyst development and a foal.

INTRODUCTION

Equine assisted reproductive technologies have evolved rapidly during the last decade and the relatively new techniques of cryopreserving immature oocytes may offer further advancement. For clinical application, it would allow postponement of the decision on the choice of stallion for intracytoplasmic sperm injection (ICSI). For research, it could provide a reliable source of immature equine oocytes in countries without access to equine slaughterhouses, such as the United States, assuming that such cells could be transported legally. Moreover, oocyte cryopreservation allows the preservation of genetics from valuable horses and endangered breeds (Smits *et al.* 2012b). However, the overall success rate of this technique in the horse is low. So far, only two papers report on the successful use of vitrified oocytes partially matured *in vivo* (Maclellan *et al.* 2002, Maclellan *et al.* 2010) while pregnancies obtained after fertilization of *in vitro* produced immature vitrified-warmed oocytes retrieved from slaughterhouse ovaries have not been reported yet. In the first study, oocytes were obtained by ovum pick-up after initiation of maturation *in vivo*, cultured in maturation medium for 2–4 h, vitrified-warmed, and then cultured for 10–12 h before subsequent transfer to inseminated mares for *in vivo* fertilization, resulting in two live born foals (Maclellan *et al.* 2002). The same authors also reported four pregnancies obtained from oocytes that were vitrified after initiation of maturation *in vivo*, fertilized by ICSI, *in vitro* cultured and transferred to recipient mares (Maclellan *et al.* 2010).

Vitrification is the most commonly used cryopreservation technique for oocytes. It is characterized by the use of high concentrations of cryoprotectants (CPAs) and the fast cooling rate (Vajta 2013). Oocytes from large domestic species are rich in cytoplasmic lipid droplets causing them to be highly sensitive to chilling (Ledda *et al.* 2001), thus requiring species specific optimization of the exposure time and concentration of CPAs.

Successful vitrification is influenced by different factors that affect oocyte cryotolerance, including the presence of cumulus cells surrounding the oocyte at the time of vitrification. While a protective effect of cumulus cells during vitrification of immature oocytes has been reported (Tharasanit *et al.* 2009, Zhou *et al.* 2010, Purohit *et al.* 2012), other studies show that cumulus cells constitute a tight multilayer barrier that reduces the entry of CPAs into the oocyte, thereby influencing the exchange of water and CPAs (Papis *et al.* 2013). Removing all cumulus cells before vitrification of immature oocytes might result in a lower maturation rate and impaired embryo development (Tanghe *et al.* 2002). Therefore, vitrification of oocytes that are surrounded only by corona radiata (CR) cells has been proposed as a sound alternative in cattle (Papis *et al.* 2013), as the CR cells allow an appropriate

exchange of water and CPAs, while the developmental capacity of the oocyte is sustained (Papis *et al.* 2013, Ortiz-Escribano *et al.* 2016).

The aim of the current study was to improve the vitrification protocol for immature equine oocytes. To this end, the effect of the number of cumulus cells layers (multiple layers of cumulus cells vs. corona radiata only) surrounding immature equine oocytes at the time of vitrification was evaluated, and two vitrification protocols were compared: one with a short exposure to a high CPA concentration, and one with a longer exposure to a lower CPA concentration.

MATERIALS AND METHODS

Media and reagents

Dulbecco´s Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12), Dulbecco´s phosphate buffered saline (DPBS), Tissue Culture Medium-199 with Hanks' salts (TCM-199) and Hoechst 33342 were purchased from Life Technologies Europe. Unless otherwise stated, all other components were obtained from Sigma (Bornem, Belgium).

Maturation medium was composed of DMEM/F12 supplemented with serum replacement (9.4%), epidermal growth factor (0.05 µg/ml), follicle stimulating hormone (9.4 µg/ml), luteinizing hormone (1.88 µg/ml), glutamine (90 µg/ml), ascorbic acid (68 µg/ml), polyvinyl alcohol (23 µg/ml), myoinositol (4.5 µg/ml), Na pyruvate (99.5 µg/ml), insulin (9.5 µg/ml), transferrin (8.6 µg/ml), selenium (10 ng/ml), cysteine (0.094 mg/ml), cysteamine (0.046 mg/ml) and lactic acid (9.4 µl/ml).

Experimental design

In three consecutive experiments, immature equine oocytes were either vitrified with a short exposure to a high CPA concentration (further referred to as short vitrification protocol), or vitrified with a longer exposure to a lower CPA concentration (further referred to as long vitrification protocol), or not vitrified (control). In experiment 1, oocytes surrounded by multiple layers of cumulus cells (further referred to as CC oocytes, Figure 1A) or by corona radiata only (further referred to as CR oocytes, Figure 1B) were used. Fresh and vitrified-warmed CC and CR oocytes were matured *in vitro* and the nuclear maturation was evaluated. In Experiment 2, fresh and vitrified-warmed CR oocytes were matured *in vitro*, and meiotic spindle configuration was assessed. In Experiment 3, fresh and vitrified-warmed CR oocytes were matured *in vitro*, and the developmental competence to

the blastocyst stage was studied after fertilization by ICSI. Blastocysts obtained after vitrification with the short vitrification protocol were transferred on day 9 after ICSI to recipient mares.

Collection of equine immature oocytes

Equine ovaries were obtained from a local slaughterhouse, and transported in an insulated box to the laboratory at room temperature within 1 h. All follicles between 5 and 20 mm were aspirated using a 16-gauge needle attached to a vacuum pump (-100 mm Hg), scraped with the aspirating needle and flushed with TCM199 (Hanks).

Recovered oocytes were classified either as CC oocytes, which were surrounded by multiple layers of cumulus cells (Figure 1A) or as CR oocytes, which were surrounded by the corona radiata only (Figure 1B). Most of the recovered oocytes (more than 2/3) were classified as CC oocytes. To increase the number of CR oocytes, the excess cumulus cells in cumulus compact CC oocytes were removed by repeated pipetting of the oocyte inTCM199 (Hanks). As such, the CR oocytes used in this study were either directly collected from the slaughterhouse ovaries (less than 1/3) or obtained after repeated pipetting of cumulus compact CC oocytes. Due to our collection aspiration technique, we were unable to identify the directly collected CR oocytes as being either expanded or cumulus compact CC oocytes. Therefore, all the collected expanded oocytes were assigned to the CC oocytes in order to obtain a balanced number of expanded and cumulus compact oocytes in both groups. Denuded and partially denuded oocytes were excluded from all experiments.

Recovered oocytes allocated to the control groups were immediately placed in maturation medium, while oocytes in the vitrified groups were first vitrified, then after one week of storage, they were warmed and incubated in maturation medium.

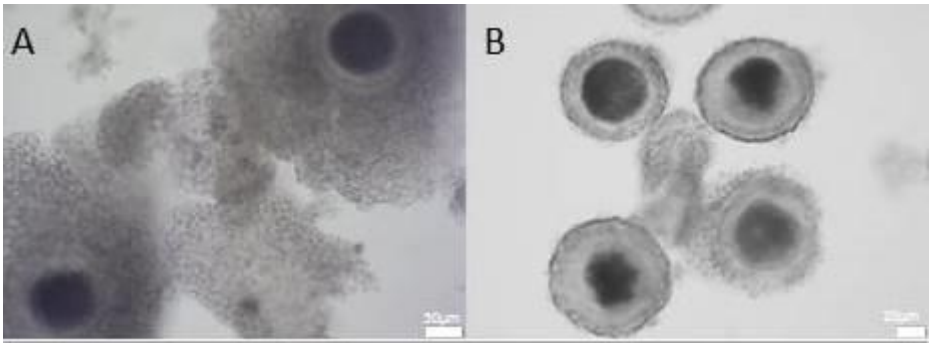

Figure 1. Representative images of oocytes surrounded by multiple layers of cumulus cells (A) and oocytes surrounded by only corona radiata cells (B).

Vitrification and warming

The composition of the vitrification and warming solutions used in the two different protocols is given in Table 1. For both protocols, vitrification and warming steps were performed on a heated plate at 37°C. A custom-adapted device (see details in figure 4 of chapter 1) was used to store the oocytes in liquid nitrogen (LN_2). Oocytes were loaded using a 130 µm pipette in order to minimize the volume surrounding the oocytes. When oocytes were deposited over the surface of the custom-adapted device, the excess medium was removed with the pipette by capillarity.

Protocol with long exposure to low concentration of CPAs

This vitrification protocol was based on that described by Kuwayama *et al.* 2005 with some modifications, and will be referred to as 'long vitrification protocol'. Briefly, four oocytes at a time were placed into one single 75 µL droplet of handling solution (HS_L; long vitrification protocol), consisting of TCM199 (Hanks) supplemented with 20% (v/v) fetal bovine serum (FBS, Greiner Bio-one, Belgium). In order to allow a gradual equilibration of the oocytes, the HS_L droplet, containing the oocytes, was merged with a first 75 µL droplet of equilibration solution (ES) containing HS_L, with 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO). After 2 min, oocytes were transferred to the first ES droplet, which was merged with a second droplet of 75 µL containing ES, and oocytes were left there for 2 min. Next, oocytes were transferred to another droplet of 75 µL containing ES, and incubated for 6 min. Subsequently, oocytes were transferred into four consecutive 50 µl droplets of vitrification solution (VS), consisting of HS_L with 15% (v/v) EG, 15% (v/v) DMSO and 0.5 M sucrose for 60 s in total, and then loaded on a custom-adapted device and plunged into LN_2 within 10–20 s (Table 1).

After one week in LN_2, the custom-adapted device containing the four oocytes was introduced into 4 mL of warming solution (W1) containing HS_L supplemented with 1 M sucrose for 1 min. Next, oocytes were moved to 4 mL of W2 containing HS_L supplemented with 0.5 M sucrose for 3 min, and finally to 4 mL of W3 containing HS_L supplemented with 0.25 M sucrose for 5 min. Finally, oocytes were placed in 4 mL of HS_L, where they were stored until all oocytes were warmed (Table 1).

Protocol with short exposure to high concentration of CPAs

The second method of vitrification was based on the protocol described by Tharasanit *et al.* 2006, with some modifications, and will be referred to as the 'short vitrification protocol'. Four oocytes at a time were placed in one single 100 µL droplet of handling solution (HS_S; short vitrification protocol) containing TCM199 (Hanks) supplemented with 0.014% (w/v) bovine serum albumin (BSA) for 1 min. The oocytes were then transferred to a 100 µL droplet of ES, consisting of HS_s supplemented with 10% (v/v) EG and 10% (v/v) DMSO. After 25 seconds, the oocytes were transferred to a 100 µL droplet of VS containing HS_s supplemented with 20% (v/v) EG, 20% (v/v) DMSO and 0.5 M sucrose. After 15 s, the oocytes were transferred to a custom-adapted device and plunged into LN_2 within 10–20 s.

After one week in LN_2, the custom-adapted device was transferred into 4 mL of W1 containing HS_s supplemented with 0.5 M sucrose where oocytes were cultured for 5 min. Next, oocytes were stored in HS_s until warming of all oocytes was completed (Table 1).

In vitro maturation

A maximum of 40 oocytes at a time were transferred to 500 µL of DMEM/F12 based maturation medium (Smits *et al.* 2010) at 38.5°C in a humidified atmosphere of 5% CO_2 in air for 28 h. For the exact composition of the maturation medium, we refer to the 'Reagents and Media' section. After maturation, oocytes were completely denuded by gentle pipetting in 0.05% (w/v) bovine hyaluronidase diluted in TCM199 (Hanks).

Table1. Composition of vitrification-warming solutions and time of exposure used in the different protocols.

	Long vitrification protocol		**Short vitrification protocol**	
	Solution	**Time**	**Solution**	**Time**
Handling solution (HS)	TCM199Hanks + 20%FBS	1'	TCM199Hanks + 0.014%BSA	1'
Equilibration solution (ES)	HS+ 7.5%EG + 7.5%DMSO	10'	HS + 10%EG + 10%DMSO	25''
Vitrification solution (VS)	HS+15%EG+15%DMSO+ 0.5M sucrose	1'	HS+20%EG+20%DMSO+0.5M sucrose	15''
Warming (W1)	HS + 1M sucrose	1'	HS + 0.5 M sucrose	5'
Warming (W2)	HS + 0.5M sucrose	3'	-	
Warming (W3)	HS + 0.25M sucrose	5'	-	

FBS: Fetal Bovine serum, EG: Ethylene Glycol, DMSO: Dimethyl sulfoxide.

Evaluation of nuclear maturation (experiment 1)

For all six groups (CC and CR oocytes vitrified according to the long vitrification protocol, CC and CR oocytes vitrified according to the short vitrification protocol and fresh control CC and CR oocytes), maturation rates (see above) were determined by nuclear staining with 10 µg/ml Hoechst 33342. Oocytes were visualized and classified as metaphase I (MI, characterized by the presence of highly condensed chromosomes and the absence of the first polar body, figure 2A), metaphase II (MII, characterized by the presence of well-organized chromosomes and the presence of the first polar body, figure 2B) or degenerated (characterized by the absence of chromosomes) by epifluorescence microscopy using a Nikon TE300 inverted microscope with a 20× objective and equipped with a Nikon DS-Ri1 camera (Nikon Benelux, Zaventem, Belgium).

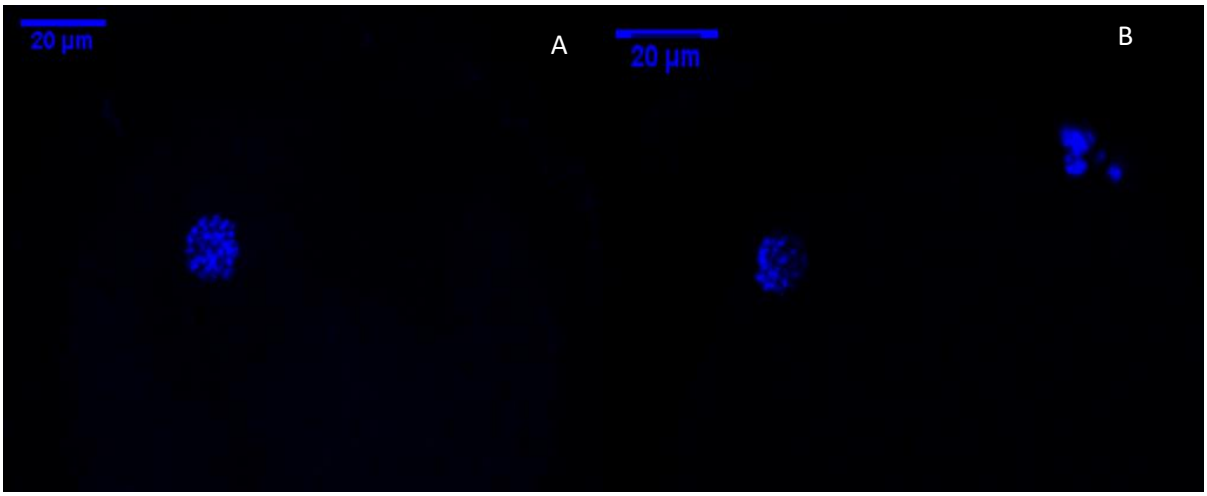

Figure 2. Representative images of an immature (A) and mature oocyte (B) stained with Hoechst.

Spindle status assay (experiment 2)

Oocytes with a visible polar body after *in vitro* maturation (see above) were fixed in 4% (v/v) paraformaldehyde for 25 min, permeabilized with 0.5% (w/v) Triton X-100 for 1 h and blocked with PBS containing 10% (v/v) goat serum and 0.5% (w/v) BSA at 4°C overnight. Subsequently, oocytes were incubated with anti-α-tubulin monoclonal antibody (Molecular Probes, Paisley, UK; 1:200 dilution) overnight at 4°C followed by incubation with anti-mouse IgG antibody Alexa Fluor 488 (Molecular Probes; 1:500) for 1 h at 25°C. Oocytes were counterstained with 10 µg/ml Hoechst 33342 for 10 min and analysed using a Leica TCS-SP8 X confocal microscope (Leica Microsystems, Wetzlar, Germany). Chromosome and microtubule distributions were classified according to Tremoleda et al. (Tremoleda *et al.* 2001). Briefly, the meiotic spindle was defined as normal when it was symmetrically barrel-shaped with the two poles and two equal sets of chromosomes aligned at

its centre (Figure 3A). On the other hand, abnormal spindles showed disorganized, clumped, dispersed or unidentifiable spindle elements (Figure 3B) with chromosome alignment defects (Figure 3C).

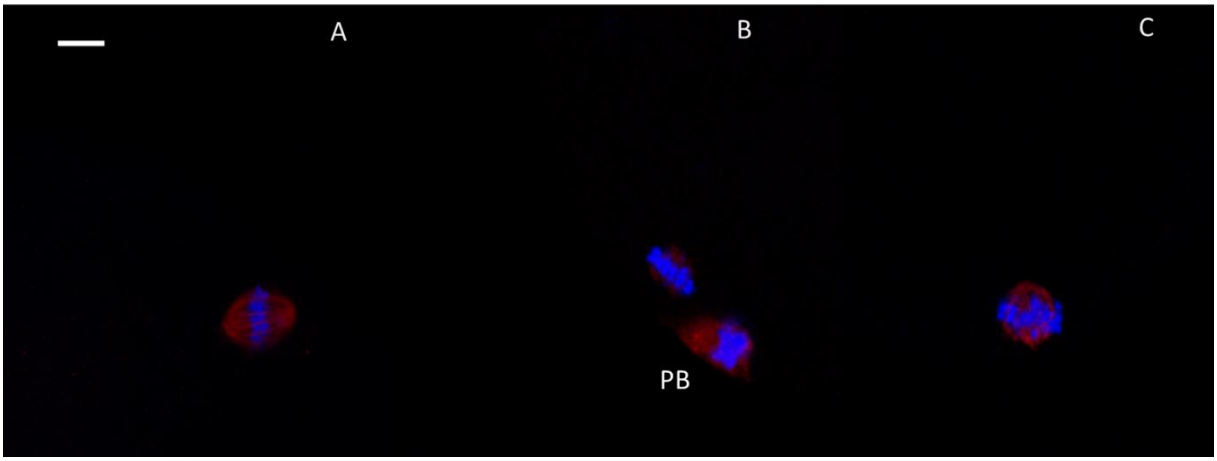

Figure 3. Confocal images illustrating cytoskeleton morphology in vitrified-warmed oocytes. Microtubules are stained in red and chromatin in blue. (A) shows a normal MII oocyte with its typical barrel-shaped metaphase II spindle configuration and the chromosomes perfectly aligned in blue. (B) shows an oocyte with a extruded polar body (PB), smaller spindle and aligned chromosomes.(C) shows oocyte with a disrupted spindle and dispersed chromosomes. Scale bar 10 µm.

Intracytoplasmic injection and embryo culture (experiment 3)

Oocytes showing an extruded polar body after *in vitro* maturation (see above) were fertilized by ICSI as described by Smits et al. (Smits *et al.* 2012a). Semen was collected of one stallion of proven fertility, and spermatozoa were selected using 45%–90% Percoll (GE Healthcare, Belgium) density gradient centrifugation for 40 min at 750 x g at 26°C. After removal of the supernatant, the sperm pellet was washed in 5 ml of Ca^{2+}-free Sperm-TALP (tyrode´s albumin-lactate-pyruvate) using centrifugation for 10 min at 400 x g at 26°C. The supernatant was removed again and the sperm pellet was re-suspended in 300 µl of Ca^{2+}-free Sperm-TALP and kept at room temperature until used for ICSI. Immediately before ICSI, a small volume of sperm suspension was added to the left side of a 5 µl droplet of 9% polyvinylpyrrolidone in PBS and the spermatozoa were allowed to swim out to the right side of the droplet where they were picked up for ICSI.

All manipulations were performed on the heated stage (37°C) of an inverted microscope. A progressively motile spermatozoon was aspirated with its tail first into a blunt piezo pipet of 6 µm (Origio, Vreeland, The Netherlands) and immobilized by applying a few pulses of a piezo drill (Prime Tech, Ibaraki, Japan, speed: 4, intensity: 3) to its tail. Oocytes were held in separate 5 µL droplets of

TCM199 (Hanks) containing 10% (v/v) FBS under mineral oil. The oocyte was fixed by aspiration with a holding pipet with an inner diameter of 15–20 µm (Origio, Vreeland, The Netherlands) keeping the polar body at 12 o'clock or 6 o'clock. The zona pellucida was drilled using the piezo (speed: 4, intensity: 3), a piece of zona was removed and after penetration of the oolemma with the piezo (speed: 3, intensity: 2), the spermatozoon was injected into the cytoplasm of the oocyte. Injected oocytes were cultured in groups of 10 to 20 in 20 µL droplets of DMEM/F12 with 10% (v/v) FBS at 38.2°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Cleavage rate was determined on day 2.5 after fertilization and blastocyst development on day 9 post-fertilization.

Embryo transfer

Blastocysts were washed twice in preheated Emcare Holding Medium (ICPbio Reproduction, USA) and placed in a 2 mL tube filled with preheated Emcare Holding Medium. During transport to the embryo transfer centre (2 h), the tube was kept in 50 mL of preheated PBS in an insulated box. Upon arrival, blastocysts were again washed in Emcare Holding Medium and transferred transvaginally to the uterus of a recipient mare at day 4 or 5 post ovulation.

