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Factors that affect oocyte vitrification in small ruminants*

Fatores que afetam a vitrificação de oócitos em pequenos ruminantes

Dowglish Ferreira Chaves,** Joanna Maria Gonçalves de Souza-Fabjan,** Pascal Mermillod,***
Vicente José de Figueirêdo Freitas**

Abstract

At the end of its growth, the mammalian oocyte, include in preovulatory follicle, is the largest cell of the organism, with about 120 µm in diameter. The size of oocyte, together with the specific arrangement of its organels and cytoskeleton, makes a real challenge for cryopreservation techniques. For such large cell, methods that do not require equilibrium to cryopreservation, such as vitrification, are more promising. It is important to highlight that the cryopreservation of oocytes is more difficult than zygotes or later stage embryos and this technique is a challenging task because of oocyte sensitive nature to chilling and toxic effects of cryoprotectants. However, the development of a reliable method for oocyte cryopreservation would be an important advance in the field of reproductive biology for the preservation of genetic resources. The vitrification of mammalian oocytes is influenced by many variables, such as different cryoprotectants, vitrification techniques, presence or absence of *cumulus* cells, the oocyte structure, metabolism (such as level of lipid storage) and meiotic stage. Thus, all these factors should be considered to optimize the techniques and adapt them to oocytes from each species. Therefore, the present review aims to describe the main factors that affect oocyte vitrification in sheep and goats, reporting the main findings in both species, as well as perspectives of future improvements.

Keywords: COC, cryopreservation, gamete, goat, sheep.

Resumo

No fim do seu crescimento, o oócito mamífero é a maior célula do organismo, com cerca de 120 µm de diâmetro. O tamanho do oócito em conjunto com a disposição específica das organelas e do citoesqueleto faz com que ele represente um verdadeiro desafio para as técnicas de criopreservação. Para grandes células, métodos que não exigem equilíbrio para criopreservação, como a vitrificação, são mais promissores. É importante ressaltar que a criopreservação de oócitos é mais difícil que de zigotos ou embriões em estádios mais tardios e esta técnica representa um desafio devido à natureza sensível do oócito ao resfriamento e aos efeitos tóxicos de crioprotetores. Entretanto, o desenvolvimento de uma técnica confiável para a criopreservação oocitária representaria um avanço importante para a preservação de recursos genéticos. A vitrificação de oócitos em mamíferos é influenciada por muitas variáveis, tais como diferentes crioprotetores, técnicas de vitrificação, presença ou ausência de células do *cumulus,* estrutura do oócito, metabolismo (como o nível de armazenamento de lipídios) e estágio meiótico. Assim, todos esses fatores devem ser considerados quando o objetivo é otimizar as técnicas e adaptá-las para oócitos de cada espécie. Assim, a presente revisão tem como objetivo descrever os principais fatores que afetam a vitrificação de oócitos em ovinos e caprinos, relatando os principais resultados em ambas as espécies, bem como as perspectivas de melhorias futuras.

Palavras-chave: CCO, caprino, criopreservação, gameta, ovino.

Introduction

At the end of its growth, the oocyte is the larger cell of the organism, with about 120 µm in diameter. The oocyte is gathering morphological and functional specificities that make difficult its cryopreservation under conventional controlled freezing methods. Indeed, the size of the oocyte, together with the specific arrangement of its organels and cytoskeleton, makes it a real challenge for cryoconservation techniques. For such large cell, non equilibrium cryopreservation methods, such as vitrification, are more promising. In addition, the cell cycle of the oocyte is finely controlled and stopped at very specific stages, requiring taking into account the specific nuclear configuration of these stages. Cryopreservation of oocytes is more difficult than zygotes and later stage embryos (Kharche et al., 2005) and this technique is a challenging task because of its sensitive nature to chilling (Bhat et al., 2013) and to toxic effects of cryoprotectants. However, this biotechnological tool has a number of potential applications such as biodiversity maintenance and development of breeding programs. In the research field, oocyte banking may be of utmost importance to increase the availability of oocytes for research applications such as genetic engineering or embryo cloning (Ambrosini et al., 2006). Therefore, the development of a reliable method for the cryopreservation of mammalian oocytes would be an important advance for the preservation of genetic resources (Bogliolo et al., 2007).

Vitrification is an approach that uses the combination of high concentrations of cryoprotective agents and rapid temperature decrease, resulting in a glass amorphous solidification solution

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*Laboratory of Physiology and Control of Reproduction, Veterinary School, State University of Ceará, Av. Dr. Silas Munguba, 1700, 60740-90, Fortaleza, CE, Brazil.
***INRA, UMR7247, Physiologie de la Reproduction et des Comportements, INRA, CNRS, Université de Tours, Haras Nationaux. 37380 Nouzilly, France.
Corresponding author. Tel.: +55 85 3101-9861. E-mail address: joannavet@gmail.com (J.M.G. Souza-Fabjan)

without the formation of ice crystals (Rall, 1987; Vajta, 2000). Interestingly, this technique is considered substantially less toxic to cells such as oocytes and early embryos which naturally exhibit high sensitivity to slow freezing (Vajta et al., 1998) and in which the size of the cells does not allow to reach the equilibrium with external milieu, required for slow freezing. Thus, this tool can be a good alternative in the establishment of female germplasm collection, facilitating the management of genetic resources and improving basic and applied research (Kharche et al., 2005; Succu et al., 2007b).

