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Review

Cryopreservation of equine oocytes: looking into the crystal ball*

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Abstract. *In vitro* embryo production has evolved rapidly in the horse over the past decade, but blastocyst rates from vitrified equine oocytes remain quite poor and further research is needed to warrant application. Oocyte vitrification is affected by several technical and biological factors. In the horse, short exposure of immature oocytes to the combination of permeating and non-permeating cryoprotective agents has been associated with the best results so far. High cooling and warming rates are also crucial and can be obtained by using minimal volumes and open cryodevices. Vitrification of *in vivo*-matured oocytes has yielded better results, but is less practical. The presence of the corona radiata seems to partially protect those factors that are necessary for the construction of the normal spindle and for chromosome alignment, but multiple layers of cumulus cells may impair permeation of cryoprotective agents. In addition to the spindle, the oolemma and mitochondria are also particularly sensitive to vitrification damage, which should be minimised in future vitrification procedures. This review presents promising protocols and novel strategies in equine oocyte vitrification, with a focus on blastocyst development and foal production as most reliable outcome parameters.

Additional keywords: blastocyst, cumulus cells, intracytoplasmic sperm injection, maturation, vitrification.

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Introduction

Cryopreservation enables the preservation of living cells and tissues for an extended period of time by applying very low subzero temperatures (less than −130°C) that stop cell metabolism (Mazur 1984; Pegg 2007). Significant progress in cryopreservation strategies has had an important effect in many fields, with possibly the most significant effect on reproductive medicine (Mandawala *et al.* 2016). The first report of oocyte cryopreservation dates back to 1958 (Sherman and Lin 1958), but this was only at −10°C for periods up to 3.5 h, and it took until the first decade of this century for efficient protocols to be developed and this was predominantly driven by research in bovine, human and murine oocytes (Saragusty and Arav 2011; Vajta 2013). Now, mature-stage oocyte cryopreservation is offered as a routine technique in human fertility centres, resulting in equal rates of clinical pregnancy and implantation compared with those obtained using fresh oocytes (Rienzi *et al.* 2010; Parmegiani *et al.* 2011). In domestic animals, live births have been obtained after oocyte cryopreservation in the mouse (Parkening *et al.* 1976), rabbit (Al-Hasani *et al.* 1989), cow (Fuku *et al.* 1992), horse (Maclellan *et al.* 2002; Ortiz-Escribano *et al.* 2017), pig (Somfai *et al.* 2014) and cat (Pope *et al.* 2012). In contrast with the situation in humans, the clinical efficiency in animal species is not satisfactory (Ducheyne *et al.* 2019; Mogas 2019). Oocyte cryopreservation is only of practical use in a particular animal species if the associated assisted reproductive technologies (i.e. IVM, IVF, *in vitro* culture and embryo transfer) have been optimised to support it.

In the horse, *in vitro* embryo production (IVEP) has become a commercial reality in the past decade and equine reproduction centres now increasingly offer ovum pick-up (OPU) followed by intracytoplasmic sperm injection (ICSI) to overcome fertility problems in mares and stallions, to increase the offspring of a single frozen sperm straw and to plan and increase the propagation of valuable female genetics (Claes *et al.* 2016; Maserati and Mutto 2016; Morris 2018). An important complement to clinical

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ICSI would be the ability to cryopreserve oocytes, which would improve overall flexibility. Oocyte cryopreservation enables: (1) decisions regarding the fertilising stallion for ICSI to be postponed; (2) oocytes to be salvaged from recently deceased animals in places where IVEP is not available; (3) oocytes to be collected and stored outside the reproductive season; and (4) good-quality germ lines of young mares to be preserved before they are enrolled in competition, because old mares generally have a lower number of follicles that can be punctured by OPU, which affects the chances of an embryo after IVEP (Cuervo-Arango *et al.* 2019). Moreover, horse oocyte banks would allow worldwide spread of valuable female genetics for commercial purposes or for breeding programs of endangered equid breeds and species (Smits *et al.* 2012). Most wild equids are currently endangered or threatened with extinction (Adams *et al.* 2009). In addition, the number of horse breeds has been shrinking due to increasing mechanisation (Bodó and Alderson 2005; Smits *et al.* 2012) and, at the other end of the spectrum, some remaining non-endangered horse breeds consist of a highly inbred population with a high burden of hereditary diseases due to intensive selection (Ducro *et al.* 2015; Leegwater *et al.* 2016). Cryopreserved oocytes could be included in a genome resource bank and could be used later to increase the gene pool in horse breed populations with a narrow genetic base, save endangered species from extinction and potentially re-establish extinct species.