Statistical analyses

Statistical analyses were conducted using the Statistical Package for the Social Sciences (IBM® SPSS® Statistics 23.0, Chicago, IL). Two-way analysis of variance (ANOVA) was performed in order to evaluate the maturation rate outcome (MI and MII) and the proportion of degenerated oocytes (dependent variables). Fixed effects (independent variables) were classified according to the number of layers of cumulus cells (CC and CR), and the vitrification treatment (control, long vitrification protocol or short vitrification protocol), and their respective first degree interactions. Variables obtained from embryo development analysis, and spindle and chromosome alignment were evaluated using one-way ANOVA. The models included the likelihood of maturation, cleavage, blastocyst rate, spindle and chromosome configuration as dependent variables. The treatment (control, long vitrification protocol and short vitrification protocol) was set as the categorical independent variable. For all outcome variables, the replicates were forced into the model to account for clustering of observations within a replicate. Results are expressed as mean ± standard error of the mean (SEM). For all the models, statistical significance level was set at $p < 0.05$.

RESULTS

Effect of the number of layers of cumulus cells and vitrification protocol on the maturation rate of vitrified equine oocytes (experiment 1).

Equine immature oocytes were used to evaluate the effect of the number of cumulus cell layers (CC vs. CR, Figure 1A–B) and the two vitrification protocols (long protocol vs. short protocol, Table 1) on the maturation rate of vitrified-warmed equine oocytes (Table 2). A strong association was observed between the number of cumulus cell layers and the vitrification treatment on maturation and degeneration rates.

The maturation rate of CR oocytes vitrified with the long vitrification protocol did not differ significantly from control oocytes. However, the maturation rate of CC oocytes vitrified with the short vitrification protocol (25.3%) was lower ($p < 0.05$) compared with control CC oocytes (53.4%), and with control CR oocytes (58.1%). Also the maturation rate of CR oocytes vitrified with the short vitrification protocol (34.4%) was lower ($p<0.05$) compared with control CR oocytes (58.1%). Furthermore, the maturation rate of CR oocytes vitrified with the long vitrification protocol was significantly higher when compared to CC oocytes vitrified with the short vitrification protocol (48.4% vs. 25.3%, $p < 0.05$).

Degeneration rate was numerically higher in vitrified oocytes when compared with control groups, but the differences with the respective control were not significant. However, when comparing CC oocytes vitrified with the short vitrification protocol with CR control, the difference was significant ($p < 0.05$).

No association between the number of cumulus cells layers and treatment was observed when analysing MI. Therefore, MI was analysed as main effect, and a higher MI rate was demonstrated in oocytes vitrified with the short vitrification protocol compare to fresh oocytes ($p < 0.05$).

Table 2. Overview of the *in vitro* maturation rates of control and vitrified immature equine oocytes surrounded by multiple layers of cumulus cells (CC) and by corona radiata cells only (CR) using two different vitrification protocols.

Treatment	Group	n	Undefined	MI(%)		MII(%)	Degenerated (%)
Control	CC	88	13	12(13.6±3.6)		47(53.4±9.7)^{a,b}	16(18.2±3.4)^{a,b}
	CR	86	16	9(10.5±1.9)		50(58.1±4)^{a}	11(12.8±2.2)^{a}
Long vitrification protocol	CC	93	9	20(21±3.4)		32(34.4±4.7)^{b,c}	32(34.4±7.5)^{a,b}
	CR	122	13	16(13.1±2.6)		59(48.4±6.3)^{a,b}	34(27.9±8.5)^{a,b}
Short vitrification protocol	CC	99	12	21(21.2±3.2)	*	25(25.3±4.1)^{c}	41(41.4±6.3)^{b}
	CR	122	20	25(20.5±6.2)		42(34.4±2.5)^{b,c}	35(28.7±5.7)^{a,b}

For MI, main effects were analyzed, and differences were only observed between the treatments. * The control group was significantly different from the group vitrified with the short vitrification protocol ($p<0.05$).^{a,b,c} Groups with different superscripts within the same column are significantly different ($p<0.05$). Data are given as mean percentages ± SEM (five replicates, n=610).

Effect of vitrification on spindle morphology of equine immature oocytes (experiment 2)

We observed fewer oocytes with a normal spindle in vitrified oocytes compared with control oocytes (53.3%–68.4% vs. 81.3%, $p < 0.05$; Figure 4). Furthermore, a lower percentage of a correct alignment of chromosomes was observed in vitrified oocytes compared with control oocytes (66.7%–68.4% vs. 87.5%, $p < 0.05$, Figure 4). However, no significant differences were observed in the spindle morphology and chromosome alignment between vitrified groups (Figure 4).

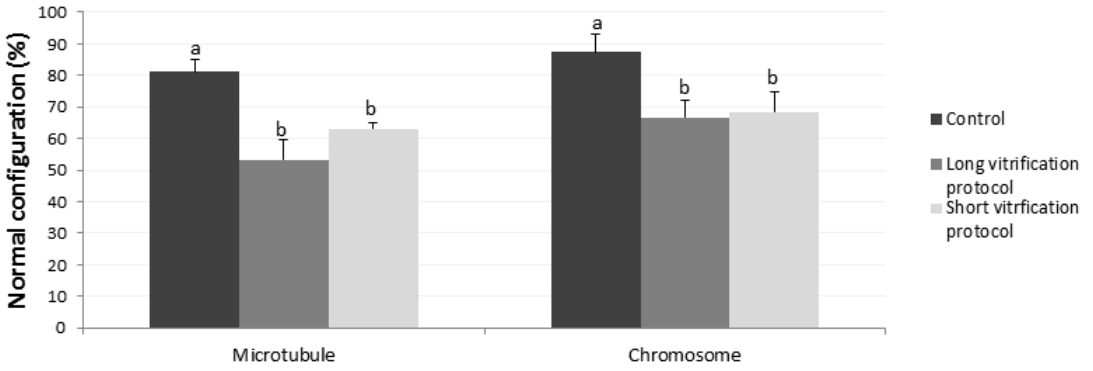

Figure 4. Percentage of normal microtubule and chromosome alignment of control and vitrified oocytes. [a,b,] Groups with different superscripts are significantly different ($p < 0.05$). Three replicates, n = 50.

Effect of vitrification protocol on embryo development of vitrified equine immature oocytes (experiment 3)

In a pilot experiment (n=583), control and vitrified oocytes used in Experiment 1 were fertilized by ICSI and cultured for 9 d. Preliminary results showed that only CR oocytes vitrified with the short vitrification protocol had the potential to develop into a blastocyst (data not shown). According to the results from Experiment 1 and the pilot study, we decided to use only CR oocytes to investigate the effect of both vitrification protocols on embryo development (Table 3). Vitrification significantly decreased the number of cleaved oocytes in both methods ($p < 0.05$). Furthermore, embryo development was significantly impaired after vitrification in both protocols ($p < 0.05$), but only the oocytes vitrified with the short vitrification protocol showed potential to develop into a blastocyst (6.9%, $p<0.05$).

All blastocysts obtained after vitrification of immature oocytes based on the short vitrification protocol were transferred on day 9 after ICSI to recipient mares at day 4 or 5 after ovulation. Initially, two blastocysts were transferred to two mares, but no pregnancies were observed. Next, two blastocysts were transferred to one mare, and ultrasound revealed one embryonic vesicle, though no embryonic heartbeat could be detected at day 21 after transfer, so the pregnancy was lost. After transferring another blastocyst to a recipient mare, an embryonic vesicle was detected 9 days after transfer and a heartbeat was confirmed at 21 days after transfer (20% pregnancy rate). The pregnancy resulted in a healthy male foal, born on May 12th 2017 (Figure 5).

Table 3. Overview of the maturation rate and embryo development of control and vitrified-warmed equine immature oocytes surrounded only by corona radiata cells, using two vitrification protocols (long vitrification protocol vs. short vitrification protocol).

Group	n	MII oocytes (%)	Cleavage (%)	Blastocyst (% of injected oocytes)	Blastocyst (% of cleaved zygotes)
Control	145	80 (54.8±3.7)[a]	61 (76.3±4.2)[a]	16 (20±3.5)[a]	16 (26.2±0.8)[a]
Long vitrification protocol	141	56 (39.7±5)[b]	17 (30.3±3.5)[b]	0[b]	0[b]
Short vitrification protocol	179	72 (40.2±1.7)[b]	30 (41.6±8.9)[b]	5 (6.9±2.6)[c]	5 (16.7±7.7)[a]

[a,b,c] Groups with different superscripts within the same column are significantly different ($p<0.05$). Data are given as mean percentages ± SEM (four replicates, n=465).

DISCUSSION

In this study, we have demonstrated that immature CR equine oocytes can be vitrified successfully using a high concentration of CPAs and a short time of exposure to the equilibration and vitrification solutions. Vitrified oocytes generally showed lower maturation rates compared with control, and the cleavage rate and blastocyst formation were significantly compromised. Nevertheless, we obtained an improved blastocyst development, and to the best of our knowledge, for the first time, a foal after transferring an *in vitro* produced (IVP) blastocyst, which was derived from a vitrified immature equine oocyte.

In a first experiment, we observed that the maturation of vitrified immature equine oocytes was strongly influenced by the number of cumulus cell layers surrounding the oocyte. In horses, a protective effect of cumulus cells during vitrification of immature oocytes has been suggested (Tharasanit *et al.* 2009), but in that study, vitrification of cumulus-intact oocytes was compared with that of denuded oocytes, from which the cumulus cells had been removed completely. As the presence of cumulus cells is indispensable for maturation (Tanghe *et al.* 2002), the low maturation rate observed after vitrification of denuded oocytes might be a consequence of the total absence of cumulus cells, rather than the effect of the vitrification process. Therefore, CR oocytes were used in this study to evaluate the effect of the number of cumulus cell layers, as described previously in cattle (Ortiz-Escribano *et al.* 2016). We observed no significant difference in maturation rate

between control fresh CC and CR oocytes, indicating that corona radiata cells have the capacity to support the oocyte reaching MII as adequately as intact CC oocytes. In our study, the presence of multiple layers of cumulus cells surrounding the oocyte combined with the use of a short vitrification protocol, i.e. less than one min., negatively affected survival and further maturation. We presumed that this effect was related to the layers of cumulus cells surrounding the oocyte, and not to the potential difference between CC and CR oocytes, because both expanded and compact oocytes were equally assigned to CC and CR oocytes. Cumulus cells may impair the movement of CPAs into the oocyte leading potentially to an inappropriate intracellular CPA concentration, as the efflux of water from oocytes occurs quickly, within 20 s, whereas the influx of CPAs takes longer (Jin *et al.* 2011).

Figure 5. A healthy male foal was born at term, here depicted at 3 days of age.

Exposing oocytes to CPAs during vitrification induces osmotic volume changes due to the migration of water and CPAs. In mature oocytes, these volume changes might cause a disruption of the spindle while in immature oocytes, microfilament organization might be disturbed (Joly *et al.* 1992, Heo *et al.* 2011). In our study, vitrification resulted in significantly higher percentages of oocytes with abnormal

spindle structures associated with disorganized microtubules when compared to control oocytes. No significant differences were observed amongst the vitrified groups, even though oocytes vitrified with the short vitrification protocol were exposed to more extreme osmotic changes as a consequence of the higher concentration of CPAs used.

Vitrification of immature oocytes is generally associated with a significant decrease in blastocyst development in mammalian species (Tharasanit *et al.* 2006, Zhou *et al.* 2010, Somfai *et al.* 2014), because cryopreservation can induce a rupture of the oolemma (Arav *et al.* 1996, Men *et al.* 2002) and cytoskeletal disorganization (Saragusty and Arav 2011). In our study, cleavage and blastocyst rates were indeed severely reduced in vitrified oocytes when compared to control oocytes. Although oocytes vitrified with both protocols showed not a significantly different cleavage rate, blastocyst development was only observed in oocytes vitrified with the short vitrification protocol. Almost 7% of the injected oocytes developed into a blastocyst, i.e. 16% of the cleaved embryos.

The short vitrification protocol used in this study, was previously described by Tharasanit et al. (Tharasanit *et al.* 2006). These authors reported 1% blastocyst formation after vitrification of immature equine oocytes. The higher blastocysts rate obtained in this study (7%) may be the result of the two modifications included. Besides the fact that we used CR oocytes, a custom adapted device similar to the cryotop was used as an alternative to the open pulled straw (OPS) that was used in the other study. Using this device, oocytes are loaded in a minimum volume of vitrification solution (<1 µl), resulting in faster cooling-warming rates than with the OPS, which in other species was demonstrated to lead higher cleavage and blastocyst rates (Liu *et al.* 2008, Morato *et al.* 2008, Li *et al.* 2012).

Surprisingly, we observed that CR oocytes vitrified with the long vitrification protocol appeared to have not very strongly reduced maturation rates (no significant difference with control fresh oocytes), but were not able to develop into blastocysts. High blastocyst rates (11%, 1/9) have been recently published by Canesin et al. 2016 using this long protocol for vitrification of equine oocytes. However, these authors used a different device to load the oocytes and different concentrations of sucrose during warming, which may explain in part, the disparate results.

More interestingly, Canesin et al. 2016 reported that the concentration and time of exposure (more than 10 min) to CPAs used in the long vitrification protocol were detrimental for the developmental competence of immature equine oocytes. Indeed, CPA toxicity is considered as the most limiting factor when developing a successful vitrification protocol. The toxicity of penetrating CPAs consistently increases with higher CPA concentrations, higher exposure temperature, and/or longer

exposure time (Szurek and Eroglu 2011). Although higher concentrations of CPAs were used in the equilibration and vitrification solutions of the short vitrification protocol, the very strongly reduced time of exposure could have resulted in the successful cryopreservation, as previously reported in bovine oocytes (Yamada *et al.* 2007).

The ultimate goal of oocyte vitrification is to preserve developmental capacity to the blastocyst stage, resulting in a successful pregnancy and a live foal. Live births have been reported after vitrifying immature bovine (Vieira *et al.* 2008) and porcine (Somfai *et al.* 2014) oocytes; however, as far as we know, no pregnancies or foals born from vitrified immature oocytes after complete IVP have been reported. In the current study, we report a successful equine pregnancy resulting in a healthy foal (Figure 5) which is a major achievement in the field of equine assisted reproduction, and of equine oocyte cryopreservation in particular.

In conclusion, we developed an improved method for the vitrification of immature equine oocytes. Although the blastocyst rate was compromised, blastocyst development using our vitrification protocol was enhanced and did result in a healthy foal. Nevertheless, further research is needed to reduce the ultrastructural spindle alterations observed in vitrified oocytes and, concurrently, to improve normal resumption of meiosis and subsequent blastocyst development.

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CHAPTER 5

INFLUENCE OF OOCYTE MATURATION IN THE PRESENCE OF SERUM ON THE VITRIFICATION OF OOCYTES AND BLASTOCYSTS IN CATTLE

CHAPTER 5.1

EFFECT OF OOCYTE MATURATION IN THE PRESENCE OF SERUM ON THE VITRIFICATION OF MATURE BOVINE OOCYTES

Part of this work was presented at:

COST: Epigenetics and Periconception Environment. Workshop 2014, Las Palmas, Spain.

Serum free maturation medium influences the vitrification of mature bovine oocytes.

Ortiz-Escribano N, Heras S, Maes D, Van den Abbeel E, Van Soom A.

Summary

The presence of serum during oocyte maturation and embryo culture influences the cryotolerance of blastocysts in a negative way. It is also known that bovine embryos produced in serum-free media contain fewer lipids and are more tolerant to vitrification. However, a drawback of such serum-free procedure is that it may induce zona pellucida hardening. The aims of this study were to compare the effect of maturation of oocytes in the presence of serum on vitrification outcome by investigating (1) the possible zona hardening effect in oocytes and (2) fertilization and blastocyst development rate. Collected oocytes were matured in serum-free medium (EGF) or serum containing medium (FBS). For every condition half of the oocytes were vitrified in 15% ethylene glycol, 15% dimethyl sulfoxide and 0.5 M sucrose and the other half were used as control (fresh oocytes). In a first experiment solubility of the zona pellucida was investigated by pronase digestion. We could not observe any increase in the zona digestion time when oocytes were cultured in serum-free medium or when oocytes were vitrified. When fertilization rate was investigated, no differences were observed between oocytes matured in both conditions. However, vitrification decreased the fertilization rate ($p<0.05$). Further studies showed that the cleavage rate of vitrified oocytes was also reduced, but interestingly, maturation in serum led to a significantly lower cleavage rate compared to oocytes matured in a serum-free medium ($p<0.05$). No differences were observed when blastocysts development was analyzed in both conditions, probably due to the fact that vitrification drastically reduced blastocysts formation ($p<0.05$). In conclusion, vitrification impaired the fertilization and development of mature oocytes. Moreover, although zona hardening did not occur and there were not differences in the fertilization rate when oocytes were matured in serum, its presence might compromise the further embryo development.

INTRODUCTION

Vitrification is one of the most promising techniques used in assisted reproductive technology (ART) since ice formation can be avoided by establishing a vitreous state during cooling (Liebermann 2012). In animals, successful oocyte vitrification would allow preserving the genetic material from females that died accidentally, the creation of a gene bank and providing a repeatable supply of oocytes for research. However, while vitrification of embryos has been successfully conducted, oocytes are notoriously difficult to preserve. Oocytes have shown a low cryotolerance to vitrification due to their membrane characteristics, their low surface-to-volume ratio, and their high lipid content, which make them particularly sensitive to chilling injury and intracellular ice formation (Nagashima *et al.* 1994, Pollard and Leibo 1994). During the last decade, considerable efforts have been made in order to increase the cryotolerance of oocytes. One of the important factors linked to the oocyte cryotolerance is their lipid content (Pereira and Marques 2008). In general, farm animals such as cattle and pig, are rich in lipid content compared to human or murine oocytes. Moreover, lipid content can be influenced by the culture medium, and some authors have confirmed that the presence of serum during culture produces a significant increase in the lipid droplets (Abe *et al.* 2002). Fortunately, culture in serum-free medium has shown to reduce the accumulation of cytoplasmic lipid droplets and increased the cryotolerance of bovine blastocysts (Abe and Hoshi 2003, Rizos *et al.* 2003, Gómez *et al.* 2008). Most studies have focused on investigating the cryotolerance of blastocysts, but as far as we know, there is no data about the direct effect of serum-maturation on the vitrification of bovine oocytes. Therefore, the aim of our study was to compare the cryotolerance of oocytes matured in two different conditions (one supplemented with serum, glutamine and pyruvate and the other medium supplemented with Epidermal Growth Factor).

MATERIALS AND METHODS

Media and reagents

Basic Eagle's Medium, Tissue Culture medium (TCM) 199, Minimal Essential Medium non-essential amino acids, kanamycin, and gentamycin were purchased from Life Technologies Europe and all other components were obtained from Sigma (Bornem, Belgium), unless otherwise stated.

Collection and *in vitro* maturation of oocytes

Bovine ovaries were obtained from a local slaughterhouse and rinsed twice in physiological saline supplemented with kanamycin (25 mg/ml). Cumulus-oocyte complexes (COCs) were aspirated from 2-8 mm follicles and matured in groups of 60 in 500 µL of bicarbonate-buffered TCM199 medium supplemented with two different formulations for 22 h at 38.5°C in 5% CO_2 in air. The first formulation (further referred to as FBS condition) was based on serum supplementation with 20% Fetal Bovine serum (FBS, Greiner Bio-one), 50 mg/ml gentamycin, 0.4 mM L-glutamine and 2 mM Na-pyruvate. In the second condition (further referred to as EGF condition), the medium was supplemented with 50 mg/ml gentamycin and 20 ng/ml epidermal growth factor (EGF). From every condition half of the oocytes were used fresh (control EGF and control FBS) and the other half were vitrified-warmed (vitrified EGF and vitrified FBS). Fertilization rate (experiment 1) and blastocyst development (experiment 2) were studied in the different groups.

Vitrification and Warming

Mature oocytes were vitrified as described by Kuwayama *et al.* 2005 with some modifications (Figure 1). The handling solution (HS) used was TCM199/ Hank's/ Hepes supplemented with 20% FBS. All the vitrification media were prepared using this HS. Vitrification and warming steps were performed at 38.5°C on a heated plate.

Vitrification was performed in two steps: equilibration and vitrification. Oocytes were equilibrated by transferring them sequentially in three drops of 75 µl of equilibration solution (ES) composed of HS with 7.5% ethylene glycol (EG) and 7.5% dimethyl-sulfoxide (DMSO). After oocytes regained their original volume, they were subsequently transferred into four consecutive 50 µl drops of vitrification solution (VS) composed of HS with 15% EG, 15% DMSO and 0.5 M sucrose. Oocytes were exposed to equilibration solution for 10-15 min and to vitrification solution for 45-60 sec. Four oocytes were loaded to a manufactured-cryotop and within 5 sec submerged in liquid nitrogen (LN_2). After one week in LN_2, oocytes were warmed by transferring them to a warming solution composed of HS with 1 M of sucrose. This was followed by a three step wash-out of the hyperosmolar sucrose reduced from 1 M to 0.5 M (washing 1, 3 min), 0.25 M (washing 2, 5 min) and 0 M in HS (washing 3, 5 min). Oocytes were washed in HS three times and then incubated in maturation medium for 2 h to allow them to recover.