Different approaches have been used to identify and overcome the main barriers of oocyte vitrification have been reported in order to obtain better results in post-warming survival, as evaluated by *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and embryo development. In this perspective, the present review aims to describe the usual factors that affect oocyte vitrification in sheep and goats, reporting the main results for these species as well as describing improvements to existing technical perspectives.

Main damages to vitrified oocytes

Oocyte vitrification may cause damages at structural, ultrastructural and molecular levels. Indeed some studies have demonstrated that the vitrification process in sheep oocytes caused damages on morphology, ultrastructure, meiotic spindle and chromosomal arrangement (Succu et al., 2007a; Ebrahimi et al., 2010b; Ebrahimi et al., 2012). These changes can be classified as either reversible or irreversible. An example of irreversible change is the hardening of zona pellucida caused by premature release of cortical granules or by inappropriate environment. This is considered as the main cause of failed fertilization in oocytes after vitrification/warming (Larman et al., 2006; Asgari et al., 2012). In contrast, changes that can be restored after warming are considered reversible, as for example some alterations at the spindle, chromosomes and perivitelline space (Succu et al., 2007a).

Bogliolo et al. (2007) found reduced viability and meiotic competence in vitrified sheep immature *cumulus* oocyte complexes (COC), possibly resulting from an extensive loss of *cumulus* cell plasma membrane integrity, as well as from a drastic reduction in gap-junction communication between oocytes and the surrounding cells during the cryopreservation procedures. In addition to these changes, it has been described that the microtubular structure of oocyte meiotic spindle is highly sensitive to physical (chilling/warming) and chemical (exposure to cryoprotectants) events. Therefore, the vitrification process can trigger the depolymerization of tubulin and microtubule disassembly, presenting deleterious effects on the chromosomal constitution of matured vitrified oocytes (Succu et al., 2007a).

In prepubertal matured sheep oocytes the vitrification resulted in drastic alterations in meiotic spindle and subsequent chromosomal dispersion. The authors suggested that ethylene glycol (EG), dimethylsulfoxide (DMSO) and sucrose (SUC) exert major toxic effects on this cell type, since oocytes only exposed to vitrification solutions showed similar damage as vitrified oocytes (Succu et al., 2007b). Conversely, using the same vitrification solutions and protocol they showed that in ovine adult matured oocyte, spindle morphological configuration was altered after vitrification but not after the only exposition to vitrification solutions (Succu et al., 2007a).

In goats, after vitrification/warming, 19% of the immature oocytes showed reduced mass of *cumulus* cells, clear ooplasm and ruputure or abnormally of zona pellucida (Kharche et al., 2005). Sharma et al. (2006) reported that 16% of caprine oocytes were damaged as a result of vitrification, showing zona nicking, zona rupture, dissolution of the zona, cytoplasm squeezing and changes in the shape of the oocytes. Vitrified oocytes had a significantly lower fertilization rate (8%) when compared to the not exposed and not vitrified control (17%), possibly due to chromosomal abnormalities caused by the vitrification process.

Cryoprotectants

The cryoprotectants (CP) commonly used for oocyte vitrification are classified as penetrating or non-penetrating. Some examples of penetrating are EG, propylene glycol (PG), glycerol (GLY) and DMSO, that are characterized by small molecules which easily cross cell membrane. These compounds act by forming hydrogen bonds with water molecules, thus preventing ice crystallization (Jain and Paulson, 2006). In contrast, SUC and trehalose (TRE) are non-penetrating CP, which remain in the extracellular environment, increasing external osmotic pressure, resulting in cell dehydration (Guignot, 2005). On warming, these compounds also act as an osmotic buffer, preventing excessive water inflow into the hyperosmotic intracellular medium, avoiding the “cell swelling” or even rupture (Jain and Paulson, 2006).

Vitrification requires high concentrations of CP. It is therefore important to minimize the damage caused to cells by the osmotic stress or chemical toxicity (Arav, 2014). Survival of sheep *in vitro* matured oocytes may be improved by step wise dilution of the cryoprotectant, which enabled high survival, cleavage and blastocyst rates of the vitrified oocytes (Bhat et al., 2013). These authors have been conducting research to improve sheep oocyte vitrification. Comparing vitrification solution with EG or DMSO, morphological changes occurred in only 10% of the oocytes when using 40% EG, while for the same concentration of DMSO changes were of 25%. These structures when subjected to parthenogenetic activation resulted in cleavage rate higher for EG than for DMSO, 51% and 34% respectively. The same was observed for blastocyst production with rates of 18% for EG and 10% for DMSO. The authors suggested that EG would be the CP of choice due its high penetration in oocytes with, in turn, a quick removal after warming (Bhat et al., 2013).

When evaluating the use of EG, PG or DMSO associated with, respectively, 0.5, 1.0 or 1.5 M of SUC, 5.5 M EG and 5.0 M PG showed a higher cleavage rate after IVF (20-21%) than 4.5 M of DMSO. DMSO provided a good survival rate post-warming, but the cleavage rate was low (16%), possibly due to cytological damages (Dike, 2009). Another explanation may be due to the toxicity or osmolarity shock caused in these oocytes exposed to CP (Purohit et al., 2012).

The strategy of CP association is commonly used to reduce the ‘solution effect’, which causes toxicity and osmotic injury to the oocytes. This mechanism is induced by high concentrations of CP. Among these methods, the use of non-penetrating CP is very useful for two reasons: 1) the shrinkage of the oocyte and consequently the amount of water inside the cell that may crystallize during rapid cooling and warming is lower and 2) the reduction of the amount of the CP that penetrates the cell thus reducing the possible toxic effect (for review see Arav, 2014).

It has been demonstrated that EG and DMSO presents negative aspects regarding the induction of complete release of cortical granules and the formation of female pronuclei in sheep oocytes. The authors noted that vitrification solutions containing DMSO and EG could induce parthenogenetic activation of *in vitro* matured ovine oocytes (Tian et al., 2007). In mice, CP such as EG, DMSO and propanediol (PrOH), when used in vitrification protocols caused an increase in intracellular calcium level in matured oocytes. However, this mechanism is not fully understood. It has been proposed that the lipophilic properties of these agents can exert nonspecific effect on the plasma membrane and other internal membranes such as the endoplasmic reticulum. This process would lead to an influx of extracellular calcium and/or liberation of internal calcium (Larman et al., 2006; 2007), resulting in parthenogenetic activation. After removing extracellular calcium, the exposure to EG and PrOH did not induce any change in the oocyte. However, the same behavior was not observed for DMSO, which seems to recruit calcium from intracellular stores (Larman et al., 2007).

The vitrification of matured prepubertal sheep oocytes showed high sensitivity to both low temperature and CP exposure (EG and DMSO) suggesting that the ability to recover injuries is determinant in the successful applications of cryopreservation procedures. Damages to meiotic spindle were observed in 64% of vitrified oocytes and in 57% of oocytes only exposed to CP, with cleavage rates of 21 and 45% and blastocyst production of 0 and 3%, respectively (Succu et al., 2007b).

Differents concentrations of GLY and EG associated with 0.5 M sucrose were evaluated in goat COC. The results demonstrated that at concentrations of 6 and 8 M of EG no morphological alterations occurred in 95% and 94% of the oocytes, respectively. Nuclear maturation and fertilization of vitrified oocytes was higher with increasing concentration of both GLY or EG up to 8 M, but at 10 M, the proportion of oocytes matured or fertilized decreased significantly. The optimum vitrification solution was up to 8 M concentration of GLY and EG, while EG is a better CP for ultrarapid freezing of goat oocytes (Garg and Purohit, 2007).

In sheep, the use of 5.0 M PG associated with 1 M or 1.5 M of SUC resulted in better cleavage rates of 29 and 33%, respectively, and lower morphological damage when compared with the association 0.5 M and 2.0 M of SUC. This study emphasized that SUC is an important CP to maintain the oocyte structure. However, the use of 2 M had a deleterious effect on cleavage rate, indicating that very high concentration can cause internal cellular damage (Dike and Obembe, 2013). In goats, the use of 40% PG associated with 0.25 M TRE promoted 94% of post-warming oocyte survival, but only 8% of them exhibited extrusion of polar body after IVF (Sharma et al., 2006).

The use of 40% PrOH for immature goat oocyte vitrification caused nuclear maturation lower than 74% obtained in control group. The authors inferred that the low results may be mainly due to detrimental effects of CP. However, the vitrification procedure was also considered detrimental since groups of oocytes only exposed to CP (not vitrified) showed higher rates than vitrified group (Kharche et al., 2005).

After oocyte vitrification, the reduction in IVM rates may indicate a lack of competence after both, exposure to CP agents and vitrification process. In sheep, both factors significantly decreased the rate of oocyte maturation compared with non-vitrified control oocytes. In addition, a high number of oocytes without signs of sperm penetration after IVF was observed (Moawad et al., 2012). The Table 1 compiles some studies on oocyte vitrification in goats and sheep using different CP and later *in vitro* analysis.

Table 1: Vitrification method, meiotic stage, equilibration solution, equilibration time, vitrification solution, vitrification time, rates of *in vitro* cleavage or *in vitro* fertilization (IVF) and blastocyst production in vitrified goat and sheep oocytes

Species	Method	Meiotic Stage	Equilibration solution	Equilibration time	Vitrification solution	Vitrification time	% Cleav or IVF*	% Blast	Reference
Goat	Covent. straw	GV	40% PG, 0.25 M TRE	10 min	40% PG, 0.25 M TRE	-	18*	-	Kharche et al. (2005)
Goat	Covent. straw	GV	4.0 M EG, 0.25 M SUC	3-5 min	8.0 M EG, 0.5 M SUC	1 min	32*	-	Garg and Purohit (2007)
Goat	Covent. straw	GV	4.0 M EG, 0.25 M SUC	3.5 min	8.0 M EG, 0.5 M SUC	2-3 min	32*	-	Purohit et al. (2012)
Sheep	Cryoloop	MII (denude)	7.5% EG, 7.5% DMSO	3 min	16.5% EG, 16.5% DMSO, 0.5 M SUC	20 sec	21	0	Succu et al. (2007b)
Sheep	New device	MII	7.5% EG, 7.5% DMSO	7 min	15% EG, 15% DMSO, 0.5 M SUC	-	47	1.5	Shirazi et al. (2012)
Sheep	Solid surface vitrification	GV	4% EG	6-10 min	35% (v/v) EG, 5% PVP, 0.4M TRE	< 60 sec	10	0,5	Moawad et al. (2012)
Sheep	Covent. straw	MII	5.0 M PrOH, 1.5 SUC	45 sec	5.0 M PrOH, 1.5 SUC	-	33	-	Dike and Obembe (2013)
Sheep	Open pulled straw	MII (denude)	10% EG, 10% DMSO, 0.5 M SUC	30 sec	15% EG, 15% DMSO, 18% Ficoll, 0.5 M SUC	25 sec	49	6.9	Mo et al. (2014)