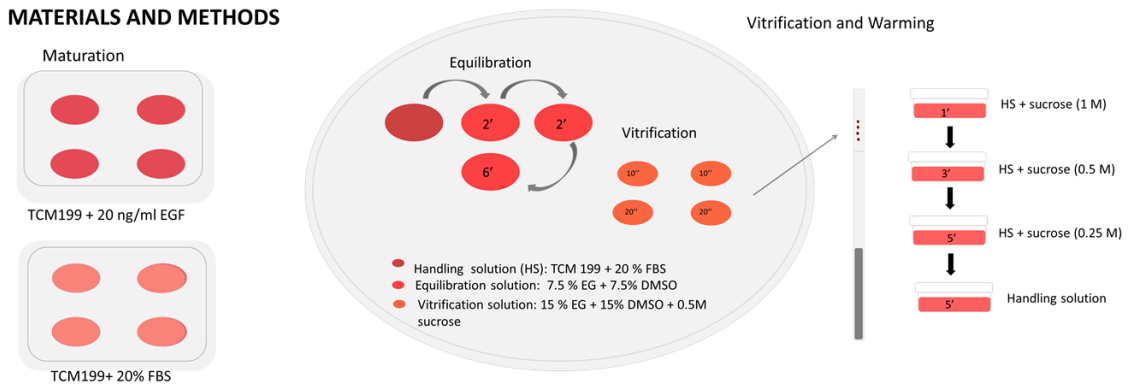

Figure 1. Schematic overview of the in vitro maturation in EGF and FBS conditions and vitrification-warming protocol.

Fertilization and culture

Control (fresh) and vitrified oocytes were fertilized in the same conditions. Frozen-thawed bull spermatozoa were separated using a Percoll gradient (45% and 90%; Pharmacia, GE Healthcare). The final sperm concentration of $1x10^6$spermatozoa/mL was adjusted in IVF Tyrode's albumin-pyruvate–lactate (TALP), consisting of bicarbonate-buffered Tyrode solution, supplemented with BSA (6 mg/ml) and heparin (25 mg/ml). At 21 h post-insemination, presumptive zygotes were vortexed to remove cumulus cells, washed and cultured in groups of 25 in 50 µl droplets of synthetic oviductal fluid medium (SOF) supplemented with ITS (5 µg/ml Insulin + 5 µg/ml Transferrin + 5 ng/ml Selenium) and 0.4% BSA, at 38.5° C in 5% CO_2, 5% O_2 and 90% N_2.

Assessment of fertilization (Experiment 1)

Fertilization rate was determined with the nuclear staining Hoechst 33342 (Molecular Probes, Invitrogen, Merelbeke, Belgium) which selectively binds to double stranded DNA. Presumed zygotes were fixed in 4% formalin and then stained with Hoechst 10 µg/ml for 10 min. The evaluation of the fertilization parameters was performed under a Leica DMR fluorescence microscope (Leica microsystems). Successful fertilization was characterized by the presence of two pronuclei (Figure 2A) and three or more pronuclei were considered as indicative for polyspermy.

Assessment of embryo development (Experiment 2)

After 48h post insemination, the embryo development was based on the cleavage rate (Figure 2B) visualized under stereo microscope. The cleavage rate was calculated based on the number of cultured zygotes. Blastocyst rate (Figure 2C) was determined after 8 days post insemination and calculated based on the number of cultured zygotes as well.

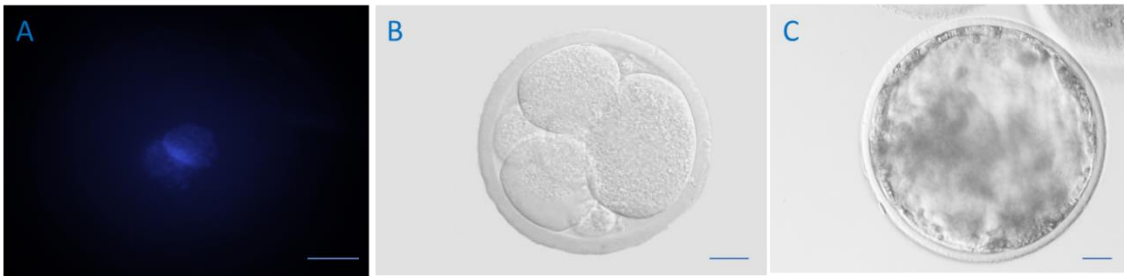

Figure 2. Development after vitrification. A) Fertilized oocyte B) Cleaved embryo and C) Blastocyst. Scale bar 20µm.

Statistical analysis

Statistical analyses were determined using Statistical Package for the Social Sciences (SPSS 23). Binomial variables obtained from the fertilization and embryo development studies were analyzed using a binary logistic regression model. A Bonferroni´s correction was applied to correct for multiple comparisons. The level of statistical significance level was set for $P < 0.05$. All data are expressed as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

The aim of this experiment was to compare the effect of two maturation media (one supplemented with serum, glutamine and pyruvate and other supplemented with EGF) on the cryotolerance of vitrified bovine oocytes. When fertilization rate was assayed, not differences were observed between oocytes matured in the two different media (Figure 3). However, vitrification drastically reduced the fertilization rate in both conditions ($p<0.05$, Figure 3). The low fertilization rate observed might be a consequence of meiotic spindle disorganization that occurs in vitrified mature oocytes (Aman and Parks 1994, Shaw *et al.* 2000, Men *et al.* 2002, Prentice *et al.* 2011).

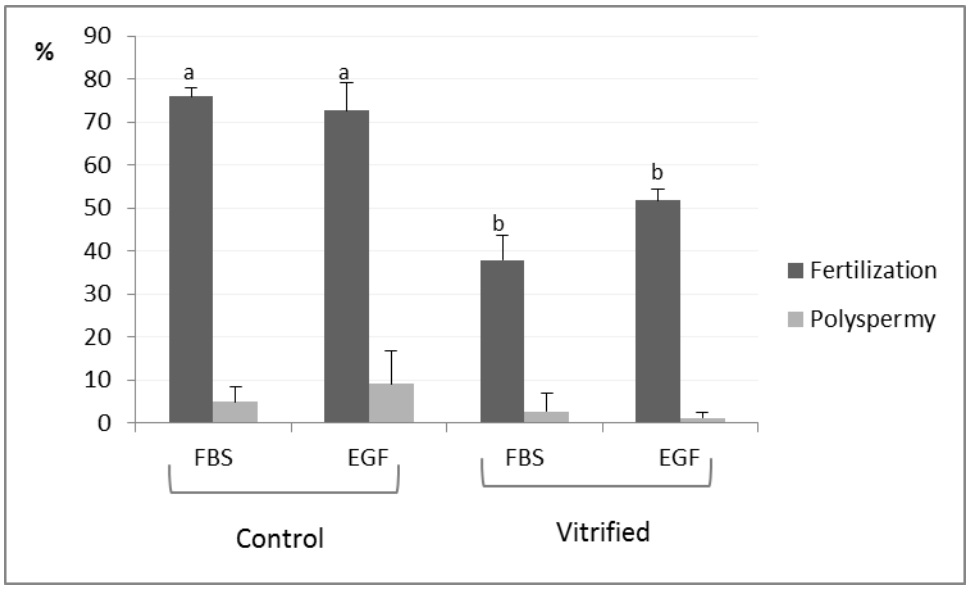

Figure 3. Fertilization and polyspermy rates of control and vitrified oocytes matured in EGF and FBS conditions. [a,b] Different superscripts indicate significant differences between the groups ($p<0.05$), n=359, 3 replicates.

Blastocyst development was further investigated in fresh and vitrified oocytes matured in FBS or EGF conditions, and we observed a reduction in the cleavage rate after vitrification ($p<0.05$, Figure 4). More interestingly, we observed that the presence of serum, glutamine and pyruvate during maturation significantly decreased the cleavage rate of vitrified oocytes ($p<0.05$, Figure 4), whereas it did not have an effect in control oocytes. For us, it was not possible to observe similar effects when blastocyst rate was analysed (Figure 5), because vitrification reduced drastically the blastocyst formation. However, we think that studies on parthenogenetic activation would allow us to gain more insights about the effect of such a component as serum on blastocyst formation.

We previously showed that the presence of certain fatty acids, like palmitic and stearic acid during oocyte maturation could lower cryotolerance of the resulting blastocysts (Shehab-El-Deen *et al.* 2009). Similarly, in this study we observed that the supplementation of serum in combination with glutamine and pyruvate has also a direct effect on the cryotolerance of vitrified oocytes. It has been reported that the serum produces the accumulation of lipids and induce the neosynthesis of tryaglicerides (Abe 2002). Moreover, in our study this effect may have been intensified by the addition of glutamine and pyruvate that serve as a source of energy for the oocyte.

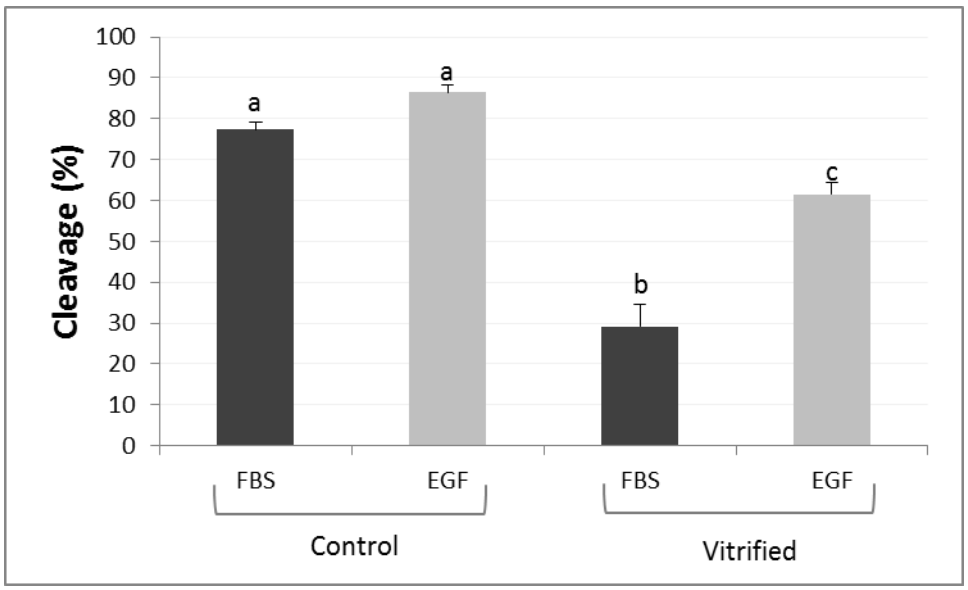

Figure4. Cleavage rate of control and vitrified oocytes matured in EGF and FBS conditions. [a,b,c] Different superscripts indicate significant differences between the groups ($p<0.05$), n=406, 3 replicates.

Although in our study we could not distinguish if the negative effect observed in oocytes matured in FBS condition was only due to the presence of serum or promoted by the presence of glutamine and pyruvate, we showed that oocyte cryotolerance is directly affected by the composition of the maturation medium.

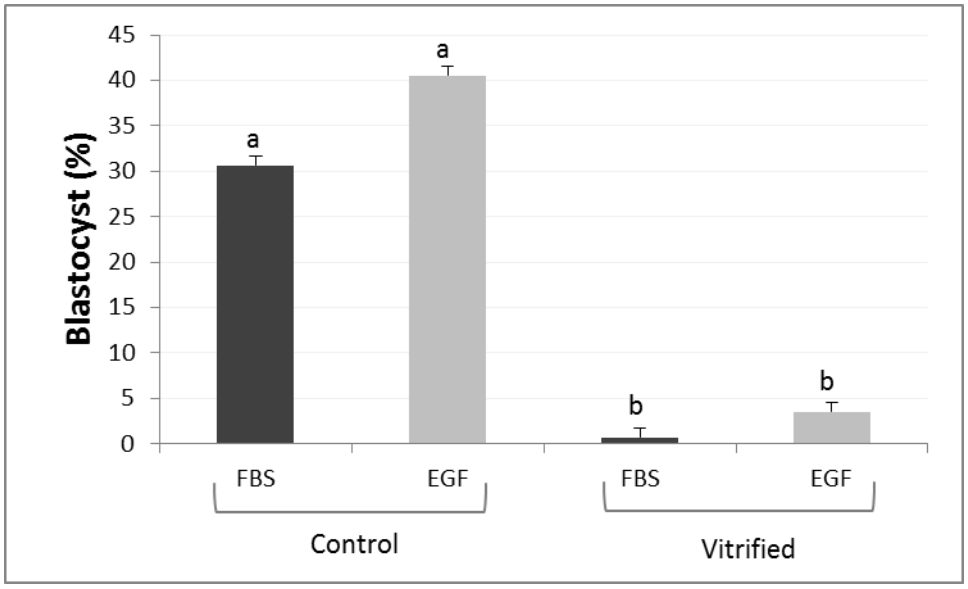

Figure 5. Blastocyst rate of control and vitrified oocytes matured in EGF and FBS conditions. [a,b] Different superscripts indicate significant differences between the groups ($p<0.05$), n=406, 3 replicates.

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CHAPTER 5.2

EFFECT OF OOCYTE MATURATION IN THE PRESENCE OF SERUM AND BLOCKING CONNEXIN CHANNELS ON THE VITRIFICATION OF BOVINE BLASTOCYSTS

Modified from:

Blocking connexin channels improves embryo development of vitrified bovine blastocysts.

Ortiz-Escribano N, Szymańska KJ, Bol M, Vandenberghe L, Decrock E,

Van Poucke M, Peelman L, Van den Abbeel E, Van Soom A and Leybaert L.
Biology of Reproduction 2017, 96 288-301

Summary

Connexins (Cxs) are required for normal embryo development and implantation. They form gap junctions (GJs) connecting the cytoplasm of adjacent cells and hemichannels (HCs), which are normally closed but open in response to stress conditions. Excessive HC opening is detrimental for cell function and may lead to cell death. We found that hatching of in vitro produced bovine embryos, matured in serum-containing conditions, was significantly improved when vitrification/warming was done in the presence of Gap26 that targets GJA1 (Cx43) and GJA4 (Cx37). Further work showed that HCs from blastocysts produced after oocyte maturation in the presence of serum, were open shortly after vitrification/warming and this was prevented by Gap26. Gap26, applied for the exposure times used, inhibited Cx43 and Cx37 HCs while it did not have an effect on GJs. Interestingly, Gap26 had no effect on blastocyst degeneration or cell death. We conclude that blocking HCs protects embryos during vitrification and warming by a functional effect not linked to cell death.

INTRODUCTION

Intercellular communication is of utmost importance during mammalian embryo development, and is established by direct cell-to-cell contact through clusters of channels formed by connexin (Cx) proteins that are present in the plasma membrane (Houghton 2005). Connexins belong to a large family of proteins with 21 isoforms being identified in humans that are named according to their corresponding molecular weight. Each Cx consists of four transmembrane segments, two extracellular loops, one intracellular loop, and an N- and C-terminal ending projecting into the cytoplasm. Six Cx proteins gather into a hemichannel (HC) configuration and two HCs belonging to the membrane of adjacent cells connect and create gap junctions (GJs). Gap junctions serve as direct passageways that allow the movement of ions (e.g. Na^+, K^+, Ca^{2+}), small molecules (e.g. ATP/ADP, glucose, glutamate), and second messengers (e.g. inositol trisphosphate [IP_3], cAMP) between neighboring cells, providing them with a crucial role in the synchronization of cellular functions and tissue homeostasis (Alexander and Goldberg 2003, Saez *et al.* 2003). In contrast to GJs, free HCs (further referred to as HCs) display a low open probability in normal physiological conditions, but they may be activated in response to stimuli that are primarily associated with cellular stress. Triggers that open HCs include a strong depolarization of the membrane potential, a decrease in extracellular Ca^{2+} concentration, an increase in intracellular Ca^{2+} concentration, alterations in the phosphorylation status, oxidative stress, ischemic conditions, exposure to pro-inflammatory cytokines and mechanical stress (Wang *et al.* 2013b). Upon opening, these channels form large poorly selective pores between the intracellular and extracellular environment that mediate the entry or escape of ions (Na^+, Ca^{2+}, K^+), and molecules (NAD^+, ATP, glutamate, prostaglandins, glutathione, IP_3) with a MW generally lower than 1.5-2 kDa (Wang *et al.* 2013a). While several physiological roles have been proposed, most of the current knowledge points to a detrimental role of HCs that may result in cellular dysfunction and may lead to cell death (Decrock *et al.* 2009, Eugenin *et al.* 2012, Xu and Nicholson 2013).

Cryopreservation is widely applied in gametes (oocytes and semen) and embryos for assisted reproductive technologies in animals and humans. In cattle breeding, the cryopreservation of blastocysts allows to optimize embryo transfer by enhancing genetic selection processes and facilitating intercontinental exchange of genetic material. Vitrification is a cryopreservation technique characterized by extremely fast cooling rates inducing solidification without ice crystal formation. Successful vitrification depends on these high cooling and warming rates, and high viscosity of the solutions, achieved by using high concentrations of cryoprotective agents (CPAs), and a small volume of the sample to be vitrified (Arav 2014). However, vitrification is a dynamic process

during which a number of physical (pH, temperature, osmolarity) and chemical factors fluctuate over a wide non-physiological range, which results in extensive cell damage (Kopeika *et al.* 2015). Moreover, it causes physical cell stress due to strong dehydration, rapid cell shrinkage in high osmolarity solutions, and cellular toxicity by the relative high CPA concentrations (Kaur *et al.* 2013).

Successful vitrification is influenced by different factors that affect embryo cryotolerance. It is known that bovine embryos cultured in the presence of serum are less tolerant to vitrification compared to embryos cultured in serum-free medium (Abe *et al.* 2002, Gómez *et al.* 2008). Also oocyte maturation conditions affect embryo freezability, as we have demonstrated earlier, with the addition of palmitic or stearic acid to the maturation medium leading to reduced cryotolerance in bovine blastocysts (Shehab-El-Deen *et al.* 2009). In this study, we compared two different oocyte maturation conditions, one with serum and the other serum free, containing only epidermal growth factor (EGF) as maturation inducing factor, whereas the resulting embryos were cultured in serum-free medium. As such, we hypothesized to obtain embryos with different cryotolerance, with lesser cryotolerance being present in the group matured in the presence of serum.

Adapting oocyte maturation or embryo culture conditions can influence embryo freezability, but fine-tuning technical factors, such as the carrier system, can also increase cryosurvival of embryos. Alternative approaches aim to increase embryo resistance to cryopreservation by applying high hydrostatic pressure (Pribenszky *et al.* 2005, Siqueira Filho *et al.* 2011, Trigal *et al.* 2013), by artificially collapsing the blastocyst (Choi *et al.* 2009, Min *et al.* 2014), by assisted hatching (Kung *et al.* 2003, Hershlag and Feng 2005, Taniyama *et al.* 2011) or by removing cytoplasmic lipid droplets (Nagashima *et al.* 1994, Accorsi *et al.* 2016). Interestingly, attempts have also been undertaken to target specific membrane proteins like aquaporins (AQPs), which form channels that facilitate water movement across the plasma membrane. Thus, overexpression of AQP3 in zebra fish embryos was used to dampen osmotic stress and facilitate the passage of CPAs to enhance cellular tolerance against rapid freezing (Hagedorn *et al.* 2002).

Vitrification is a chemically stressful process that may potentially lead to HC opening. In contrast to AQPs, HCs do not allow the passage of water (Hansen *et al.* 2014), but as pointed out above, they rather act as toxic pores that may impair embryo development. As such, they facilitate ion flow that consequently may impact water flow. This contrasts to the well-known physiological roles of GJs that are essential for embryo and blastocyst development (Houghton 2005). In previous work, we demonstrated that blocking Cx channels with the Cx-targeting peptide Gap27 (which mimics a sequence of the second extracellular loop of the Cx protein), protected human blood vessels against freezing/thawing induced cell death of endothelial and smooth muscle cells (Bol *et al.* 2013). We

here set out to investigate whether blocking Cx channels, in particular HCs, with Gap26, which mimics a sequence of the first extracellular Cx loop, could improve the outcome for bovine blastocysts undergoing the vitrification/warming process.

MATERIALS AND METHODS

Reagents and Media

Culture medium (TCM) 199, basal medium eagle, minimal essential medium, Dulbecco Modified Eagle medium (DMEM), Hanks balanced salt solution (HBSS), TCM199/Hanks/HEPES, RNAse-free PBS, gentamycin, penicillin, streptomycin, fungizone, anti-Cx37 antibody, secondary antibody Alexa 488, fetal bovine serum (FBS) used in cells, propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Life Technologies Europe. Dextran-fluorescein was obtained from Invitrogen. Anti-Cx43 antibody, FBS used in embryo production was obtained from Greiner Bio-One (Belgium), and all other components were obtained from Sigma (St. Louis, MO). The Cx channel inhibitor peptides Gap26 (VCYDKSFPISHVR), Gap26 tagged with fluorescein isothiocyanate (FITC) at its carboxy-terminal end (Gap26-FITC), and Gap19 (KQIEIKKFK) were synthesized by Pepnome Limited (Jida Zhuhai, China) at >90% purity.