[#] Convent. straw: Conventional straw; PrOH: propanediol; PG: Propylene glycol; TRE: trehalose; EG: Ethylene glycol; SUC: sucrose; DMSO: dimethylsulfoxide; PVP: polyvinylpyrrolidone

Techniques of vitrification

Several techniques have been used for vitrification of sheep and goat oocytes, such as conventional straw, open pulled straw (OPS), hemistraw, solid surface vitrification (SSV), cryotop and cryoloop, to increase rates of post-warming survival and subsequent *in vitro* development (Ebrahimi et al., 2010a; Fernández-Reyez et al., 2012; Rao et al., 2012; Bhat et al., 2013). The objective of these techniques is to reduce the volume of solution and improve the contact with nitrogen to increase the cooling speed and allows the occurrence of vitrification of solution even with lower concentrations of CP (less toxic). However, some aspects still need to be clarified, as demonstrated by the few studies comparing different techniques in small ruminant oocytes (Ebrahimi et al, 2012; Rao et al., 2012).

For vitrification of sheep oocytes, it is suggested that depending on the technique, a reduced volume of CP results in an increased viability after warming, allowing improved IVM, IVF and embryo development rates (Arav et al., 2002; Fernández-Reyez et al., 2012), probably due to accelerated cooling rate of reduced volume. In this sense, the negative effects of CP can be reduced by using lower concentrations and a minimum volume and direct contact in liquid nitrogen, allowing fast cooling rate and, in turn, the vitrification of low CP concentration solution. Another important aspect is to ensure that, during immersion, the oocytes are surrounded with liquid nitrogen and not vapor, providing a better caloric exchange (Moawad et al., 2012).

The OPS, hemistraw, cryotop, SSV and cryoloop were used for the vitrification of goat oocytes. COC vitrified using cryotop in the same cryoprotectant solution used for other techniques showed an increase in the potential to complete meiotic maturation post-warming, inferred by higher proportion of polar body extrusion and visualization of the metaphase II plate. Oocytes vitrified with OPS and conventional straw showed lower survival, maybe due to larger volume of vitrification solution (Rao et al., 2012), as reported in sheep (Ebrahimi et al., 2010a).

Regarding the induction of cell death through apoptosis, it is possible to monitor the proportion of BCL2 (anti-apoptotic) and BAX (pro-apoptotic) genes to evaluate the efficiency of different techniques. Goat COC vitrified by either OPS or conventional straw showed an increase in expression of BAX, promoting degeneration and, consequently, low oocyte IVM rates. In contrast, the expression of BCL2 in oocytes vitrified with cryotop and hemistraw was superior, with a low rate of degeneration and higher IVM rate (Rao et al., 2012).

Ovine oocytes vitrified using cryotop technique presented apoptotic genes expression similar to control oocytes. These results demonstrated that the use of this technique was not sufficient to activate the apoptotic process. However, induced numerical chromosomal abnormalities that reduced IVM rates was observed (Ebrahimi et al., 2010b). Succu et al. (2007a) also reported chromosomal damages in 93% of oocytes vitrified with OPS, 83% with cryotop, and 63% with cryoloop when vitrified in the same concentration of cryoprotectants.

Cumulus cells: COC vs denuded oocytes

For oocyte vitrification there is the option to use either COC or denuded oocytes and to evaluate the most suitable and least harmful way is imperative for the success of the technique. Few investigators have paid sufficient attention to the consequences of the presence or absence of *cumulus* cells on oocyte survival following cryostorage, and the results are still controversial (Bogliolo et al., 2007). However, it is well known that the presence of viable *cumulus* cells and functional junctions are necessary for meiotic resumption and the success of cytoplasmic maturation (Shirazi et al., 2007). The vitrification in small ruminant oocytes has been performed at different moments of its development, and with or without *cumulus* cells (Figure 1).

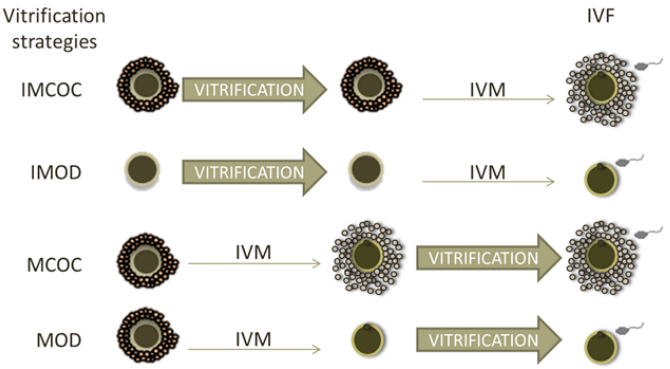

Figure 1: Strategies for oocyte vitrification in small ruminants regarding the stage of *in vitro* maturation (IVM) and the presence of *cumulus* cells. Vitrification: immature *cumulus* oocyte complexes (COC) after collection (IMCOC); immature oocytes and denuded after collection (IMOD); COC previously submitted to IVM (MCOC) and oocytes denuded after IVM (MOD).

Cumulus cells are functionally and physically connected to the oocyte, establishing a sophisticated network of mutual interaction, which ultimately confers full development competence to the oocyte (Bogliolo et al., 2007). The presence of *cumulus* cells can minimize the migration of cortical granules and avoid premature hardening reaction of the zona pellucida, which consequently improve the fertilization rates after cryopreservation/warming (Vincent et al., 1990).

The effect of *cumulus* cells on the quality of vitrified oocytes was well described in the literature (Kharche et al., 2005; Nema et al., 2007; Zhang et al., 2009; Purohit et al., 2012), whereas Bogliolo et al. (2007) stated that denudation of immature ovine oocytes before IVM and prior to vitrification produced an increase in oocyte survival and maturation ability. In goats, in matured oocytes, *cumulus* cells demonstrated to be important when attached to oocytes offering some protection against cryodamages due to vitrification, leading to a better post-warming morphological maintenance and oocyte survival (Purohit et al., 2012). The presence of *cumulus* cells was also important for enhancing IVM rates, with superior results for goat COC in comparison to denuded oocytes (Nema et al., 2007; Purohit et al., 2012). However, after IVM, the number of vitrified oocytes with abnormal spindle and chromatin configuration was significantly higher compared to control oocytes, independently of the presence or absence of *cumulus* cells (Bogliolo et al., 2007).

No effect of *cumulus* cells on vitrification of ovine matured oocytes was detected on survival, cleavage and blastocyst rates (Zhang et al., 2009). Mo et al. (2014) also reported similar results for survival, cleavage and blastocyst formation rates for ovine matured oocytes with or without *cumulus* cells after warming. However, Shirazi et al. (2012) reported that when vitrification solution contained 20% fetal bovine serum, higher rate of cleavage was observed for sheep COC (53%) than for

denuded oocytes (41%). Purohit et al. (2012) showed that after vitrification of immature goat COC, IVF rates were similar (32%) to those found in the non-vitrified control group (43%). These results demonstrated that *cumulus* cells were beneficial to oocyte vitrification, since the groups which have been denuded before or after IVM showed lower rates for IVF, 25 and 17%, respectively.

Meiotic stage: matured vs immature

The oocyte vitrification can be performed using two different meiotic stages: germinal vesicle stage (GV, immature) or MII (metaphase II, matured) oocytes (Fernández-Reyez et al., 2012).

When matured oocytes are used for vitrification better membrane stability during chilling may occur. However, exposure to low temperature induces damage determining changes in the meiotic spindle, which in turn may result in chromosomal aberrations, increasing in polyploidy and problems of fertilization (Chen et al., 2003; Tharasanit et al., 2006; Dike, 2009). On the other hand, chromosomes of immature oocytes would not be directly affected by the meiotic spindle of MII, since at GV stage, the genetic material remains uncondensed and confined within the nucleus (Bogliolo et al., 2007). However, there are still few studies that assess the effects of oocyte vitrification comparing the stages of maturation, especially in sheep and goats (Moawad et al., 2011).

The vitrification of immature ovine oocytes induced significant changes in spindle and chromatin configuration after IVM (Bogliolo et al., 2007). These results agree with Moawad et al. (2012) who mentioned that this form of vitrification negatively affected the IVM rate, resulting in meiotic resumption failure in 63% of oocytes. Shirazi et al. (2012) found that despite the lack of difference in survival rate of ovine oocytes vitrified at MII or GV, matured oocytes showed a higher resistance to vitrification with cleavage rate of 53%, higher than the rate obtained for immature oocytes (37%). Mo et al. (2014) also demonstrated that the meiotic status determines the ability of sheep oocytes to survive vitrification while MII oocytes showed the highest survival rates and developmental competence after vitrification.

Purohit et al. (2012) reported that immature *cumulu*s compact goat oocytes survived better to vitrification than immature ones. Their study showed that the proportion of fertilized oocytes after vitrification was higher in immature oocytes. The authors stated that although the effect of nuclear stage at cryopreservation is not fully understood, some reports suggested that GV stage is more resistant to cryodamage due to their smaller size, lack of cortical granules and a longer period to recover from cryoinjury.

In contrast, Quan et al. (2014) compared the vitrification of immature with MII goat oocytes and described that the rate of vitrified/thawed MII oocytes with normal morphology and cleavage rate after parthenogenetic activation were significantly higher than vitrified/thawed GV oocytes. These authors suggest that the tolerance of MII oocytes to vitrification and thawing may be more than that of the GV oocytes, so MII oocytes may be more suitable for vitrification. However, further studies are necessary to improve the vitrification procedure for small ruminant oocytes and their development to normal offspring.

Stabilizers of cytoskeleton

In an attempt to improve the efficiency of oocyte cryopreservation, some valuable research has been applied on the use of cytoskeleton stabilizers. In this perspective, cytochalasin B (CCB), an inhibitor of cytokinesis by disruption of microfilaments, can reduce microtubule damage during cryopreservation (Silvestre et al., 2006). Another cytoskeleton stabilizer is taxol, which has been reported to significantly improve the post warming development of vitrified oocytes in humans (Fuchinoue et al., 2004) and swine (Shi et al., 2006), probably because taxol inhibits microtubules depolymerization and thus prevent chromosome dispersion observed in bovine (Morató et al., 2008). It has been reported that cytoskeletal inhibitors like CCB can be used to reduce cryodamages in oocytes and cumulus cells. In the vitrification of bovine matured oocytes, CCB reduced damage and improved the stability of microtubules (Rho et al., 2002). Moreover, on bovine immature oocytes, CCB preserved the functionality of gap junctions between oocytes and cumulus cells (Vieira et al., 2002).