Hepes-TALP contained (in mM) NaCl (114), KCl (3.1), NaH_2PO_4 (0.3), $CaCl_2$ (2.1), $MgCl_2$ (0.4), $NaHCO_3$ (2), sodium pyruvate (0.2), sodium lactate (10), HEPES (10) and further supplemented with 10 µg/ml gentamycin sulfate and 3 mg/ml bovine serum albumin (BSA). The final pH of this medium was set at 7.4. Phosphate-buffered saline (PBS) contained (in mM): NaCl (137), KCl (2.68), CaCl2 (0.90), $MgCl_2.6H_2O$ (0.334), KH_2PO_4 (1.47), and $Na_2HPO_4.2H_2O$ (6.46) at a final pH of 7.4. Fert-TALP consisted of HEPES-TALP solution, supplemented with 6 mg/ml BSA and 4 mM $NaHCO_3$. Synthetic oviduct fluid (SOF) consisted of (in mM): myo-inositol (2.8), sodium citrate (0.3), NaCl (107.6), KCl (7.2), KH_2PO_4 (1.2), $MgSO_4$ $5H_2O$ (1.5), Na lactate (7.1), $NaHCO_3$ (28.4), Na pyruvate (0.7), $CaCl_2.2H_2O$ (1.8), glutamine (0.4), and supplemented with 50 µg/ml gentamycine, 0.4 % (W/v) BSA, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium (Wydooghe *et al.* 2014).

The media used during the vitrification were as follows: handling medium (HM) consisting of TCM199/Hanks/HEPES (cat no. 22350-029) supplemented with 20% (v/v) FBS, equilibration solution (ES) consisting of HM supplemented with 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO), and the vitrification solution (VS) consisted of HM supplemented with 15% (v/v) EG, 15% (v/v) DMSO and 0.5 M sucrose.

Divalent-free (DF) ion solution (used for HC dye uptake studies) contained (in mM) NaCl (137), $Na_2HPO_4.2H_2O$ (0.18), KCl (5.36), KH_2PO_4 (0.44), D-glucose (5.55) and HEPES (25). Solution for scrape-

loading and dye transfer (SLDT solution) contained (in mM): NaCl (137), KCl (5.36), $MgCl_2$ (0.81), D-glucose (5.55), HEPES (25) at a final pH of 7.4.

All media were filtered through a sterile 0.22 µm filter (Millipore Corporation, New Bedford, MA) prior to use.

Cells

Wild-type (WT) and HeLa cells stably transfected with murine GJA1 (further referred to as Cx43) and GJA4 (further referred to as Cx37) were used for HC dye uptake and SLDT studies, and were kindly provided by Dr. Klaus Willecke (Molecular Genetics and Cell Biology, University of Bonn, Bonn, Germany). Cells were grown in DMEM supplemented with 10% (v/v) FBS, 10 U/ml penicillin, 10µg/ml streptomycin and 2.5 µg/ml fungizone in 10% CO_2 at 37°C. HeLa-Cx43 culture medium additionally contained puromycin (1µg/ml).

Embryo production

In vitro produced bovine blastocysts were derived from immature oocytes that were collected from slaughterhouse ovaries (Figure 1). Cumulus oocytes complexes (COCs) were recovered from follicles with a diameter of 2-8 mm using an 18 gauge needle attached to a 10 ml syringe. Oocytes with homogeneous dark cytoplasm and compact cumulus cells were selected, and matured in groups of 60 in 500 µL of bicarbonate-buffered TCM199 medium supplemented with two different formulations. The first formulation was based on serum supplementation with 20% (v/v) FBS, 50 mg/ml gentamycin, 0.4 mM L-glutamine and 2 mM Na-pyruvate; this condition is further referred to as the FBS condition. In the second, serum-free condition, further referred to as EGF condition, the medium was supplemented with 50 mg/ml gentamycin and 20 ng/ml EGF. In both conditions, oocytes were matured for 22 h at 38.5°C in 5% CO_2 in air. For fertilization, frozen-thawed bovine sperm was separated over a Percoll gradient (45% and 90%; GE Healthcare), washed, and diluted in Fert-TALP to a final sperm concentration of $1x10^6$ spermatozoa/ml. The matured COCs were washed in 500 ml Fert-TALP and co-incubated with sperm for 21 h. After fertilization, presumptive zygotes were first vortexed to remove the excess of the sperm and cumulus cells, and then cultured in groups of 25 in 50 µl droplets of SOF medium (Wydooghe *et al.* 2014) at 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2.

Blastocyst vitrification, storage, and warming

In vitro produced bovine blastocysts were vitrified on days 7 and 8 post insemination with a slightly modified protocol published earlier (Kuwayama *et al.* 2005). Vitrification was performed at room temperature (RT, 22-26°C) in two steps: an equilibration step and a vitrification step. For equilibration, blastocysts were set in 70 µl of HM solution, and this droplet was merged with a second 70 µl droplet also containing ES (for HM and ES composition see the section 'Reagents and media'). After 2 min, blastocysts were transferred to a second and third 70 µL ES droplet (each 2 min) and then kept for 6 min in a fourth ES droplet (Figure 1). The vitrification steps included sequential transfer to five 50 µl droplets of VS composition (see the section 'Reagents and media'). Incubation in the last droplet was 30 s, and this droplet contained Gap26 (200 µM) in the Gap26 treatment condition. Finally, two to three blastocysts surrounded by a small volume (< 1 µl) were placed in a custom-adapted device and then transferred to liquid nitrogen (LN_2). The custom-adapted device consisted of a 0.25 mL straw, with the end was cut, creating a surface that allows loading blastocysts with a minimal volume (<1 µL). At the opposite end, a metal wire was inserted to avoid floating of the device in LN2 (Figure 1).

After one week of storage in LN_2, the custom-adapted device containing blastocysts was warmed by transferring it quickly into a 38.5°C warm HM solution supplemented with 1 M sucrose, and the blastocysts were further incubated for 1 min. This was followed by a three steps sucrose wash-out procedure (3, 5, and 5 min). In the Gap26 treatment condition, the peptide concentration was gradually diluted, halving the concentration with each transfer step: 200 µM; 100 µM, 50 µM (Figure 1). Hereafter, blastocysts were washed three times in HM and then cultured individually in 20 µl SOF droplets overlaid with mineral oil at 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2. Blastocyst staging was determined after 1 and 2 days of individual culture, and classified as non-hatching, hatching, hatched and degenerated (Figure 5A). For the non-vitrified controls, blastocysts were selected at day 7 and 8 and individually transferred to 20 µl of SOF droplets at the same conditions that vitrified groups.

Non-vitrified blastocysts produced after oocyte maturation in EGF or FBS conditions were cultured with Gap 26 dissolved in HM at the same concentration, and for the same duration of exposure as vitrified ones. Then, blastocysts were cultured individually in 20 µL of SOF droplets and further development (to hatching) was determined after 1 and 2 days of individual culture.

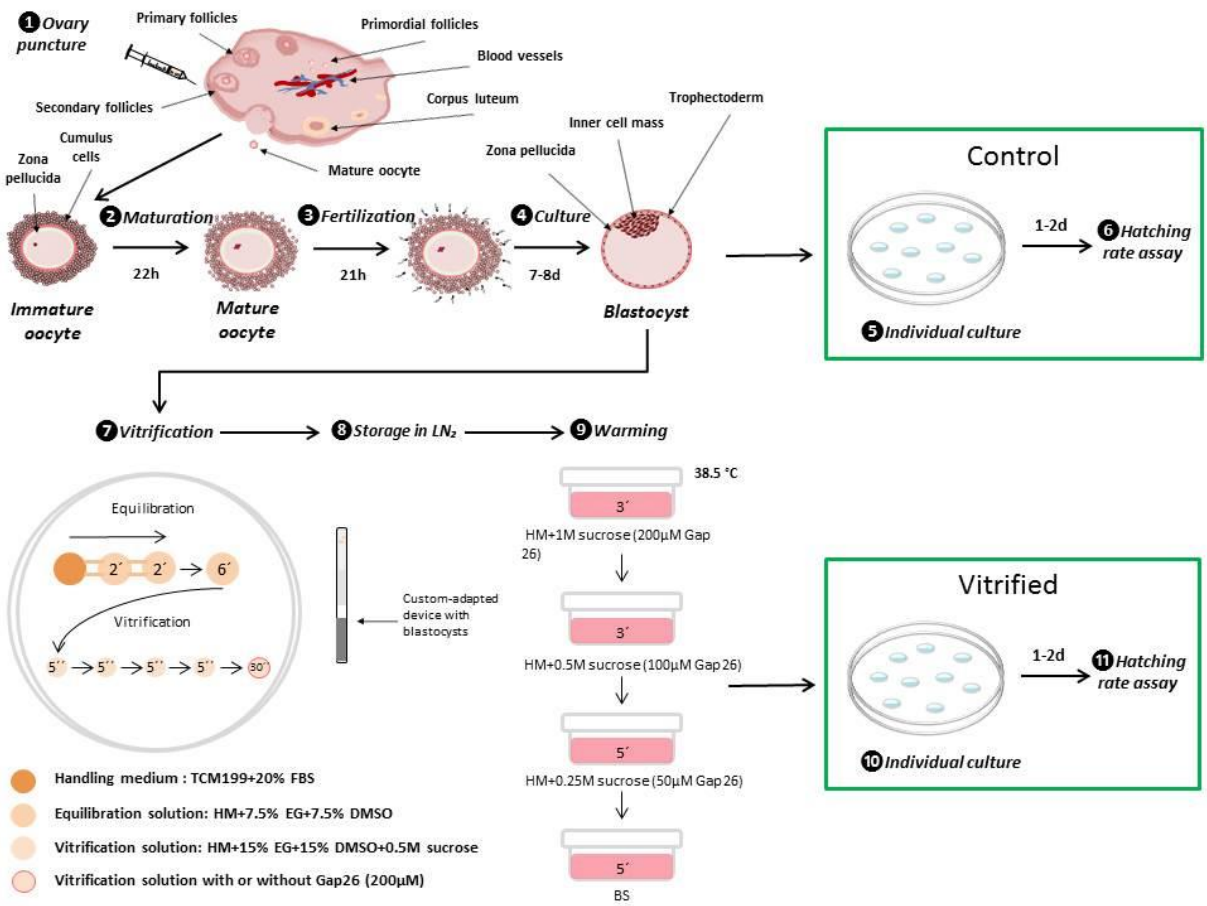

Figure 1. Schematic overview of in vitro production of bovine embryos and subsequent treatment. (1) Slaughterhouse ovaries were punctured to obtain immature oocytes. **(2)** Immature oocytes were matured in EGF or FBS medium for 22 hours. **(3)** Oocytes were co-incubated with spermatozoa for 21 hours and **(4)** subsequently cultured in SOF for 7-8 days (group culture). Normal and expanded blastocysts were randomly divided into control and vitrified groups. **(5)** Control blastocysts were immediately cultured in individual drops, and vitrified blastocysts were first vitrified-warmed and then cultured in the same condition as the control group. **(6)** Hatching/hatched rates were assessed after one and two days of individual culture. **(7)** For vitrification, blastocysts were exposed to equilibration solution for a period of 10 minutes and then to vitrification solution for 20 sec (4 x 5 sec) plus 30 sec (last droplet). In the treated group, Gap26 was added in the last droplet. **(8)** Two or three embryos were loaded into a custom-adapted device in a small volume (< 1µl) of vitrification solution and stored in liquid nitrogen (LN_2) for one week. **(9)** Blastocysts were warmed and washed in decreasing concentrations of sucrose. Exposure to Gap26 was also gradually decreased. **(10)** Vitrified blastocysts were cultured in individual drops and **(11)** hatching/hatched rates were assessed as for the controls **(6)**. All steps were performed at room temperature except the first warming step which was performed on a heated plate at 38.5°C.

Reverse transcription polymerase chain reaction

Blastocysts were produced as described above, either after oocyte maturation in FBS or in EGF medium. Twenty blastocysts from each condition were washed three times in 3 ml of RNAse-free PBS, transferred to 20 µl of lysis buffer consisting of 5 mM DTT (Dithiothreitol; Promega, The Netherlands), 4 U/µL RNasinPlus RNase inhibitor (Promega), and 0.64 µM Igepal in RNase free water, and immediately stored at −80 °C. RNA was extracted using the RNeasy Micro kit (Qiagen, Belgium) following the manufacturer´s instructions, including a genomic DNA removal step. The extracted RNA was dissolved in 14 µL RNase-free water. A minus RT control was performed with GAPDH primers (119pb) (sense) 5'-TTCAACGGCACAGTCAAGG-3' and (antisense) 5'-ACATACTCAGCACCAGCATCAC-3' to check for contamination of genomic DNA (Goossens *et al.* 2005). Reverse transcription was performed with oligo-dT primers on the total amount of RNA using the iScriptcDNA synthesis kit (BioRad, Belgium) according to the manufacturer's instructions and cDNA was diluted 2.5-fold for downstream PCR.

The oligonucleotide sequences to amplify the GJA1 (Cx43) used were (sense) 5'-TGGCATTGAAGAGCACGGCA-3' (anti-sense) 5'-TCAGCAAGAAGGCCACCTCGA-3'. For GJA4 (Cx37) the following sequences were used (sense) 5'-AGCCCGTGTTTGTGTGCCAG-3' and (antisense) 5'-ACCAGGGAGATGAGTCCGACCA-3'. The primers were designed using Primers3Plus (Untergasser *et al.* 2007) based on their conserved bovine mRNA sequences (GJA1; Acc. No.: NM_174068.2, 104 bp and GJA4; Acc. No.: NM_001083738.1, 121bp), avoiding secondary structures as indicated by MFold (Zucker 2003). BiSearch software was used to confirm specificity (Arányi *et al.* 2006).

All PCR reactions were performed in a volume of 10 µl containing 0.5 U FastStartTaq DNA Polymerase and 1 µl 10x reaction buffer (Roche, Belgium), 200 µM dNTPs (Bioline Reagents, UK), 500 nM of each primer (IDT, Belgium) and 2 µl cDNA. The PCR program consisted of initial denaturation at 95°C for 4 min, followed by 40 cycles of 20 s at 95°C, 20 s at 64°C and 40 s at 72°C, with a final 2 min elongation at 72°C. The PCR products were verified by electrophoresis.

Immunofluorescence microscopy

In vitro produced blastocysts derived from EGF or FBS maturation conditions were fixed in 4% paraformaldehyde (PFA) for 20 min at RT. Blastocysts were permeabilized with 0.5 % Triton X-100 for 1 h and blocked with a solution consisting of 10% goat serum and 0.5% BSA prepared in PBS, at 4°C overnight. Blastocysts were then incubated with primary antibody, anti-Cx43 polyclonal AB (1:500) or anti-Cx37 polyclonal (1:250) diluted in blocking solution at 4°C overnight. Blastocysts were rinsed three times with PBS and incubated for 1 h at RT, with the appropriate secondary antibody conjugated with Alexa-488 (1:500). Next, blastocysts were rinsed three times in PBS, counterstained with DAPI 10 µg/ml for 10 min and mounted with DABCO mounting medium. Images were obtained using a Nikon C1si confocal microscope (Nikon BeLux, Brussels, Belgium), with a Plan Apo VC 20x-63x oil immersion objective (Nikon).

Gap26-fluorescein isothiocyanate uptake measurements

For blastocyst Gap26-FITC uptake measurements, blastocysts were incubated with Gap26-FITC (200 µM) for 1 h at RT. Next, blastocysts were counterstained with DAPI 10 µg/ml for 10 min, mounted with DABCO mounting medium, and visualized with a Leica TCS-SP8 X confocal microscope (Leica Microsystems, Wetzlar, Germany) with a 63x1 water immersion objective with 1.2 NA.

Hemichannel dye uptake studies

Hemichannel dye uptake studies were performed on HeLa cells and blastocysts. For HeLa cells, cells were seeded at 40,000 cells per cm² density in cell culture treated, polystyrene, 4-well culture dishes (SPL Life Sciences, Korea); the assay was performed the next day. Cultures were rinsed once in HBSS followed by three times in DF solution (see the section ‘reagents and media’). Cells were subsequently incubated for 10 min in DF solution containing the HC-permeable dye PI (668.4 Da; 1 mM), after which cells were washed three times with HBSS, fixed for 15 min with 4% PFA and stained with 1 µg/ml DAPI. All manipulations were carried out at RT. Images were acquired with a Nikon TE300 epifluorescence microscope equipped with a 10× objective (Plan APO, NA 0.45; Nikon), and a Nikon DS-Ri1 camera (Nikon Belux, Zaventem, Belgium). In each culture, nine images were taken at fixed locations across the well. The number of PI-positive cells was determined using ImageJ version 1.48 (plugin: Analyze Particles) after applying a threshold that corresponds to the upper level of the background signal, and was expressed relative to the total number of DAPI-positive nuclei.

For blastocyst dye uptake studies, blastocysts were washed three times in HEPES-TALP medium and subsequently incubated for 25 min at RT with PI solution (1 mM). PI is also taken up by dead cells, and we therefore performed additional studies with a HC-impermeable dextran-fluorescein dye (10 kDa; 200 µM). After 25 min, blastocysts were washed in HEPES-TALP and fixed for 25 min in 4% PFA. Nuclei were counterstained with DAPI 10 µg/ml for 10 min and mounted with DABCO mounting medium. Three dimensional images were reconstructed from the blastocyst after acquiring Z-stack images using a Leica TCS-SP8 X confocal microscope (Leica Microsystems, Wetzlar, Germany). The number of PI-positive and dextran-negative cells was expressed relative to the total number of cells per blastocyst. The number of PI-positive cells was determined using ImageJ version 1.48 (plugin: Analyze, Cell Counter) and was expressed relative to the total number of DAPI-positive nuclei.

Scrape-loading and dye transfer studies

SLDT studies were done on confluent HeLa cell cultures seeded on four-well dishes. Cultures were rinsed two times with SLDT solution and then exposed for 1 min to SLDT buffer containing the GJ-permeable dye 6-carboxy fluorescein (6-CF, 0.4mM, Life Technologies), after which a linear scratch was made through the cell culture with a syringe needle. After 1 min, cells were washed with HBSS-HEPES and left for 15 min in the dark to allow the dye to spread to neighboring cells. Sixteen images were acquired from each well, along the right and left side of the scrape with a Nikon TE300 epifluorescence microscope equipped with a 10× objective (Plan APO, NA 0.45; Nikon) and a Nikon DS-Ri1 camera. Gap junctions communication was quantified by fitting the fluorescence diffusion profile to a mono-exponentially decaying function. A spatial constant of dye spread reflecting the degree of GJ coupling was determined with GraphPad Prism 6 (GraphPad Software, San Diego, CA).

Blastocyst cell death studies

For cell death assays, blastocysts were fixed in neutral buffered 4% PFA, and then permeabilized with 0.5% Triton X-100 at RT for 5 min. Cell death was detected by in situ terminal deoxynucleotidyltransferase (TdT)-mediated deoxyuridine triphosphate nick end-labeling (TUNEL), using a commercial In Situ Cell Death Detection Kit (Roche, Belgium). Blastocysts were incubated with the TUNEL reaction mixture for 1 h at 37 °C. Blastocysts treated with DNase for 10 min were used as a positive control, and blastocysts not exposed to TdT enzyme as a negative control. Thereafter, blastocysts were rinsed three times in PBS and stained with DAPI 10 µg/ml for 10 min. Slides were examined by epifluorescence microscopy using a Nikon TE300 inverted microscope with a x10 objective and equipped with a Nikon DS-Ri1 camera (Nikon Belux, Zaventem, Belgium).

Statistical analysis

Statistical analysis was determined using Statistical Package for the Social Sciences (SPSS 23). Binomial variables obtained from the embryo development studies were analyzed using a binary logistic regression model. A Bonferroni´s correction was applied to correct for multiple comparisons. In order to compare the means between groups an ANOVA test was performed (cell death and dye assay studies). A non-parametric, Kruskal-Wallis test was used to analyze dye uptake in blastocysts, since the results were not normally distributed. The level of statistical significance level was set for $P < 0.05$. All data are expressed as mean ± standard error of the mean (SEM).The number of blastocysts used in each experiment and condition, as well as the number of replicates is given in Table 1.

Table 1. Overview of the number of blastocysts and replicates used for the experiments in this study.

Experiment	Condition	Number of blastocysts	Number of replicates
Imunofluorescence	EGF	8	3
	FBS	12	
RT-PCR	EGF	60	3
	FBS	60	
Scrape-loading Transfer study	-	-	4-5
HC dye uptake study in cells	HeLa Cx43	-	6
	HeLa Cx37		7
Blastocyst vitrification	EGF	504	10
	FBS	367	8
Blastocyst HC dye uptake study	EGF	118	3
	FBS	137	
Blastocyst cell death study	EGF	92	4
	FBS	105	

RESULTS

Characterization of connexin expression in bovine blastocysts

We used in vitro bovine blastocysts derived from oocytes matured under FBS or EGF conditions in order to study the Cx expression at mRNA and protein level. We found evidence for Cx37 and Cx43 at the messenger RNA level in the blastocysts derived from both FBS and EGF conditions (Figure 2A). Immunohistochemical analysis further confirmed the presence of both Cxs at the protein level (Figure 2B).