In sheep, the first reports of the use of CCB in oocyte vitrification have been described using COC of prepubertal sheep. In this study, no effect of CCB was detected on oocyte maturation capacity for groups vitrified or just exposed to vitrification solution (Silvestre et al., 2006).

However, a subsequent study using adult sheep immature oocytes revealed that CCB reduced significantly oocyte survival after vitrification. For the authors, a plausible explanation for the decrease in viability could not be directly linked to a cytotoxic effect, but the morphological changes induced in the structure of the cytoskeleton and oocyte plasma membrane that could result in an increased sensitivity to the process of oocyte vitrification (Bogliolo et al., 2007). Shirazi et al. (2012) reported that the pre-treatment with CCB had no effect on survival rate of adult sheep oocytes after warming. However, with respect to the cleavage rate, this study demonstrated a negative effect of CCB. Furthermore, the resulting blastocysts were only obtained with oocytes vitrified in the absence of CCB.

In contrast, Zang et al. (2009) showed that CCB pre-treatment of ovine matured oocytes following vitrification and IVF improved embryo developmental rate to blastocyst stage. The treatment with 7.5 or 10 µg/mL for 20-25 min was beneficial to ovine matured oocytes submitted to vitrification. For taxol, the same study reports that pre-treatment with 0.5 µM taxol for 20-25 min can significantly improve the development to the blastocyst stage of vitrified ovine mature oocytes, but higher concentrations of taxol (1 or 5 µM) may be harmful to ovine mature oocyte.

In a recent study, CCB had no significant effect on the developmental potential of vitrified ovine mature oocytes. However, when oocytes were pre-treated with taxol for 30 min before vitrification, the survival rate after vitrification and the developmental competence were improved. This study showed that the cryosurvival and cleavage rates of taxol treated oocytes were higher than for those treated with and control oocytes (Mo et al., 2014).

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Conclusions

It is reasonable to assume that to date there is no efficient method available for small ruminant oocytes vitrification and more research is necessary to improve the vitrification techniques. The ultra rapid techniques seem to provide some benefits for the quality of thawed oocytes in these species, probably by allowing reducing the concentration of cryoprotectants while maintaining the vitrification capacity of the solution. Regarding the cryoprotectants and meiotic stage, further studies are essential in order to guarantee which conditions would be better. The use of cytochalasin B still remains controversial, whereas taxol has been shown as a promising tool for the vitrification of sheep and goat oocytes. Therefore, oocyte physiology needs to be better understood in order to improve the success of this important technique in small ruminants.

References

AMBROSINI, G.; ANDRISANI, A.; PORCU, E.; REBELLATO, E.; REVELLI, A.; CASERTA, D.; COSMI, E.; MARCI, R.; MOSCARINI, M. Oocytes cryopreservation: state of art. *Reproductive toxicology*, v. 22, n. 2, p. 250-262, 2006.

ARAV, A. Cryopreservation of oocytes and embryos. *Theriogenology*, v. 81, n. 1, p. 96-102, 2014.

ARAV, A.; YAVIN, S.; ZERON, Y.; NATAN, D.; DEKEL, I.; GACITUA, H. New trends in gamete's cryopreservation. *Molecular and Cellular Endocrinology,* v. 187, n. 1-2, p. 77-81, 2002.

ASGARI, V.; HOSSEINI, S.M.; OSTADHOSSEINI, S.; HAJIAN, M.; AZHDARI, Z. T.; MOSAIE, M.; NASR-ESFAHANI, M.H. Specific activation requirements of *in vitro*-matured sheep oocytes following vitrification-warming. *Molecular Reproduction and Development*, v. 79, n. 7, p. 434-444, 2012.

BHAT, M.H.; YAQOOB, S.H.; KHAN, F.A.; WAHEED, S.M.; SHARMAB, V. ; VAJTAC, G.; GANAIA, N.A.; SHAHA, R.A. Open pulled straw vitrification of *in vitro* matured sheep oocytes using different cryoprotectants. *Small Ruminant Research*, v. 112, n. 1-3, p. 136-140, 2013.

BOGLIOLO, L.; ARIU, F.; FOIS, S.; ROSATI, I.; ZEDDA, M.T.; LEONI, G.; SUCCU, S.; PAU, S.; LEDDA, S. Morphological and biochemical analysis of immature ovine oocytes vitrified with or without *cumulus* cells. *Theriogenology*, v. 68, n. 8, p. 1138-1149, 2007.

CHEN, S.U.; LIEN, Y.R.; CHAO, H.N.; HO, Y.S.; YANG, T.Y.; LEE, T.Y. Effects of cryopreservation on meiotic spindles of oocytes and its dynamics after thawing: clinical implications in oocyte freezing – a review article. *Molecular and Cellular Endocrinology,* v. 202, n. 1, p. 101-107, 2003.

DIKE, I.P. Efficiency of intracellular cryoprotectants on the cryopreservation of sheep oocytes by controlled slow freezing and vitrification techniques. *Journal of Cell and Animal Biology*, v. 3, n. 3, p. 44-49, 2009.

DIKE, I.P.; OBEMBE, O.O. Effect of sucrose and propylene glycol on the vitrification of sheep oocytes. *Journal of Cell and Animal Biology*, v. 7, n. 3, p. 25-30, 2013.