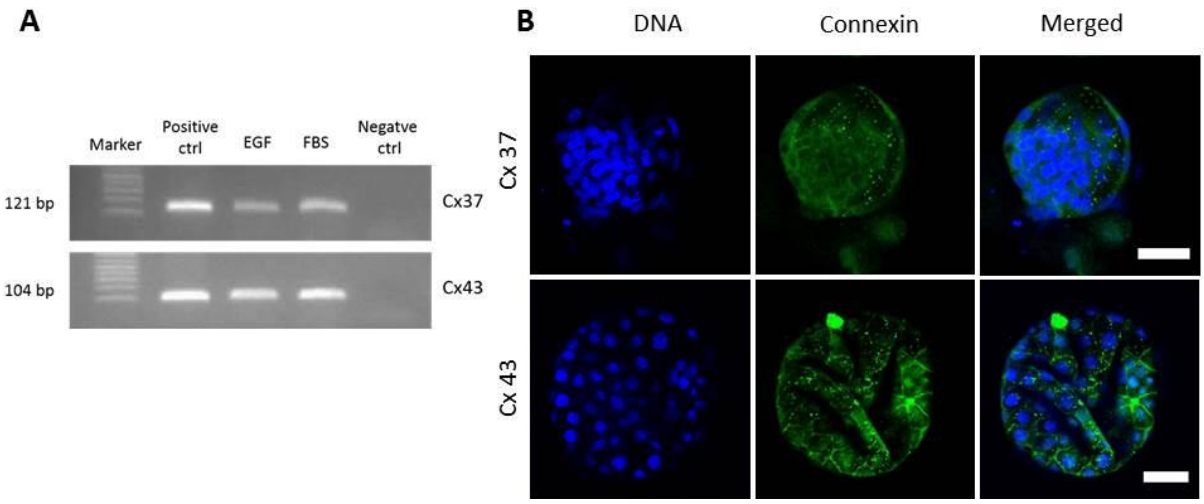

Figure 2. Expression and localization of Cx37 and Cx43 in in vitro produced bovine blastocysts. **A.** PCR amplifications of Cx37 and Cx43 from cDNAs sample showing that Cx37 and Cx43 are expressed in in vitro produced blastocysts which were matured in EGF or FBS conditions. Genomic DNA was used as positive control. **B.** Confocal images of connexin protein localization in bovine blastocysts (FBS condition) shown in green with nuclear DAPI staining in blue. Representative images for both FBS and EGF conditions. Scale bar 50 µm.

Gap26 effects on Cx43 and Cx37 channels

We tested the effect of Gap26 on the function of channels composed of the two Cxs found in the blastocysts. To this end, we used HeLa cells stably transfected with Cx43 and Cx37 (HeLa-Cx43 and HeLa-Cx37 cells respectively). We first investigated the effect on GJ channels, making use of SLDT assays with the GJ-permeable low molecular weight dye 6-CF (376 Da, charge:-2). Dye spread to neighboring cells was observed in HeLa-Cx43 cells and was absent in HeLa WT cells. The presence of Gap26 (200 µM; 1 h pre-incubation and inclusion of the peptide during the 16 min of the SLDT procedure) did not influence GJ-mediated dye transfer (Figure 3). By contrast, 24 h pre-incubation did significantly inhibit dye transfer, as previously observed with Gap27 (Decrock *et al.* 2009) . We did not test Gap26 effects on Cx37 GJs because HeLa-Cx37 cells showed little dye spread with 6-CF, as reported by others (Weber *et al.* 2004, Kameritsch *et al.* 2005, Ek-Vitorin and Burt 2013). Exposure of the blastocysts to Gap26 in the vitrification/warming experiments described below was ~10 min (see Figure 1). As 1 h and 16 min Gap26 incubation did not affect GJ coupling in HeLa-Cx43, we can confidently conclude that shorter Gap26 exposure times will not have any effect on coupling.

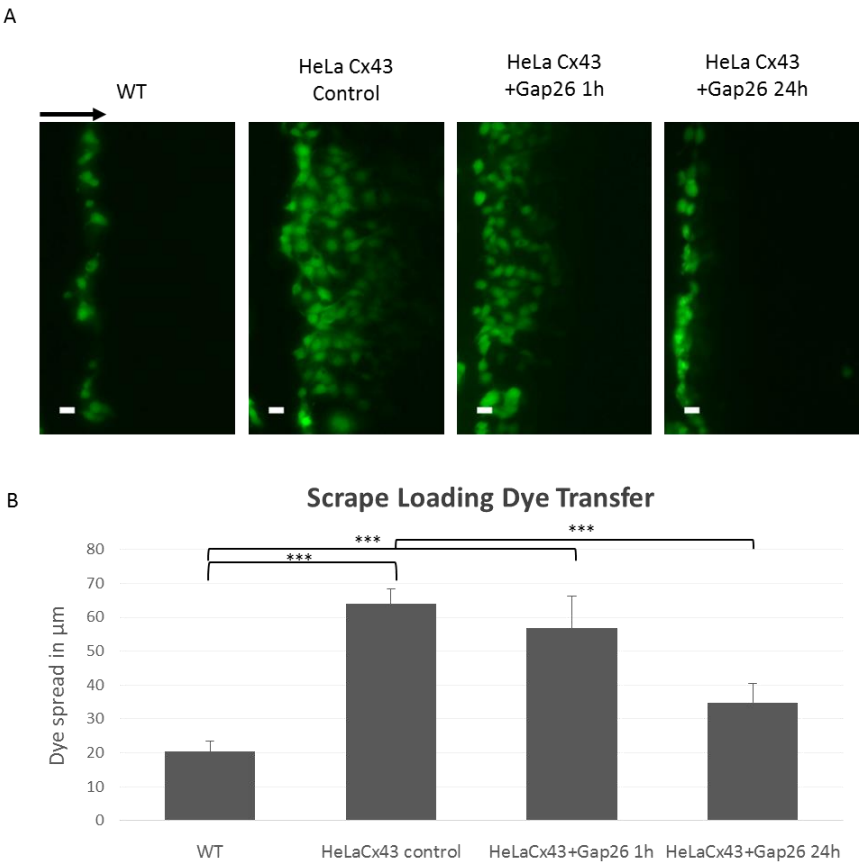

Figure 3. Effect of Gap26 on GJ coupling in HeLa-Cx43 cells measured with SLDT. **A.** Representative example images showing no dye (6-CF) spread in HeLa WT, dye spread over several cell layers in HeLa-Cx43 (arrow shows direction of dye spread from the scrape), no effect on dye spread of 1 h exposure to Gap26 and reduction of dye spread with 24 h Gap26 exposure. Scale bar 25 µm. **B.** Summary data after quantification of experiments as illustrated in A, demonstrating that only 24 h incubations with Gap26 inhibit dye spread but not 1 h incubation. Dye spread is expressed as the spatial constant of exponential decrease of 6-CF fluorescence intensity. n=4-5, *** P<0.001

We next tested the effect of Gap26 on HCs based on dye uptake studies with the HC-permeable dye PI (MW 668 Da). Exposure of HeLa-Cx43 cells to a nominally Ca^{2+}- and Mg^{2+}-DF solution that triggers HC opening, induced cellular PI uptake that was significantly inhibited by Gap26 (200 µM; 1 min pre-incubation and inclusion of the peptide during the 10 min of DF stimulation, Figure 4A). Gap19 (200 µM), a specific blocker of HCs composed of Cx43 (Wang *et al.* 2013c), also inhibited dye uptake in an equally strong way. Non-specific dye uptake via larger pores or disrupted membranes was determined in dye uptake experiments with 10 kDa Dextran FITC, which showed one or two cells per inspected field (0.3604 µm²); because of the very low counts, these were not further quantified. We next performed PI uptake studies in HeLa-Cx37 cells and found that DF-triggered dye uptake was, as observed for Cx43, also significantly inhibited by Gap26 (Figure 4B).

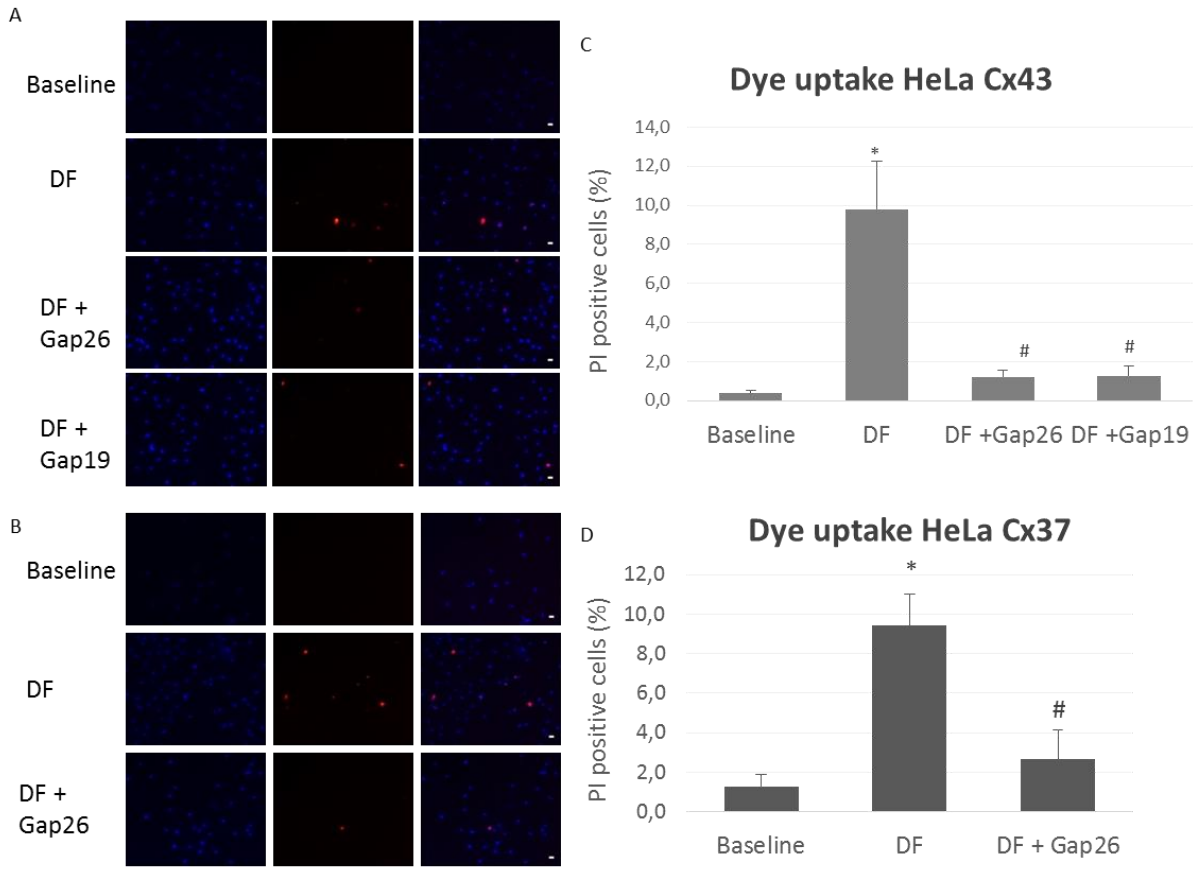

Figure 4. Effect of Gap26 on HC dye uptake in HeLa-Cx43 and HeLa-Cx37 cells. **A-B.** Representative images of PI dye uptake experiments in HeLa-Cx43 and HeLa-Cx37 cells. PI is red; blue represents nuclear DAPI staining. Scale bar 25µm. **C-D.** Summary data of experiments as illustrated in A-B, demonstrating significant Gap26 inhibition of PI uptake in both HeLa-Cx43 (n=6) and HeLa-Cx37 (n=7). *comparison to baseline, P<0.05; # comparison to DF, P<0.05.

Effects of Gap26 treatment on embryo development of vitrified/warmed blastocysts

In vitro produced blastocysts were collected and vitrified on day 7 and 8 post-insemination (see Figure 5A for the blastocyst formation rate from both conditions). At day 7, blastocysts and expanded blastocysts of code 1 (excellent and good according to IETS criteria 1998), were selected for this study. Embryos of this quality have a symmetrical and spherical mass with individual blastomeres that are uniform in size, color and density (Bo and Mapletoft 2013). Early blastocysts were kept an additional day in culture and collected on day 8. Overall, less than 2% finally remained at the early blastocyst stage (see Figure 5B for the percentages of early blastocysts, blastocysts and expanded blastocysts obtained at day 7 and 8).

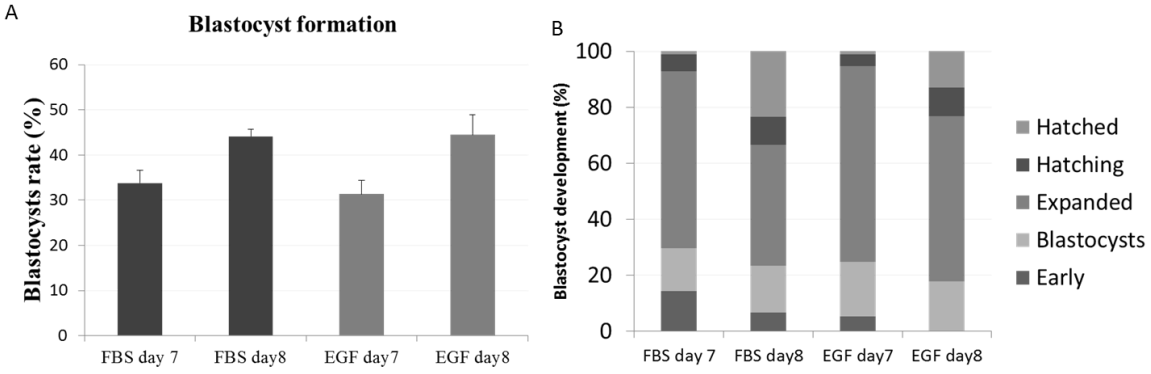

Figure 5. (A) Percentage of blastocysts at day 7 and 8 post insemination, produced from oocytes matured in serum containing (FBS) or serum-free (EGF) medium (mean ± SEM; n = 1174, three replicates). **(B)** Distribution of different blastocysts stages (according to IETS code) derived from EGF and EFBS conditions at day 7 and 8 post-insemination (n=1174, three replicates).

Selected blastocysts were randomly allocated into six different groups: two control groups (non-vitrified EGF and FBS) and four vitrified groups (EGF, EGF + Gap26, FBS and FBS + Gap26, Table 1). Non-vitrified groups were cultured in individual droplets of SOF for two days and the development to hatching was assessed on days 1 and 2. Vitrified groups were first warmed, and then analyzed for hatching/hatched rate on day 1 and day 2, as for the non-vitrified condition. Figure 6A illustrates the different outcomes, while the repartition between these different classes is shown in Figure 6B. In the presence of Gap26, blastocyst degeneration rate appeared to be decreased in the FBS group, but this visible difference did not attain statistical significance as compared to the other groups ($p=0.07$), (Figure 7A). We next compared hatching/hatched rates on day 1 but no significant differences were observed at that stage (Figure 7B). However, significantly higher hatching/hatched rates were observed on day 2 in the vitrified FBS + Gap26 group as compared to vitrified FBS ($p<0.05$). Gap26 did not have any effect in the EGF + Gap26 group as compared to the EGF group. Interestingly, the hatching/hatched rate in the FBS + Gap26 group was significantly higher than in the EGF + Gap26 group and also exceeded the hatching/hatched rates in the non-vitrified FBS or EFG groups significantly (Figure 7B). Importantly, Gap26 had no effect on the hatching/hatched rate of non-vitrified blastocysts (Figure 7C).

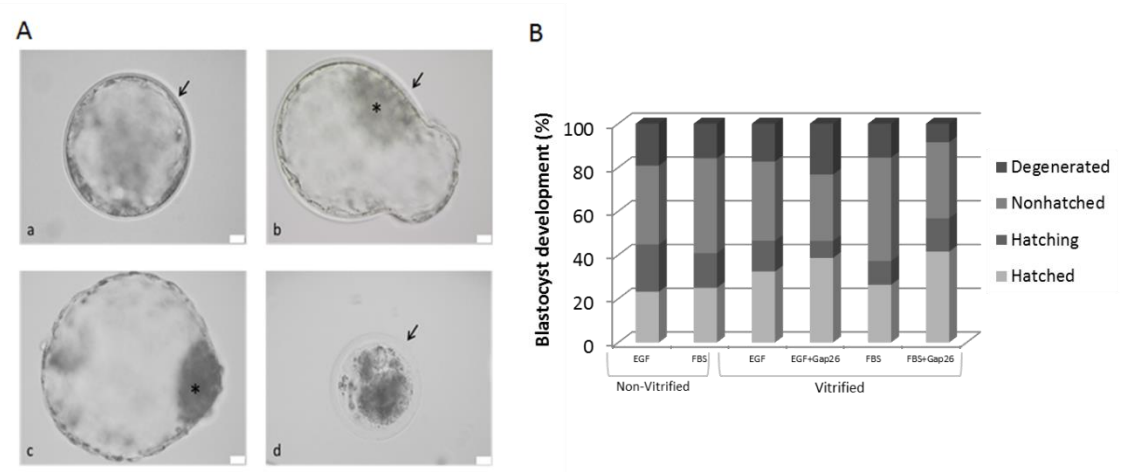

Figure 6. Various blastocyst stages after two days of individual culture**. A.** Representative images depicting (a) an expanded non-hatched blastocyst, (b) a hatching blastocyst, (c) a hatched blastocyst and (d) a degenerated blastocyst. Arrows indicate zona pellucida and asterisks indicate the inner cell mass (scale bar 20 µm). **B.** Repartition of the various blastocyst stages in the different experimental groups used.

We further tested the possibility that Gap26 protection in the vitrified FBS group was the result of better Gap26-uptake in the blastocysts as compared to the EGF group. Experiments with fluorescent Gap26-FITC however demonstrated no differences of Gap26 uptake in the EGF group compared to FBS conditions (Figure 8).

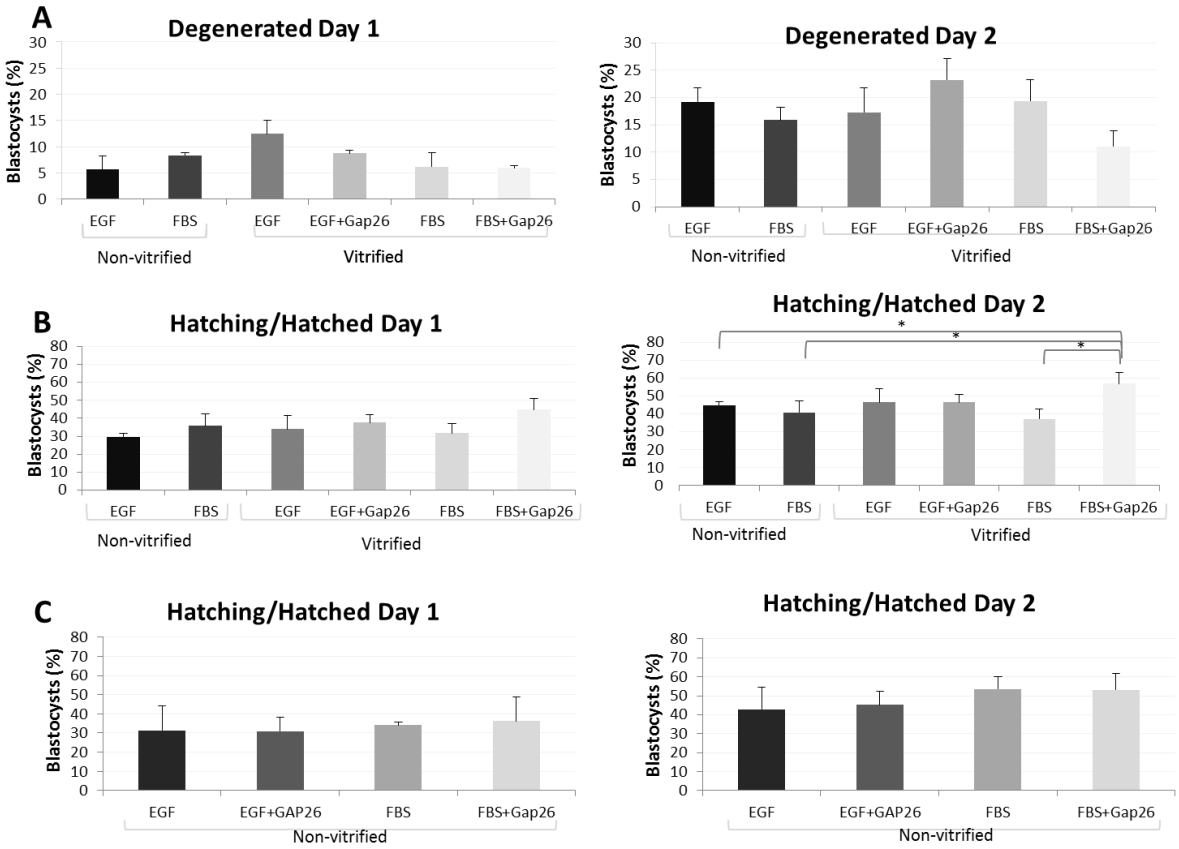

Figure 7. Effect of Gap26 on degeneration and hatching/hatched rates of FBS and EGF derived blastocysts**. A.** Percentage of degenerated blastocysts after day 1 and day 2 of individual culture. **B.** Percentage of hatching/hatched blastocysts after day 1 and day 2 of individual culture (EGF = 504, 10 replicates and FBS = 367, 8 replicates). * P<0.05. **C.** Percentage of hatching/hatched rates of non-vitrified blastocysts derived from EGF and FBS conditions, and further cultured for 1 and 2 days in individual culture (n=165, 4 replicates).

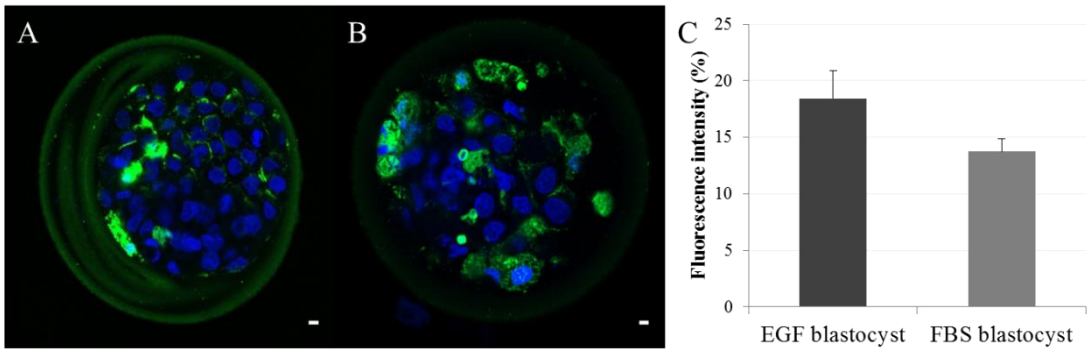

Figure 8. Gap26 accumulation in blastocysts derived from EGF or FBS conditions.
A-B. Representatives confocal images after incubation of blastocysts derived from EGF (A) and FBS (B) conditions. Gap 26-FITC stains green while nuclear DAPI staining is blue. Scale bar is 15 µm. **C. Average data of experiments as shown in panels A and B.** The bar chart shows the relative fluorescence intensity of Gap26-FITC signal; there were no significant differences (n = 13).

Gap26 treatment does not influence cell death in vitrified/warmed blastocysts

In order to test if Gap 26 reduces cell death in blastocysts, we performed TUNEL staining in non-vitrified and vitrified blastocysts after two days of individual culture in SOF, (Table 1). Blastocysts qualified as degenerated were not used for TUNEL staining. Cell death was always in the range of 5%-10% (relative to the number of DAPI positive nuclei) and was not different between the different groups (Figure 9).

Vitrification/warming of blastocysts induces hemichannel opening that is inhibited by Gap26

We tested whether vitrification/warming triggers the opening of HCs in the blastocyst cells by making use of dye uptake studies with HC-permeable PI and HC-impermeable 10 kDa Dextran FITC. Blastocysts derived from EFG and FBS conditions were divided into three groups: non-vitrified, vitrified and vitrified + Gap26 (Table 1). Immediately after warming, blastocysts were exposed to PI and 10 kDa Dextran FITC for dye uptake experiments. Strikingly, the percentage of PI-positive and 10 kDa dextran-FITC-negative cells was significantly higher in vitrified blastocysts from the FBS group compared to those kept in non-vitrified conditions ($p<0.05$, Figure 10). Additionally, Gap26 (also present during the assay) significantly reduced the number of PI-positive/dextran-negative cells. In contrast to this, blastocysts from the EGF group did not show increased PI uptake after vitrification/warming, and there were no differences with the Gap26 treatment condition (Figure 10). In the FBS group, the number of dextran-positive cells was low (non-vitrified: 0.2% ± 0.09, vitrified: 0.5% ± 0.1 and vitrified + Gap26: 0.3% ± 0.07; n=43-47), pointing to low levels of non-HC linked membrane leakage. None of the dextran-positive cell counts were different from each other, indicating that membrane disruption linked to cell death was not affected by Gap26, in line with the conclusions regarding cell death based on TUNEL staining.

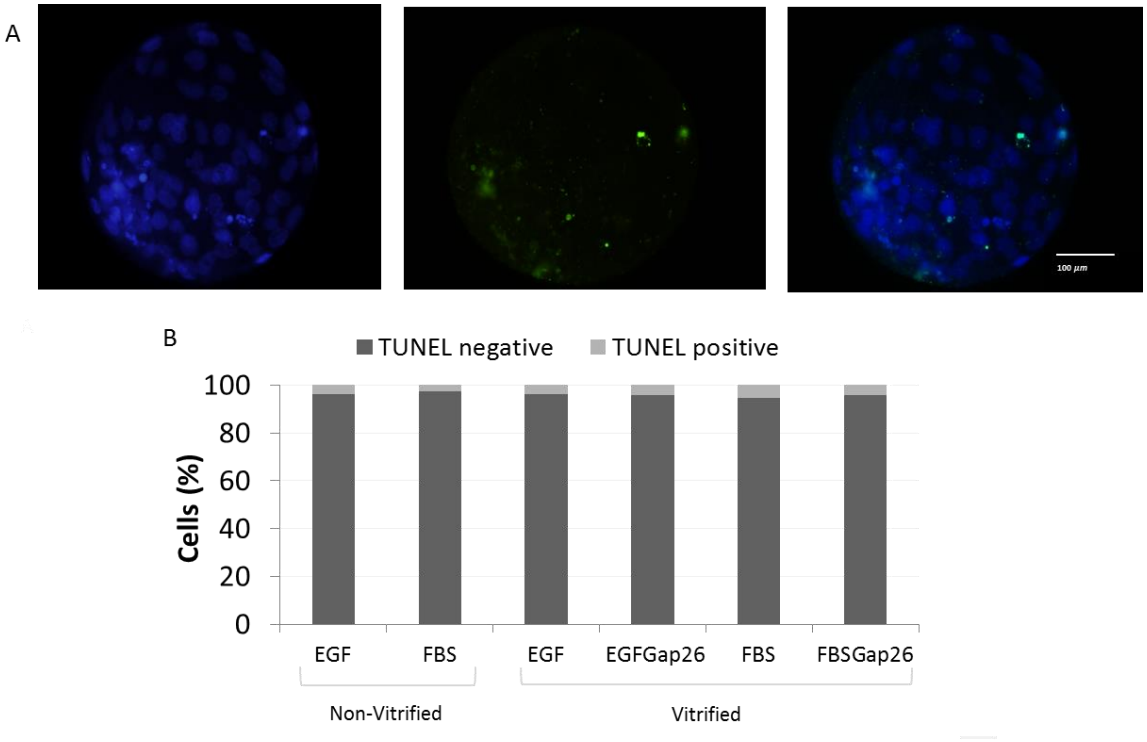

Figure 9.TUNEL staining of non-degenerated blastocysts in the different experimental groups.A. Representative example image showing TUNEL-positive DNA fragmentation in green and nuclear DAPI staining in blue. Image representative for both EGF and FBS conditions. **B.** Percentage of TUNEL positive and negative cells after two days of individual culture (EGF, n=92; FBS n=105, 4 replicates).

DISCUSSION

This study demonstrates that inclusion of the Cx channel-inhibiting peptide Gap26 during vitrification/warming of blastocysts, improves the blastocyst outcome by promoting the hatching process. The effect was only observed in blastocysts derived from oocytes matured in FBS conditions, but not in those from oocytes matured in EGF conditions. The protective effect of Gap26 on blastocysts from the FBS group exposed to vitrification/warming was linked to an inhibitory effect on Cx HCs. Indeed, HCs opened in response to vitrification/warming in blastocysts from the FBS group, and this response was inhibited by Gap26. By contrast, blastocysts from the EGF group did not show HC opening after vitrification/warming. The exposure to Gap26 during vitrification/warming was too short (~10 min) to have effects on GJs, pointing to an involvement of HCs rather than GJs. Blocking HCs did not significantly inhibit cell death (TUNEL assays) or plasma membrane disruption (10 kDa Dextran FITC assays), indicating that the protective effect of Gap26 is not linked to cell injury/cell death, but rather results from functional effects that do not kill the blastocyst cells but hamper their developmental progress towards hatching.

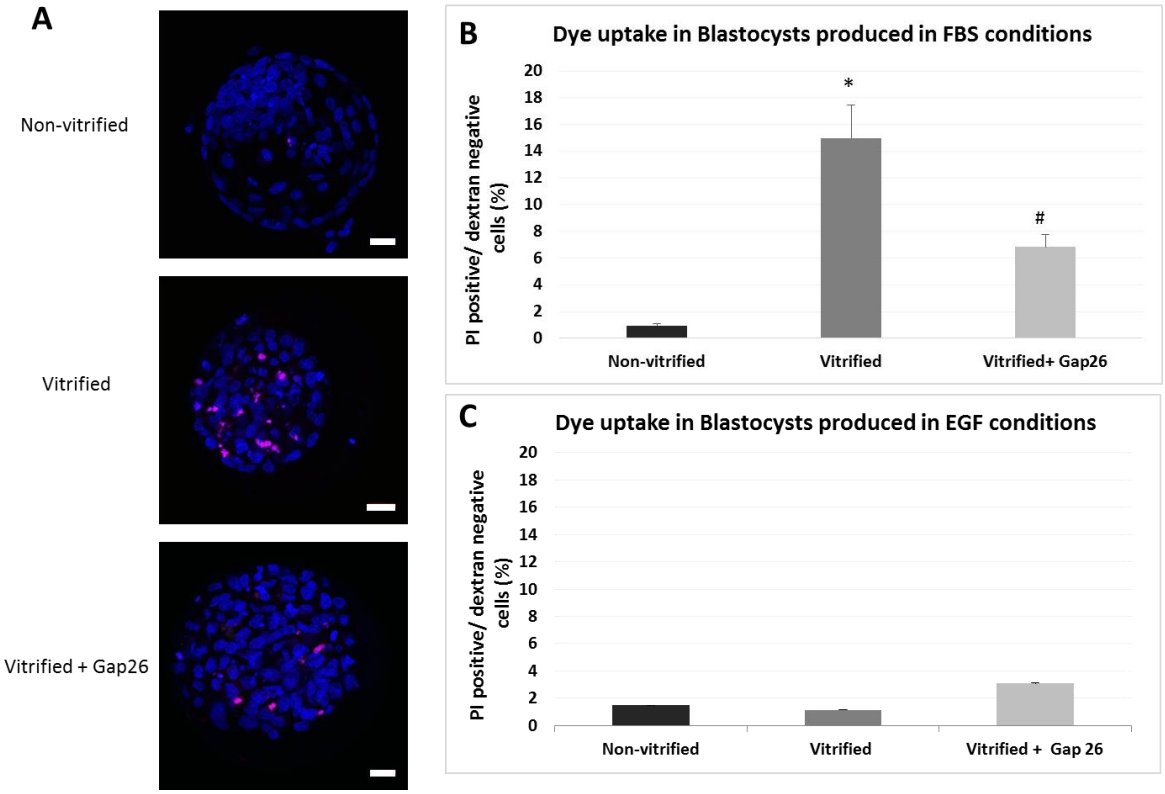

Figure 10. Hemichannel-mediated dye uptake in blastocysts after vitrification/warming. **A.** Representative images of PI (red) and dextran-FITC (green) uptake in blastocysts derived from the different conditions (non-vitrified, vitrified and vitrified + Gap26); blue represents nuclear DAPI staining. Scale bar 25µm. **B.** Summary data of the percentage of PI-positive cells/dextran-negative cells relative to the total number of nuclei per blastocysts (n=137, 3 replicates). C. Blastocysts from the EGF condition did not show PI uptake after vitrification/warming and Gap26 had no effect (n=118, 3 replicates). *comparison to control, P<0.05; # comparison to vitrified, P<0.05.

Connexins proteins that assemble into HCs and GJs, are abundantly expressed in the cytoplasm during early embryo development and in the plasma membrane after compaction of blastomeres at the morula stage (De Sousa *et al.* 1993). Cxs play an important role during preimplantation development and embryo implantation as shown in rodents (Houghton *et al.* 2002) and humans (Bloor *et al.* 2004). Various Cxs have been described at different stages of development in rodent embryos (Cx30, Cx30.3, Cx31.1, Cx31, Cx36, Cx40, Cx43, Cx45 and Cx57; Davies *et al.* 1996, Houghton *et al.* 2002), in human embryos (Cx26, Cx31, Cx32 Cx43 and Cx45; (Hardy *et al.* 1996, Bloor *et al.* 2004), and in bovine embryos (Cx30, Cx31, Cx32, Cx36, Cx43 and Cx45; Wrenzycki *et al.* 1996, Rizos *et al.* 2002, Balasubramanian *et al.* 2008). Cx43 is the most prevailing Cx and its aberrant expression or channel function may affect embryo survival (Bloor *et al.* 2004). Here, in bovine blastocysts, we

found evidence for Cx37 that is present at both messenger and protein level; immunohistochemical analysis demonstrated Cx43 and Cx37 in the cytoplasm and the plasma membranes of blastocysts matured in EGF or FBS. The importance of the currently reported presence of Cx37 is that HCs composed of this Cx have a single channel conductance of ~620 pS, which is the highest single channel conductance of all Cxs (see table 6 in Harris 2001; HC conductance is twice the single channel conductance of GJs). Thus, Cx37 and Cx43 form the building blocks of two high conductance leakage pathways characterized by a single channel conductance of ~620 and ~220 pS respectively, potentially contributing to significant complications when open during the vitrification/warming process. Recent works in embryonic neurons and smooth muscle cells have demonstrated that even the opening of a single HC per cell is sufficient to alter cell function (Moore *et al.* 2014, Bol *et al.* 2016).

Vitrification has evolved into the most commonly used method for preservation of cells and tissues. It involves exposure to high osmolarity CPA solutions, combined with an extremely fast temperature drop to -196 °C (Saragusty and Arav 2011). The high extracellular osmolarity extracts water from the cell and concentrates the permeated CPAs, together with the millisecond temperature drop, this limits ice crystal formation. Intracellular ice crystallization, however can still occur during the warming process. This is a major toxic event as it is associated with mechanical, chemical, and osmotic stresses induced by the expelling of ions from the water crystals (Watson 2000, Arav 2014). This is collectively leading to cellular stress, in combination with fluctuations over a wide non-physiological range of physical and chemical parameters such as osmotic and hydrostatic pressure, ionic composition, pH and temperature (Kopeika *et al.* 2015). Cellular stress induce HCs opening that can be inhibited by Gap26 or Gap27 mimetic peptides (Evans and Boitano 2001, De Wit and Griffith 2010, De Bock *et al.* 2011, Desplantez *et al.* 2012, Wang *et al.* 2013a) and these peptides improve the survival of cells and tissues when exposed to stress conditions (Decrock *et al.* 2009, Hawat *et al.* 2012, Wang *et al.* 2012, Davidson *et al.* 2013, Decrock *et al.* 2015).

To test the effect of peptide Gap26 during vitrification/warming, bovine blastocysts were produced from oocytes matured in serum-containing medium (FBS condition) or serum-free medium (EGF condition). Serum is routinely used in the field for oocyte maturation (Ahn *et al.* 2002, Shirazi *et al.* 2012) or embryo culture (Leibo and Loskutoff 1993, Gómez *et al.* 2008), although it is known to reduce the cryo-tolerance. The reduced cryo-tolerance of blastocysts produced in serum conditions has been explained to result from a higher amount of cytoplasmic lipid droplets that accumulate during the culture period (Abe *et al.* 2002). In the present work, FBS exposure was limited, with FBS only present during oocyte maturation, and not during embryo culture, which took place in SOF

medium supplemented with BSA and ITS (Wydooghe *et al.* 2014). While embryos cultured in serum-free media showed higher survival and hatching rates after post-thaw culture than those cultured with serum (Abe and Hoshi 2003, Gómez *et al.* 2008), limited exposure to FBS during oocyte maturation leads to hatching/hatched rates that are comparable to those from blastocysts derived from EGF condition. In line with this, the data in Fig. 6 show that hatching/hatched rates after vitrification/warming were not different between FBS and EGF conditions. Most notably, hatching/hatched rates were not significantly below the rates in non-vitrified blastocysts, indicating a well optimized cryopreservation procedure.

Most interestingly, significantly higher hatching/hatched rates were observed when Gap26 was supplemented to blastocysts derived from oocytes matured with FBS while no effect was observed for the corresponding EGF condition. This was not due to impaired Gap26 penetration into EGF-derived blastocysts, but because HCs did not open in response to vitrification/warming in the EGF condition while they opened in blastocysts derived from FBS condition. Thus, inclusion of Gap26 protects against cryopreservation stress and improves embryo development in culture leading to significantly higher hatching/hatched rates in FBS compared to EGF conditions. Of note, hatching/hatched rates in FBS + Gap26 were also higher than in control non-vitrified blastocysts. This may be related to the fact that the non-vitrified groups were cultured in total for 10 days, which is the maximum period in vitro. The reason why vitrification/warming gives distinct HC responses in FBS versus EGF conditions is not clear at this moment; it may be linked to differences in the intracellular Ca^{2+} concentration, the redox status or the phosphorylation state. Hemichannel opening is induced by slight elevation of the intracellular Ca^{2+} concentration (De Vuyst *et al.* 2006, Bol *et al.* 2016), oxidizing conditions and dephosphorylation, (reviewed in Sáez *et al.* 2005, Pogoda *et al.* 2016)).

We previously reported that the protective effect of Cx channel-blocking peptides against cryo-injury in human blood vessels is mediated by a reduction of cell death (Bol *et al.* 2013). We thus looked at several markers of embryonic injury/cell death, including blastocyst degeneration, TUNEL stainings and 10 kDa FITC-dextran dye uptake. Figure 7A illustrates that the percentage of blastocysts degenerated on day 2 fluctuated around ~20%, with the exception of the FBS + Gap26 condition where a ~10% degeneration was observed; however, this difference did not attain statistical significance. In a next step, we verified cell TUNEL positivity and 10 kDa FITC-dextran dye uptake in non-degenerated blastocysts. Figure 9 illustrates that 5-10% of the cells were TUNEL-positive, in line with reports of others (Morató *et al.* 2010). The flat TUNEL positivity rates over all groups probably results from a highly optimized vitrification protocol including a very reduced volume of the solution surrounding the blastocyst (< 1 µl), which facilitates fast cooling and warming rates. Moreover, there

were no significant differences between groups, including the FBS + Gap26 group. Apart from the TUNEL assay which mainly reports apoptosis, we also evaluated cell membrane integrity by assessing 10 kDa FITC-dextran uptake, which reflects cell injury or necrosis. We observed that ≤ 0.5 % of the blastocyst cells had taken up 10 kDa FITC-dextran, meaning they had disrupted plasma membranes; and then again, no differences with the Gap26 treated group were observed. Taken together, these results indicate that Gap26 did not inhibit cell death in the blastocysts. Gap26 non-significantly reduced the number of degenerated blastocysts in the FBS + Gap26 group but the bar chart in Figure 5B demonstrates that most of the protective effect of Gap26 results from the increased hatching/hatched rates. Collectively, we conclude from these observations that Gap26 protection is not mediated by protection against cell death but results from a functional effect of Gap26 during the vitrification/warming of blastocysts, which improves their hatching success. The vitrification process is a well optimized and extremely fast procedure; by contrast, more stress is to be expected from the warming phase during which ice crystal formation may occur. In line with this, we found evidence for HC opening in the blastocyst cells shortly after warming. Inhibiting HC opening with Gap26 may prevent the dissipation of transmembrane ionic gradients and the excessive entry of calcium ions, and may counteract the loss of important metabolites from the embryonic cells. Taken together, these actions of Gap26 may promote the physiology of cell function and signaling thereby promoting the hatching process and further embryo development.

This study is one of the few studies that investigated specific plasma membrane protein targets to determine their contribution and protective potential for improving cellular outcome following exposure to cryopreservation stress and injury. Most of the attempts in this field have been directed to fine tuning the composition of the solutions while targeting specific proteins or channels has been unsolicited. The only other plasma membrane channel that has been investigated in relation to cryopreservation is the family of the AQP channels (Hagedorn *et al.* 2002). These channels facilitate fast water movement and are highly specific for ions and small molecules. Aquaporins play important roles in follicle development, fertilization, blastocyst formation, and implantation (Huang *et al.* 2006). Moreover, they permit faster exchange of water and CPAs like EG, propylene glycol and glycerol during embryo cryopreservation that enhance tolerance against freezing/thawing stress (Edashige *et al.* 2007, Jin *et al.* 2011). Furthermore, the alteration of AQP3 genes improved the cryopreservation process in zebra fish embryos (Hagedorn *et al.* 2002). Although this seems to be a good strategy for cryopreservation in fish, induction of AQP expression in mammalian embryos would involve an extensive pre-treatment procedure that might prove difficult to implement because the oocytes and embryos are also aging in culture.