EBRAHIMI, B.; VALOJERDI, M.R.; EFTEKHARI-YAZDI, P.; BAHARVAND, H.; FARROKHI, A. IVM and gene expression of sheep *cumulus*-oocyte complexes following different methods of vitrification. *Reproductive Biomedicine Online*, v. 20, n. 1, p. 26-34, 2010a.

EBRAHIMI, B.; VALOJERDI, M.R.; EFTEKHARI-YAZDI, P.; BAHARVAND, H. *In vitro* maturation, apoptotic gene expression and incidence of numerical chromosomal abnormalities following cryotop vitrification of sheep *cumulus*-oocyte complexes. *Journal of Assisted Reproduction and Genetics*, v. 27, n. 5, p. 239-246, 2010b.

EBRAHIMI, B.; VALOJERDI, M.R.; EFTEKHARI-YAZDI, P.; BAHARVAND, H. Ultrastructural changes of sheep *cumulus*–oocyte complexes following different methods of vitrification. *Zygote*, v. 20, n. 3, p. 103-115, 2012.

FERNÁNDEZ-REYEZ, F.; DUCOLOMB, Y.; ROMO, S.; CASAS, E.; SALAZAR. Z.; BETANCOURT, M. Viability, maturation and embryo development *in vitro* of immature porcine and ovine oocytes vitrified in different devices. *Cryobiology*, v. 64, n. 3, p. 261-266, 2012.

FUCHINOUE, K.; FUKUNAGA, N.; CHIBA, S.; NAKAJO, Y.; YAGI, A.; KYONO, K. Freezing of human immature oocytes using cryoloops with Taxol in the vitrification solution. *Journal of Assisted Reproduction* and *Genetics.* v. 21, n. 3, p. 307-309, 2004.

GARG, N.; PUROHIT, G.N. Effect of different cryoprotectant concentrations for ultrarapid freezing of immature goat follicular oocytes on their subsequent maturation and fertilization *in vitro*. *Animal Reproduction*, v. 4, n. 3-4, p. 113-118, 2007.

GUIGNOT, F. Cryoconservation des embryons des espèces domestiques. *INRA Production Animales*, v. 18, n. 1, p. 27-35, 2005.

JAIN, J.K.; PAULSON, R.J. Oocyte cryopreservation. *Fertility and Sterility*, v. 86, n. 4, p. 1037-1046, 2006.

KHARCHE, S.D.; SHARMA, G. T.; MAJUMDAR, A.C. *In vitro* maturation and fertilization of goat oocytes vitrified at the germinal vesicle stage. *Small Ruminant Research*, v. 57, n. 1, p. 81-84, 2005.

LARMAN, M.G.; SHEEHAN, C.B.; GARDNER, D.K. Calcium-free vitrification reduces cryoprotectant induced zona pellucid hardening and increases fertilization rates in mouse oocytes. *Reproduction*, v. 131, n. 1, p. 53-61, 2006.

LARMAN, M.G.; KATZ-JAFFE, M.G.; SHEEHAN, C.B.; GARDNER, D.K. 1,2- propanediol and the type of cryopreservation procedure adversely affect mouse oocyte physiology. *Human Reproduction*, v. 22, n. 1, p. 250-259, 2007.

MO, X.H.; FU, X.W.; YUAN, D.S.; WU, G.Q.; JIA, B.Y.; CHENG, K.R.; DU, M.; ZHOU, Y.H.; YUE, M.X.; HOUA, Y.P.; LI, J.J.; ZHU, S.E. Effect of meiotic status, *cumulus* cells and cytoskeleton stabilizer on the developmental competence of ovine oocytes following vitrification. *Small Ruminant Research*, v. 117, n. 2-3, p. 151-157, 2014.

MORATÓ, R.; MOGAS, T.; MADDOX-HYTTEL, P. Ultrastructure of bovine oocytes exposed to Taxol prior to OPS vitrification. *Molecular Reproduction and Development*. v. 75, n. 8, p. 1318-1326, 2008.

MOAWAD, A.R.; CHOI, I.; ZHU, J.; CAMPBELL, K.H.S. Ovine oocytes vitrified at germinal vesicle stage as recipient cytoplasts for somatic cell nuclear transfer (SCNT), *Cellular Reprogramming*, v. 13, n. 4, p. 289-296, 2011.

MOAWAD, A.R.; FISHER, P.; ZHU, J.; CHOI, I.; POLGAR, Z.; DINNYES, A.; CAMPBELL, K.H.S. *In vitro* fertilization of ovine oocytes vitrified by solid surface vitrification at germinal vesicle stage. *Cryobiology*, v. 65, n. 2, p. 139-144, 2012.

NEMA, S.; JAIN, S.K.; GEHLAUT, B.S.; SARKHEL, Vitrification of immature goat oocytes by solid surface vitrification technique. *Indian Journal of Animal Sciences*, v. 77, n. 7, p. 556-558, 2007.

PUROHIT, G.N.; MEENA, H.; SOLANKI, K. Effects of vitrification on immature and *in vitro* matured, denuded and *cumulus* compact goat oocytes and their subsequent fertilization. *Journal of Reproduction and Infertility*, v. 13, n. 15, p. 53-59, 2012.

QUAN, G.B.; LI, W.J.; LAN, Z.G.; WU, S.S.; SHAO, Q.Y.; HONG, Q.H. The effects of meiotic stage on viability and developmental capability of goat oocytes vitrified by the Cryoloop method. *Small Ruminant Research*, v. 116, n. 1, p. 32-36, 2014.

RALL, W. F. Factors affecting the survival of mouse embryos cryopreserved by vitrification. *Cryobiology*, v. 24, n. 5, p. 387-402, 1987.

RAO, B.S.; MAHESH, Y.U.; CHARAN, K.V.; SUMAN, K.; SEKHAR, N.; SHIVAJI, S. Effect of vitrification on meiotic maturation and expression of genes in immature goat *cumulus* oocyte complexes. *Cryobiology*, v. 64, n. 3, p. 176-184, 2012.

RHO G.J.; KIM, S.; YOO, J.G; BALASUBRANIAN, S.; LEE, H.J.; CHOE, S.Y. Microtubulin configuration and mitochondrial distribution after ultra-rapid cooling of bovine oocytes. *Molecular Reproduction and Development*, v. 63, n. 1, p. 464-470, 2002.

SHARMA, G.T.; KHARCHE, S.D.; MAJUMDAR, A.C. Vitrification of in vitro matured goat oocytes and the effect on in vitro fertilization. *Small Ruminant Research*, v. 64, n. 1-2, p. 82–86, 2006.

SHI, W.Q.; ZHU, S.E.; ZHANG, D.; WANG, W.H.; TANG, G.L.; HOU, Y.P.; TIAN, S.J. Improved development by Taxol pretreatment after vitrification of in vitro matured porcine oocytes. *Reproduction*, v. 131, n. 4, p. 795-804, 2006.

SHIRAZI, A.; SHAMS-ESFANDABADI, N.; HOSSEINI, S.M.; KARIMI, I. The presence of cumulus cells on nuclear maturation of sheep oocytes during in-vitro maturation. *Small Ruminant Research*, v. 68, n. 3, p. 291-295. 2007.

SHIRAZI, A.; TAHERI, F.; NAZARI, H.; NORBAKHSH-NIA, M.; AHMADI, E.; HEIDARI, B. Developmental competence of ovine oocyte following vitrification: effect of oocyte developmental stage, *cumulus* cells, cytoskeleton stabiliser, FBS concentration, and equilibration time. *Zygote*, v. 14, n. 1, p. 1-9, 2012.

SILVESTRE, M.A.; YÁNIZ , J.; SALVADORA, I.; SANTOLARIA, P.; LÓPEZ-GATIUS, F. Vitrification of pre-pubertal ovine *cumulus*-oocyte complexes: effect of cytochalasin B pre-treatment. *Animal Reproduction Science*, v. 93, n. 1-2, p. 176-182, 2006.

SUCCU, S.; LEONI, G.G.; BEBBERE, D.; BERLINGUER, F.; MOSSA, F.; BOGLIOLO, L.; MADEDDU, M.; LEDDA, S.; NAITANA, S. Vitrification devices affect structural and molecular status of *in vitro* matured ovine oocytes. *Molecular Reproduction and Development*, v. 74, n. 10, p. 1337-1344, 2007a.

SUCCU, S.; LEONI, G.G.; BERLINGUER, F.; MADEDDU, M.; BEBBERE, D.; MOSSA, F.; BOGLIOLO, L.; LEDDA, S.; NAITANA, S. Effect of vitrification solutions and cooling upon *in vitro* matured prepubertal ovine oocytes. *Theriogenology*, v. 68, n. 1, p. 107-114, 2007b.

THARASANIT, T.; YAMAJI, Y.F.; KLEINHANS, F.W.; MAGOSABURO, K.; COLEN-BRANDER, B.; STOUT, T.A.E. Effect of maturation stage at cryopreservation on post-thaw cytoskeleton quality and fertilizability of equine oocytes. *Molecular Reproduction and Development*, v. 73, n. 5, p. 627-637, 2006.

TIAN, S.-J; YAN, C.-L.; YANG, H.-X.; ZHOU, G.-B.; YANG, Z.-Q.; ZHU, S.-E. Vitrification solution containing DMSO and EG can induce parthenogenetic activation of *in vitro* matured ovine oocytes and decrease sperm penetration. *Animal Reproduction Science*, v. 101, n. 3-4, p. 365-371, 2007.

VAJTA, G. Vitrification of oocytes and embryos of domestic animals. *Animal Reproduction Science*, v. 60-61, n. 2, p. 357-364, 2000.

VAJTA, G.; HOLM, P.; KUWAYAMA, M.; BOOTH, P.J.; JACOBSEN, H.; GREVE, T.; CALLESEN, H. Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Molecular Reproduction and Development*. v. 51, n. 1, p. 53-58, 1998.

VIEIRA, A.D.; MEZZALIRA, A.; BARBIERI, D.P.; LEHMKUHL, R.C.; RUBIN, M. I.; VAJTA, G. Calves born after open pulled straw vitrification of immature bovine oocytes. *Cryobiology*, v. 45, n. 1, p. 91-94, 2002.

VINCENT, C.; PICKERING, S.J.; JOHNSON, M.H. The hardening effect of dimethylsulphoxide on the mouse zona pellucida requires the presence of an oocyte and is associated with a reduction in the number of cortical granules present. *Journal of Reproduction and Fertility*, v. 89, n. 1, p. 253-259, 1990.

ZHANG, J.; NEDAMBALEB, T.L.; YANGC, M.; LIA, J. Improved development of ovine matured oocyte following solid surface vitrification (SSV): effect of *cumulus* cells and cytoskeleton stabilizer. *Animal Reproduction Science*. v. 110, n. 1-2, p. 46-55, 2009.