In conclusion, we demonstrate here for the first time that the vitrification/warming of blastocysts that were exposed earlier in development to serum during oocyte maturation, leads to opening of the HCs that impair subsequent embryo development. The inclusion of a Cx channel targeting peptide during vitrification/warming alleviates this event and improves the functional embryo outcome. Thus, targeting Cx channels provides a novel strategy to optimize the functionality of the embryo and improve its subsequent development.

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CHAPTER 6

GENERAL DISCUSSION

Although vitrification is a promising technique for application in assisted reproductive technologies (ART), the competence of vitrified oocytes and embryos is compromised after warming. A lot of work has been performed in order to gain a better understanding of some aspects of fundamental cryobiology. However, as previously described in the introduction, different variables affecting the cryobiology of both oocytes and embryos need to be addressed before vitrification results can be improved. The research described in this dissertation was conducted with the purpose to study two of these variables: (1) the effect of cumulus cells surrounding the oocyte during vitrification and their influence on the vitrification protocol **(Chapter 3 and 4)**, and (2) the effect of maturation in the presence of serum on the vitrification of oocytes and blastocysts **(Chapter 5)**.

Effect of cumulus cells on vitrified oocytes and their influence on the vitrification protocol

As previously described in the introduction, the effect of cumulus cells surrounding the oocyte during vitrification needs further investigation. Even though cumulus cells are necessary for maturation, fertilization and embryo development (Zhang *et al.* 1995), we hypothesized that they might decrease the 'vitrifyability' of oocytes by reducing the entry of cryoprotectants (CPAs). We have investigated the effect of cumulus cells on the vitrification of bovine mature oocytes (**Chapter 3)** and equine immature oocytes (**Chapter 4**).

Our first finding was that the survival and developmental competence were compromised when bovine **(Chapter 3)** and equine **(Chapter 4)** oocytes were surrounded by multiple layers of cumulus cells during vitrification. Our results corroborated the reports of Chian *et al.* 2004, but did not confirm the findings of Dinnyes *et al.* 2000, Tharasanit *et al.* 2009 and Zhou *et al.* 2010. This discrepancy may be due by the fact that these research groups used different materials and methods for the vitrification. Firstly, in all our studies, we have used a modified method in which oocytes are gradually exposed to CPAs, which might result in smaller osmotic volume changes. Secondly, whereas we have used a custom adapted device imitating the Cryotop with an extremely fast cooling rate (Figure 1), Tharasanit *et al.* 2009 used the Open Pulled Straw (OPS) for vitrified equine oocytes. As we previously described in the introduction, the use of different cryo-devices leads to different cooling and warming rates and different results (Figure 1, Saragusty and Arav 2011). Although the Leidenfrost effect might still occur when the custom adapted device is used, the higher cooling and warming rates obtained during the vitrification may be key for the observed improvement.

	Open Pulled Straw	Custom-adapted device
Volume	1.5µL	0.1µL
Cooling rate	20,000°C/min	23,000°C-42,000°C/min

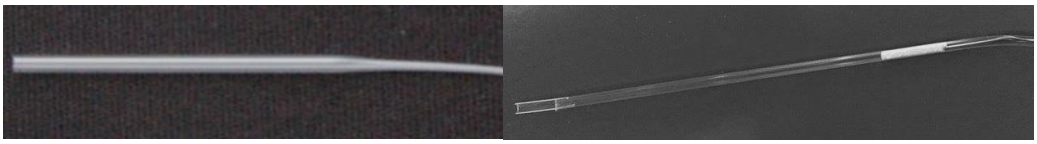

Figure 1. Comparison of Open Pulled Straw vs. the adapted device imitating the Cryotop used in this study.

We observed that bovine mature oocytes vitrified without any cumulus cells showed similar survival rates compared to their fresh counterparts **(Chapter 3)**. However, the removal of cumulus cells drastically reduced the fertilization rate and developmental competence of vitrified bovine oocytes **(Chapter 3)**, as it occurs in fresh oocytes (Tanghe *et al.* 2003, Luciano *et al.* 2005). Therefore, in our study, we used three different approaches to solve the confounding of effect on 'fertilizability' and 'vitrifyability' in bovine oocytes (**Chapter 3**). Firstly, vitrified denuded oocytes were fertilized with fresh COCs, because it is known that in this way their fertilization and blastocyst development can be restored (Tanghe *et al.* 2003, Luciano *et al.* 2005). Secondly, vitrified corona radiata (CR, Figure 2) oocytes were used as a compromise between COCs and denuded oocytes. Finally, oocytes were parthenogenetically activated taking the fertilization out from the equation, since fertilization was compromised in denuded/cumulus poor oocytes.

In the first experiment, we observed that the fertilization rate of vitrified denuded oocytes could be reverted when they were fertilized in presence of intact COCs. Thus, denuded oocytes are sufficiently protected during vitrification because CPAs may have easier access to the oocyte, and possibly enter at adequate concentrations into the oocyte (Figure 2). However, denuded oocytes still display a low embryonic developmental capacity as an indirect consequence of the absence of cumulus cells during the fertilization process. In the second experiment, we observed that the mere act of removing the outer cumulus cells was already reducing the fertilization rate of bovine fresh oocytes compared to the ones surrounded by multiple layers of cumulus cells. Human ART has reported successful fertilization after vitrification of CR oocytes (Tong *et al.* 2014). Interestingly, in our study, we could not find differences in the fertilization and blastocyst development rate between vitrified CR oocytes compared to COCs. Moreover, in the third experiment, we observed that vitrified CR

oocytes had a similar capacity to become blastocysts compared to fresh oocytes after parthenogenetic activation. As far as we know, it is the first time that the embryonic development of vitrified CR oocytes has been studied after parthenogenetic activation; this allowed us to observe that the developmental competence was not affected by vitrification, only fertilization was decreased as a consequence of cumulus cell removal.

These experiments allowed us to gain insight in the role of cumulus cells during vitrification, and two theories might explain the negative effect observed when oocytes were surrounded by multiple layers of cumulus cells during the vitrification. Firstly, cumulus-oocyte-complexes were more difficult to load over the custom adapted device compared to denuded or corona radiata oocytes. Therefore, a larger volume of medium was surrounding the specimen, and lower cooling and warming rates were achieved, as described in the introduction. Secondly, even though we have not measured permeability for water and CPAs, or measured flows of water and CPAs, or even compared shrinking and swelling curves of COCs vs. denuded oocytes, it seems a probable assumption that the layers of cumulus cells pose a significant extra barrier (Figure 2) to the exchange of water and CPAs between the ooplasm and the outside medium.

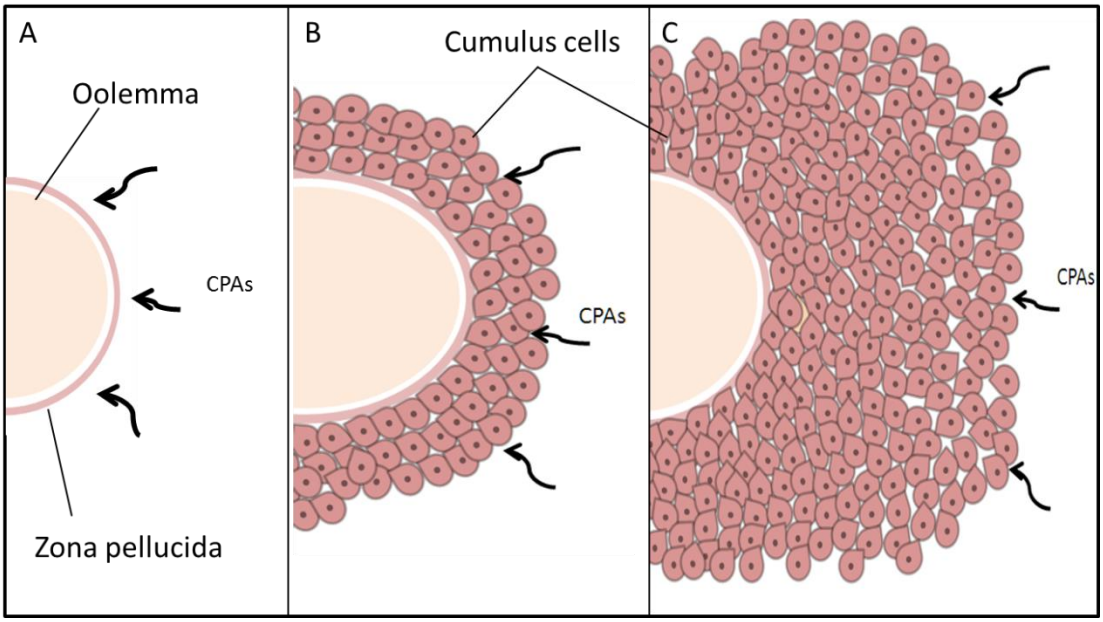

Figure 2. Diagram representing the contact of the cryoprotectants in a denuded oocyte (A), in a corona radiata oocyte (B) and in a cumulus oocyte complex (B).

The permeability of the plasma membrane is a very important variable that needs to be considered during cryopreservation, particularly during vitrification. This is due to the fact that during vitrification, a high concentration of CPAs is used, producing a potential toxic effect in cells. Therefore, it is recommendable to limit the time of exposure to high concentrations of CPAs. However, if the time of exposure is not long enough to produce the permeation, CPAs might not enter in the oocytes. This brings us to put forward two main questions: (1) how do CPAs act to protect the oocyte and (2) is the entry of permeable CPAs into the oocyte necessary during vitrification. Firstly, the addition of CPAs leads to an increase in the extracellular total solute concentration. As a consequence, the 'concentration' of water (the chemical potential of water) inside the cells is much higher than outside. This difference drives the water out of the cells. At the same time, membrane permeable CPAs will flow inside the cells, driven by the concentration difference. As the water movement is much faster than that of CPAs, cells will shrink until the water 'concentration' inside the cells equals that outside the cells. That means that at that point, the ice forming tendency inside the cells has become as low as that in the extracellular medium. Allowing time for entry of the membrane-permeable CPAs and the re-entry of most of the water brings the cells back to their original cell volume, which contributes to their survival after subsequent vitrification. On the other hand, if we do not allow the entry of CPAs, dehydration would occur that may be sufficient for intracellular vitrification, but might lead to structural and mechanical damage.

Therefore, we can assume that a certain amount of permeable CPAs need to enter into the oocyte during vitrification. Certainly, the permeability of oocytes and embryos of various species differs for different CPAs, and its relevance to vitrification was recently reviewed by (Edashige 2016) . This allows scientists and practitioners to choose the most appropriate CPA for each cell and species. If oocyte permeability for a given CPA is low, the shrinking and swelling cycle may not be finished in time and may lead to damage of the oocyte as a consequence of excessive shrinking. In the present study, we applied two of the most commonly used CPAs, dimethyl sulfoxide (DMSO) and ethylene glycol (EG), both of which are quickly penetrating into the cytoplasm. Especially in bovine oocytes, ethylene glycol shows high permeability (35 µm/min) whereas its permeability in mice is quite low (6 µm/min) compared to other CPAs such as propylene glycol (17 µm/min) at 25°C (Edashige 2016).

Ethylene glycol is considered a good CPA for oocytes because of its penetrating capacity and its low toxicity (Bautista and Kanagawa 1998). However, it does not seem to be appropriate for preservation of cumulus cells (Lindley *et al.* 2001). The use of EG as a sole CPA leads to a high percentage of dead cumulus cells after cryopreservation (Lindley *et al.* 2001). Moreover, if cumulus cells are damaged, this would influence the survival of oocytes (Lindley *et al.* 2001). This occurs because oocytes are

connected with cumulus cells via gap junctions. These connections are fundamental for growth and maturation of the oocyte (Feng *et al.* 2013). However, under apoptotic conditions, physiological messengers such as inositol 1,4,5 trisphosphate (IP_3), can pass through gap junction channels, and may act as a cell death- messenger (Decrock *et al.* 2012) between cumulus cells and oocyte. We hypothesised that a higher concentration of CPA could help speeding up the entry of CPAs into the cumulus cells, thus allowing a complete protection of the oocyte and the cumulus cells. Therefore, **in chapter 4**, the variable related to the number of cumulus cell layers (COCs and CRs) was addressed together with the concentration of CPAs for vitrified equine immature oocytes. The duration of exposure was also taken into account, as the CPA toxicity increases with its concentration.

We showed that there is a strong association between the number of cumulus cells layers and the time of exposure to CPAs. Firstly, we observed a low maturation competence in COCs when they were vitrified after a short exposure time to a high concentration of CPAs (<1min). As previously described, we presume that the presence of multiple layers of cumulus cells surrounding the oocyte did not allow a correct movement of CPAs (Figure 2), even though higher concentrations of CPAs were used. Whereas the efflux of water was produced immediately after CPA addition, there was not enough time to allow the entry of the CPAs into the oocyte. The presumed lower concentration of CPAs inside the COCs, and the consequent reduced reswelling, i.e. the stronger shrinking of the cells, may have led to cell damage.

Secondly, we observed that the situation could be reversed with the use of CR oocytes or with the exposure of COCs to CPAs for a longer period of time. These findings were indirectly confirming our hypothesis that the presence of multiple layers of cumulus cells surrounding the oocyte prevents the movement of CPAs and water, thus requiring longer time of exposure for a correct protection. However, we could not demonstrate that higher concentrations of CPAs may protect COCs. In our study, we did not investigate cell death in cumulus cells, but another study in equine reported that it is mainly detected in peripheral cumulus cells (Tharasanit *et al.* 2009). Cells in the external layers are less attached to the cumulus oocyte complex unit, being more sensitive to vitrification. Therefore, removing those external cells can only lead to better results due to the fact that (1) if they die, they do communicate a signal for cell death and (2) when they are gone, a better exchange of water and CPAs is possible.

The use of CR oocytes might thus explain in part, the improved results that we showed in our research (**Chapter 4**). When we started our investigation, there were just a few reports about the vitrification of immature equine oocytes and only one blastocyst (1% of injected oocytes) had been reported (Tharasanit *et al.* 2006). Although we did use the same protocol as Tharasanit and

colleagues, the inclusion of two modifications, such as the use of an adapted device imitating the cryotop and the vitrification of CR oocytes may have been key for an improvement of this method, leading to 7% blastocyst formation in injected vitrified-warmed immature oocytes. As well as the blastocysts development improvement, our study in the horse represents a breakthrough in equine oocyte cryopreservation, because we have achieved a first live foal after in vitro production of a blastocyst from vitrified immature equine oocytes.

In our study, using the long protocol, we also observed that a higher number of vitrified oocytes matured in a similar rate compared to fresh oocytes, but were not able to become a blastocyst. These results are in agreement with those of Canesin *et al.* 2016 who reported that exposure of oocytes to CPAs without freezing (toxicity experiment) reduced the developmental competence of oocytes, while no effect was detected on maturation rate. On the other hand, using the short vitrification protocol resulted in a lower maturation rate, but in similar blastocyst development compared to fresh oocytes. Therefore, it seems that the use of high concentration of CPAs and/or long exposure to CPAs may have led to toxic effects as suggested by several studies (Arav *et al.* 1993, Yamada *et al.* 2007, Tharasanit *et al.* 2009). Certainly, toxicity is considered as the most limiting factor when developing a successful vitrification protocol (Szurek and Eroglu 2011). Similar to our study, it was reported that the toxicity effect could not be detected after warming, but was intensified with in vitro culture (Men *et al.* 2003). It is possible that besides the toxicity that CPAs cause at a molecular and protein level, they also have an effect at DNA level as was reported in spermatozoa (Yildiz et al. 2007). Therefore, when the embryonic genome is activated, it may result in failed development.

Effect of the vitrification process on oocytes and blastocysts previously matured in presence of serum

Not only the toxicity caused by CPAs reduces the embryonic development, but vitrification itself is also considered as a stress process that induces an abnormal increase in reactive oxygen species (ROS) activity (Gupta *et al.* 2010, Zhao *et al.* 2011). An increase in the ROS level results in mitochondrial permeability transition, which consequently reduces the mitochondrial membrane potential (Liu *et al.* 2000). This reduction could induce rupture of the mitochondrial membrane, which may facilitate the release of some proteins that activate the caspase cascade (Suen *et al.* 2008).

Moreover, vitrification might induce opening of hemichannels (Bol *et al.* 2013, Wang *et al.* 2013b). Hemichannels can be forming gap junctions, which contribute to allow passage of cell death-

messengers to the oocyte and surrounding cumulus cells during vitrification, as previously discussed. Moreover, we can find them also as unapposed hemichannels in cell membranes. These hemichannels are normally closed (under resting conditions, Figure 3), but a stress process induces opening of hemichannels, allowing the escape of important ions (Na^+, Ca^{2+}, K^+), and molecules (NAD^+, ATP, glutamate, prostaglandins, glutathione, IP_3) (Wang *et al.* 2013a). These molecules are fundamental for the cells and their loss may compromise cell survival (Bol *et al.* 2013). Fortunately, hemichannels can be closed by using mimetic peptides (Evans and Boitano 2001, Desplantez *et al.* 2012, Wang *et al.* 2013a), which bind to domains in the extracellular loops of connexins. A recent study showed that the inclusion of mimetic peptide during cryopreservation increases cell viability of blood cells (Bol *et al.* 2013). We hypothesized that (1) vitrification may induce opening of hemichannels in vitrified blastocysts, and (2) the use of a mimetic peptide can alleviate this problem. Therefore in **Chapter 5.2,** the effect of vitrification was studied in blastocysts previously matured in presence of serum or epidermal growth factor (EGF). Moreover, we investigated if the inclusion of a mimetic peptide, Gap26, during the vitrification improved the embryonic development of these blastocysts.

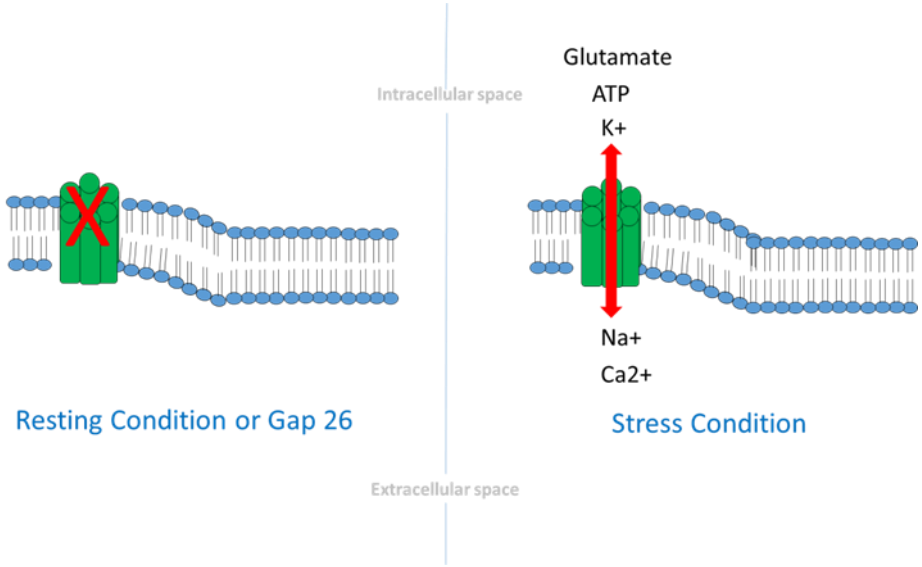

Figure 3. Diagram representing the hemichannels activity in a resting /Gap26 condition (A) and in a stress condition (B).

Firstly, we showed that vitrification induces the opening of hemichannels in blastocysts previously matured as oocytes in serum medium **(Chapter 5.2)**. Studies in somatic cells showed that opening of hemichannels may lead to apoptosis in connected cells (Bol *et al.* 2013). Apoptosis is a cellular response to stress in suboptimal conditions and the index of apoptotic cell is usually determined in order to know the embryo viability and developmental potential after vitrification (Morató *et al.* 2010). However, in our study, vitrified blastocysts did not show an increase in the number of apoptotic cells when compared to fresh blastocysts.

We further observed that the inclusion of a mimetic peptide, Gap 26 during vitrification/warming improved the hatching rate of vitrified blastocysts previously matured as oocytes in serum medium as compared to the ones not treated with the mimetic peptide (**Chapter 5.2**). Probably the opening of hemichannels did not lead to cell death, but it compromised the developmental potential of these blastocysts compared to control ones (not treated with the mimetic peptide).

Surprisingly, we also observed that opening of hemichannels could be alleviated when oocytes were matured in presence of EGF instead of serum because the vitrification did not open hemichannels in those blastocysts. This brings us to an important question: What is the effect of EGF on the activity of hemichannels? The answer is still uncertain, but a recent publication showed that EGF modifies calcium levels, which may have an effect on hemichannel activity (Hao *et al.* 2016). Therefore, further studies are needed to elucidate this topic, because this finding opens new challenges for improving vitrification.

It is also remarkable that until now, the low cryotolerance observed in embryos cultured in serum containing media was attributed to the accumulation of lipids, and the resulting changes in the lipid composition of the cell membranes (Abe *et al.* 2002, Abe and Hoshi 2003). However, the loss of functionality that might occur when hemichannels are open is also a possible explanation for the lower developmental competence observed in blastocysts matured as oocytes in serum. A carry-over effect was thus demonstrated, like in our other study where we showed lower cryosurvival of blastocysts matured as oocytes in the presence of palmitic or stearic acid (Shehab-EL-Deen et al. 2009).

In our study, we also investigated the competence of vitrified oocytes matured in presence of serum or epidermal growth factor (**Chapter 5.1**). Our data showed that the maturation in presence of serum, glutamine and pyruvate compromises the embryonic development of vitrified oocytes. However, further research would be needed in order to elucidate if vitrification induces opening of

hemichannels in oocytes and if the use of the mimetic peptide or culture in EGF can alleviate this effect.

In conclusion, this thesis has contributed to gain more insights in some of the variables that influence the vitrification process. Firstly, the critical role of cumulus cells during the vitrification of oocytes has been elucidated. Secondly, we have showed that other variables such as permeability, concentration and/or time exposure to CPAs need to be addressed in order to achieve a correct protection. Finally, our study demonstrated that vitrification can induce, depending on the culture conditions, opening of hemichannels, and that the use of a mimetic peptide or the presence of EGF during maturation can alleviate this problem.

Future perspectives

It can be concluded that the use of CR oocytes leads to a better vitrification compared to COCs or denuded oocytes. Although the fertilization of fresh bovine CR was compromised, we showed that, after parthenogenetic activation, these oocytes have the capacity to become blastocysts in a similar rate than the fresh ones. To date, major efforts have been made in order to develop a repeatable and simple method for fertilizing denuded oocytes. However, as far as we know, no such attempts were made for CR oocytes. Our results open new challenges for developing a system that supports the fertilization of CR oocytes.

Moreover, from a practical point of view, the use of CR shows also some advantages over COCs. We observed that CR oocytes are easier to manipulate, allowing us to load a higher number of them in a lower volume. Its application in clinical laboratories might lead to a more efficient vitrification process, brought about by the increase in the cooling/warming rate.

Our findings also indicate that a successful vitrification can be achieved providing that the protocol is optimized and taking into account the influencing variables. We have demonstrated the necessity for adjusting the presence of cumulus cells in combination with variables such as the time of exposure and concentration of CPAs. However, other variables such as the temperature or the permeability to CPAs also need to be addressed in further research. In our study, we used two of the CPAs with higher permeability for bovine oocytes (Edashige 2016), but further studies on altering the membrane permeability are needed in order to improve the exchange of water and CPAs and to reduce the concentration or time of exposure to CPAs (Clark and Swain 2013), as our results indicate that toxicity of CPAs is one of the most critical steps when a protocol is optimized. Moreover, studies would be necessary to investigate the effect of CPAs on embryonic genome activation or signal transduction pathways in order to understand the causes of failure in embryonic development observed in vitrified oocytes (Xu *et al.* 2014).

Previous reports on oocytes and embryos pointed out that vitrification drastically reduces their competence after warming. In our study, we observed that vitrification induces opening of hemichannels under certain conditions. This is an important discovery in vitrification, which opens new challenges to improve the process. Further studies are needed to gain more insight in this topic in order to avoid the conditions that lead to opening of hemichannels. Moreover, research on elucidating the mechanism behind EGF would help us minimize the damage produced after warming and improve the vitrification.

Finally, we have proved that the inclusion of a mimetic peptide improves embryo development of blastocysts matured in serum. However, further research is needed to investigate its application in practice because it may also lead to a better pregnancy rate not only in cattle but also in other species. Likewise, the inclusion of a mimetic peptide could improve the vitrification of oocytes by increasing their embryonic development.

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SUMMARY

Cryopreservation of gametes and embryos is an important part of assisted reproductive technologies, since it can be used to preserve genetic material for a longer time. The introduction of vitrification resulted in a promising technique for oocyte and embryo cryopreservation. However, the developmental competence of the oocytes and embryos is compromised after warming. An overview of the difficulties encountered in the vitrification process and a description of the different variables influencing the vitrification process and the cryotolerance of oocytes and embryos is given in **Chapter 1**.

The general aim of this thesis was to increase the effectiveness of vitrification for oocytes and embryos in cattle and horses (**Chapter 2**). To this purpose, we investigated two variables influencing the vitrification: (1) the presence of cumulus cells surrounding the oocyte during vitrification and their influence on the vitrification protocol and (2) the effect of maturation in the presence of serum on the vitrification of oocytes and blastocysts.

In **chapter 3**, specific attention was given to the role of cumulus cells surrounding mature bovine oocytes during their vitrification. It is already known that cumulus cells play a fundamental role during maturation, fertilization and blastocyst development. However, they might decrease the exchange of water and cryoprotectants (CPAs), which leads to an inadequate protection of the oocyte during vitrification. Our results showed that the presence of multiple layers of cumulus cells surrounding the oocytes compromised their survival after vitrification. On the other hand, we observed that total removal of cumulus cells led to similar survival rates in vitrified denuded oocytes compared to their fresh counterparts.

In bovine, it is known that removal of cumulus cells decreases the fertilization rates of the oocytes. Therefore, in our study, we used three different approaches in order to distinguish 'vitrifiability' from 'fertilizability'. Firstly, vitrified denuded oocytes were fertilized in the presence of intact fresh cumulus complex oocytes (COCs). Secondly, we used corona radiata (CR) oocytes as a compromise between COCs and denuded oocytes. Finally, vitrified oocytes were activated parthenogenetically. Our results showed that removal of cumulus cells before vitrification reduced the fertilization in vitrified denuded oocytes. Interestingly, this situation could be reverted when vitrified denuded oocytes were fertilized in the presence of fresh COCs. Secondly, we observed that the mere act of removing only the outer cumulus cells (CR oocytes) was already reducing the fertilization of fresh oocytes compared to the ones surrounded by multiple layers of cumulus cells. Such an effect was not detected in vitrified CR oocytes, which showed similar fertilization and embryonic development compared to vitrified COCs. Surprisingly, vitrified CR oocytes showed similar capacity to become blastocysts compared to fresh COCs after parthenogenetic activation.

Together, these results clearly show that the presence of cumulus cells might reduce the entrance of CPAs, compromising the vitrification of mature bovine oocytes and therefore it is advisable to remove some of the external layers of cumulus cells.

If cumulus cells reduce the exchange of CPAs, the use of high concentrations of CPAs could help speeding up their entry into the cells. Therefore, in **Chapter 4,** two protocols were evaluated (high concentrations of CPAs for a short time of exposure and lower concentrations of CPAs for a long time of exposure) for the vitrification of immature equine oocytes surrounded by some (CR) or multiple layers of cumulus cells (COCs). In our study, we observed a low maturation competence when COCs were vitrified in high concentrations of CPAs for a short time of exposure. Fortunately, this situation could be reverted with the use of CR oocytes or with the exposure of COCs to CPAs for a longer period of time. These findings support our observation in **Chapter 3**, where the presence of multiple layers of cumulus cells surrounding the oocytes prevent the movement of CPAs and water, implying the need for a longer time of exposure for a correct protection.

Consequently, COCs were discarded from our following studies and only CR oocytes were used to investigate the effect of the two different protocols on the spindle configuration and the embryonic development of vitrified immature equine oocytes (**Chapter 4**). Our data showed that both vitrification protocols resulted in higher rates of aberrant spindle configuration in vitrified oocytes compared to fresh ones in the horse. More interestingly, we observed that blastocyst development only occurred in oocytes vitrified in high concentrations of CPAs for a short time of exposure. Pregnancies were established after transfer of such blastocysts, and a healthy male foal was born on May 12, 2017, a wordl's first. The longer time of exposure to CPAs used in the other protocol might lead to toxic effects, which impaired the further embryonic development of vitrified oocytes.

These findings strongly indicate that the presence of cumulus cells might be adjusted with other variables, such as the concentration of CPAs and time of exposure, which are critical for the development of a successful vitrification protocol.

In **Chapter 5**, another variable, which influences the vitrification of oocytes and embryos, was evaluated. It is known that the presence of serum during culture reduces the cryotolerance of oocytes and blastocysts. In a first part of our study, we investigated the vitrification of bovine oocytes matured in two conditions: in presence of serum, glutamine and pyruvate or in absence of serum but with epidermal growth factor (EGF, **Chapter 5.1**). Our results showed that fertilization is not influenced by the maturation condition, but it was drastically decreased after vitrification. When

embryonic development was studied in a second experiment, we observed how the presence of serum reduced the cleavage rate of vitrified oocytes.

Vitrification is considered a stressful process that leads to hemichannels opening in cells. This produces the loss of different molecules and ions that are important for cell survival. It has been demonstrated that the inclusion of a mimetic connexin peptide closes these hemichannels, improving cells cryopreservation. In analogy with the previous study, we further investigated if such an effect also occurred in vitrified blastocysts resulting from oocytes matured in serum or EGF conditions, and we included a mimetic peptide in order to improve the vitrification of blastocysts (**Chapter 5.2**). We observed that vitrification induces opening of the hemichannels in blastocysts derived from oocytes matured in the presence of serum. Although opening of hemichannels is related to increased apoptotic cell rates, we could not observe such an effect when hemichannels were opened. Interestingly, the inclusion of the mimetic peptide closed hemichannels during vitrification, improving the embryonic development of vitrified blastocysts matured in the serum condition. On the other hand, vitrification did not result in hemichannels opening in blastocysts matured in EGF condition. Apparently, EGF might have an effect on hemichannels by modifying their activity. Additional experiments are required to confirm this effect and to investigate the opening of hemichannels under different conditions.

Finally, in **chapter 6**, the main results of this thesis are summarized and discussed. Research was focussed on the optimization of the vitrification conditions for bovine mature oocytes, equine immature oocytes and bovine blastocysts. Our results indisputably demonstrated that the presence of cumulus cells impaired the vitrification of oocytes in both cattle and horses and that their effect needs to be addressed with other variables such as the concentration and time of exposure to CPAs. Therefore, partial removal of the cumulus cells (CR) is advised for the vitrification of bovine mature oocytes in order to allow penetration of CPAs, while maintaining fertilization potential. In the horse, blastocyst development was achieved in immature CRs vitrified by short exposure to high concentrations of CPAs. Finally, vitrification induced opening of hemichannels in bovine blastocysts matured in the presence of serum, impairing their competence. This could be alleviated by the use of a mimetic peptide or by replacing serum by EGF during maturation.

SAMENVATTING

Cryopreservatie van gameten en embryo's vormt een belangrijk onderdeel van de geassisteerde voortplantingstechnieken omdat het kan gebruikt worden om genetisch materiaal voor langere tijd te bewaren. De introductie van vitrificatie resulteerde in een veelbelovende techniek voor de cryopreservatie van eicellen en embryo's. De capaciteit van eicellen en embryo's om verder te ontwikkelen is echter aangetast na het opwarmen. In **Hoofdstuk 1** wordt een overzicht gegeven van de moeilijkheden die bij de vitrificatieprocedure komen kijken, evenals een beschrijving van de verschillende variabelen die deze procedure en de invriesbaarheid van eicellen en embryo's beïnvloeden.

De algemene doelstelling van deze thesis was om de effectiviteit van de vitrificatie van eicellen en embryo's te verbeteren bij het rund en het paard (**Hoofdstuk 2**). Daartoe hebben we twee variabelen onderzocht die de vitrificatie beïnvloeden: (1) de aanwezigheid van cumuluscellen rondom de eicel gedurende de vitrificatie en hun invloed op de invriesprocedure en (2) het effect van eicelrijping in de aanwezigheid van serum op de vitrificatie van eicellen en blastocysten.

In **Hoofdstuk 3** werd specifieke aandacht gegeven aan de rol van cumuluscellen rondom de rijpe rundereicel tijdens de vitrificatie van deze eicel. Het is reeds geweten dat cumuluscellen een fundamentele rol spelen tijdens de eicelrijping, de bevruchting en de ontwikkeling van blastocysten. Ze zouden echter de uitwisseling van water en cryoprotectanten (CPAs) kunnen verhinderen, wat zou leiden tot onvoldoende bescherming van de eicel tijdens de vitrificatie. Onze resultaten toonden aan dat de aanwezigheid van meerdere lagen cumuluscellen rondom de eicel een nadelig effect had op de overleving van deze eicel na vitrificatie. Anderzijds werden na totale verwijdering van de cumuluscellen gelijkaardige overlevingspercentages geobserveerd bij de gevitrificeerde, gedenudeerde eicellen en hun verse tegenhangers.

Bij het rund is het geweten dat het verwijderen van cumuluscellen de bevruchtingspercentages van de eicellen vermindert. Daarom hebben we in onze studie drie verschillende benaderingen gebruikt om onderscheid te maken tussen 'vitrificeerbaarheid' en 'bevruchtbaarheid'. In eerste instantie werden gevitrificeerde, gedenudeerde eicellen bevrucht in de aanwezigheid van intacte cumulus-eicel-complexen (COCs). In tweede instantie werden eicellen met een corona radiatia (CR) gebruikt als compromis tussen COCs en gedenudeerde eicellen. Tenslotte werden gevitrificeerde eicellen parthenogenetisch geactiveerd. Onze resulaten toonden aan dat het verwijderen van cumuluscellen voor vitrificatie leidde tot een verminderde bevruchting van de gevitrificeerde, gedenudeerde eicellen. Een interessante bevinding hierbij was dat deze situatie hersteld kon worden wanneer de gevitrificeerde, gedenudeerde eicellen bevrucht werden in de aanwezigheid van verse COCs. Ten tweede zagen we dat het verwijderen van de buitenste cumuluslagen op zich (CR eicellen) reeds

resulteerde in een verminderde bevruchting van verse eicellen in vergelijking met eicellen die omgeven worden door meerdere lagen cumuluscellen. Zo'n effect werd niet gedetecteerd bij de gevitrificeerde CR eicellen, waarbij de bevruchting en de embryonale ontwikkeling gelijkaardig waren aan deze van de gevitrificeerde COCs. Verrassend genoeg was de capaciteit om tot blastocyst te ontwikkelen van gevitrificeerde CR eicellen gelijkaardig aan deze van verse eicellen na parthenogenetische activatie.

Over het geheel bekeken, tonen onze resultaten duidelijk aan dat de aanwezigheid van cumuluscellen het binnendringen van CPAs kan verminderen met een minder goede vitrificatie van rijpe rundereicellen tot gevolg en daarom is het aan te raden om de buitenste lagen cumuluscellen te verwijderen.

Als cumuluscellen de uitwisseling van CPAs verminderen, zou het gebruik van hogere concentraties CPAs het binnendringen in de cellen kunnen versnellen. Daarom werden in **Hoofdstuk 4** twee protocollen geëvalueerd (korte blootstelling aan hoge concentraties CPAs en lange blootstelling aan lagere concentraties CPAs) voor de vitrificatie van onrijpe paardeneicellen, omgeven door enkele (CR) of meerdere lagen cumuluscellen (COCs). In onze studie zagen we een verminderde eicelrijping wanneer COCs werden gevitrificeerd door korte blootstelling aan hoge concentraties CPAs. Gelukkig kon deze situatie hersteld worden door het gebruik van CR eicellen of door blootstelling van COCs aan lagere concentraties CPAs voor langere tijd. Deze bevindingen ondersteunen onze observaties van **Hoofdstuk 3**, waarbij de aanwezigheid van meerdere lagen cumuluscellen rondom de eicel de beweging van CPAs en water verhindert, waardoor een langere blootstelling nodig is voor een correcte bescherming.

Bijgevolg werd in de volgende studies niet verder gewerkt met COCs en werden enkel CR eicellen gebruikt om het effect van twee verschillende protocollen op de spoelfiguur en de embryonale ontwikkelin van gevitrificeerde onrijpe paardeneicellen na te gaan (**Hoofdstuk 4**). Onze gegevens toonden aan dat beide protocollen voor vitrificatie resulteerden in hogere percentages abnormale spoelfiguren bij de gevitrificeerde eicellen dan bij de verse. Een interessantere bevinding was dat de ontwikkeling tot blastocyst enkel plaatsvond bij de eicellen die gevitrificeerd waren door middel van korte blootstelling aan hoge concentraties CPAs. De transplantatie van dergelijke blastocysten resulteerde in drachten, met uiteindelijk de geboorte van een gezond hengstveulen tot gevolg, op 12 mei 2017, een wereldprimeur. De langere blootstelling aan CPAs die werd gebruikt in het andere protocol zou kunnen leiden tot toxische effecten die de verdere embryonale ontwikkeling van de gevitrificeerde eicellen verhinderden.

Deze bevindingen wijzen erop dat naast de aanwezigheid van cumuluscellen ook andere variabelen, zoals de concentratie CPAs en de blootstellingstijd, van kritiek belang zijn bij de ontwikkeling van een succesvol protocol voor vitrificatie.

In **Hoofdstuk 5** werd nog een andere variabele die de vitrificatie van eicellen en embryo's beïnvloedt bestudeerd. Het is geweten dat de aanwezigheid van serum tijdens de cultuur de invriesbaarheid van eicellen en blastocysten vermindert. In het eerste deel van onze studie hebben we de vitrificatie onderzocht van rudereicellen na rijping onder twee verschillende omstandigheden: in aanwezigheid van serum of in afwezigheid van serum maar na toevoeging van epidermale groeifactor (EGF, **Hoofdstuk 5.1**). Onze resultaten toonden aan dat bevruchting niet beïnvloed door de omstandigheden voor eicelrijping, maar was ze drastisch gedaald na vitrificatie. Wanneer de embryonale ontwikkeling werd bestudeerd in een tweede experiment, zagen we hoe de aanwezigheid van serum de deling van de gevitrificeerde eicellen verminderde.

Vitrificatie wordt beschouwd als een stressvol proces dat leidt tot de opening van 'hemichannels' in cellen. Hierdoor gaan verschillende moleculen en ionen verloren die van belang zijn voor het overleven van de cel. Er werd aangetoond dat de toevoeging van een mimetisch connexin peptide deze 'hemichannels' kan sluiten met een betere invriesbaarheid van cellen tot gevolg. Naar analogie hiermee hebben we verder onderzocht of zo'n effect ook optreedt bij gevitrificeerde blastocysten die voortkomen uit eicellen gerijpt met serum of EGF en we hebben hierbij gebruik gemaakt van een mimetisch peptide om de vitrificatie van de blastocysten te verbeteren (**Hoofdstuk 5.2**). We zagen dat vitrificatie leidt tot de opening van de 'hemichannels' bij blastocysten die voortkomen uit eicellen die gerijpt werden in aanwezigheid van serum. Hoewel opening van 'hemichannels' gerelateerd werd aan hogere percentages apoptotische cellen, hebben wij dit effect niet geobserveerd. Interessant was dat de toevoeging van het mimetisch peptide resulteerde in de sluiting van de 'hemichannels' tijdens de vitrificatie, met een verbeterde embryonale ontwikkeling van de gevitrificeerde blastocysten na rijping in serum tot gevolg. Anderzijds leidde de vitrificaite van blastocysten na rijping in EGF niet tot de opening van de 'hemichannels'. Blijkbaar zou EGF de activiteit van de 'hemichannels' kunnen beïnvloeden. Verder onderzoek is nodig om dit effect te bevestigen en om de opening van 'hemichannels' onder verschillende omstandigheden te onderzoeken.

Tenslotte worden de belangrijkste resultaten van deze thesis samengevat en bediscussieerd in **Hoofdstuk 6**. Het onderzoek was gericht op de optimalisatie van de omstandigheden voor vitrificatie van rijpe rundereicellen, onrijpe paardeneicellen en runderblastocysten. Onze resultaten tonen duidelijk aan dat de aanwezigheid van cumuluscellen de vitrificatie van eicellen nadelig beïnvloedde bij zowel het rund als het paard en dat bij dit effect ook rekening moet gehouden worden met

andere variabelen zoals de concentratie CPAs en de duur van de blootstelling hieraan. Daarom wordt geadviseerd om de cumuluscellen gedeeltelijk te verwijderen (CR) voor de vitrificatie van rijpe rundereicellen omdat op die manier de CPAs de eicel kunnen binnendringen, terwijl ook de bevruchtingscapaciteit behouden blijft. Bij het paard werd de ontwikkeling van blastocysten bekomen na vitrificatie van onrijpe CRs door korte blootstelling aan hoge concentraties CPAs. Tenslotte leidde vitrificatie van runderblastocysten na rijping in aanwezigheid van serum tot de opening van 'hemichannels', met een verminderde ontwikkeling tot gevolg. Dit effect kon tegengegaan worden door gebruik te maken van een mimetisch peptide of door serum te vervangen door EGF tijdens de eicelrijping.

CURRICULUM VITAE

Nerea Ortiz Escribano was born on January 6th, 1988 in Murcia, Spain. She completed her secondary studies in I.E.S Valle del Segura in 2006, in Murcia. In 2011, she graduated in Biology at the University of Murcia.

In 2012, Nerea ended successfully a post graduate study in ´Biology and Technology of Reproduction in Mammals´ at the Faculty of Veterinary from the University of Murcia. That same year, she was awarded with an Erasmus scholarship from the European Commission, in order to conduct the research part of the postgraduate thesis as student at the Faculty of Veterinary Medicine at Ghent University (Belgium) for three months. In 2013, she successfully obtained a grant from IWT (Agentschap voor Innovatie door Wetenschap en Technologie) for a full doctoral training program at the Faculty of Veterinary Medicine at Ghent University (Belgium).

Nerea is author and co-author of several studies published in international peer reviewed journals. Her experimental work has been presented at various international conferences.

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