Finally, storage of oocytes from abattoir-derived ovaries could provide continuous availability of material for future research and cloning purposes for countries where oocyte availability is limited (Hinrichs 2018). Cloning is used commercially to increase or restore the reproductive capacity of animals with a high genetic value, but nuclear transfer efficiency remains low and the procedure consumes many oocytes (Olivera *et al.* 2016; Gambini and Maserati 2017). Specialised laboratories could potentially use cryobanked equine oocytes as a source for oocyte-consuming cloning procedures. The production of blastocysts and the birth of healthy clones from cryopreserved oocytes in mouse (Sung *et al.* 2010; Hirata *et al.* 2011), cattle (Hou *et al.* 2005; Park *et al.* 2015) and sheep (Moawad *et al.* 2011) support this assumption, but no reports on cloned equine embryos using cryopreserved oocytes have been published so far. A summary of potential applications is outlined in Fig. 1.

Up to now, the efficiency of equine oocyte cryopreservation has been limited and only a few foals have been born resulting from mature or immature oocytes cryopreserved by vitrification (Maclellan *et al.* 2002; Ortiz-Escribano *et al.* 2017). Further research is needed to allow clinical application. This review discusses the techniques used to cryopreserve equine oocytes and the outcomes of different attempts over 25 years of research. Different strategies to increase efficiency rates aiming at technical or biological factors in the horse and promising strategies used in other species are assessed. The final part of the review focuses on the application potential and future perspectives of equine oocyte cryopreservation.

The (equine) oocyte and vitrification: a better match

Two cryopreservation techniques have primarily been used for the preservation of biological samples: slow freezing and vitrification. In slow freezing, a relatively low concentration of cryoprotective agents (CPAs; 5–10%) is used and extracellular ice formation is initiated, or at least allowed to happen. Extracellular freezing initiates an efflux of water from the cells, which, if cooling is adequately slow, can be sufficient to prevent damaging intracellular ice formation. In vitrification, high CPA levels (30–40%) combined with a high cooling rate (e.g. 70 000°C min^{-1}; Paredes and Mazur 2013) are used to prevent intra- and extracellular ice formation altogether.

Although initial successes in oocyte cryopreservation were obtained by 'slow-freezing' methods, notably in mice and human, research on vitrification has accelerated in recent years and vitrification has now gained a foothold. Results achieved with the vitrification of oocytes of different species are generally better compared with slow freezing (Edgar and Gook 2012; Rienzi *et al.* 2017). Here, we focus only on oocyte vitrification because, in the horse, so far only vitrified oocytes have been shown to support embryo development (Table 1).

Vitrification means that a liquid is transformed to an amorphous (i.e. not crystalline) glass-like solid state. In aqueous systems, this means that a solid state is reached without intra- or extracellular ice formation. Stable vitrification of aqueous solutions can be achieved by lowering the water 'concentration' (officially the chemical potential of water), which can be done by increasing the concentration of solutes to very high levels. At certain very high CPA concentrations, there is no thermodynamic tendency for ice formation until glass transition temperature is reached (stable vitrification), and therefore there is no need for rapid cooling or warming. At low cooling rates and atmospheric pressure, the required solute concentration may be as high as 80% w/w (Fahy *et al.* 1987; Chen *et al.* 2000). In order to achieve such solute concentration, the 'classical' membrane permeating CPAs, such as glycerol, ethylene glycol, propylene glycol and dimethyl sulfoxide (DMSO), complemented by non-permeating solutes, such as sugars, often referred to as non-permeating CPAs, are often used. However, at these very high concentrations, CPAs, or the very low water concentration itself, are often toxic for cells. At lower solute concentrations, ice formation can occur, but can be 'outrun' at very high cooling rates because glass transition may be reached before ice nucleation or significant growth of ice primordia have occurred (metastable vitrification). Vitrified oocytes can then be stored at −196°C in liquid nitrogen, a temperature so low that translational motion of molecules has completely ceased (Fig. 2). A major advantage of vitrification is the low risk of intracellular ice formation. Other advantages are the speed of the whole process compared with slow freezing and the fact that there is no need for expensive equipment.

In the horse, it seems that oocytes can tolerate the CPA concentrations used in current protocols (30–40%) for only a short period of time (Ortiz-Escribano *et al.* 2017; Canesin *et al.* 2018). Although some recent protocols for equine oocytes reported similar maturation and cleavage rates for fresh and vitrified oocytes (Canesin *et al.* 2017; Ortiz-Escribano *et al.* 2017), blastocyst rates were always severely affected after oocyte vitrification (Table 1). Only Maclellan *et al.* (2010) have obtained high blastocyst (40%) and pregnancy (66%) rates after vitrification of *in vivo*-matured oocytes. However, using *in vivo*-matured

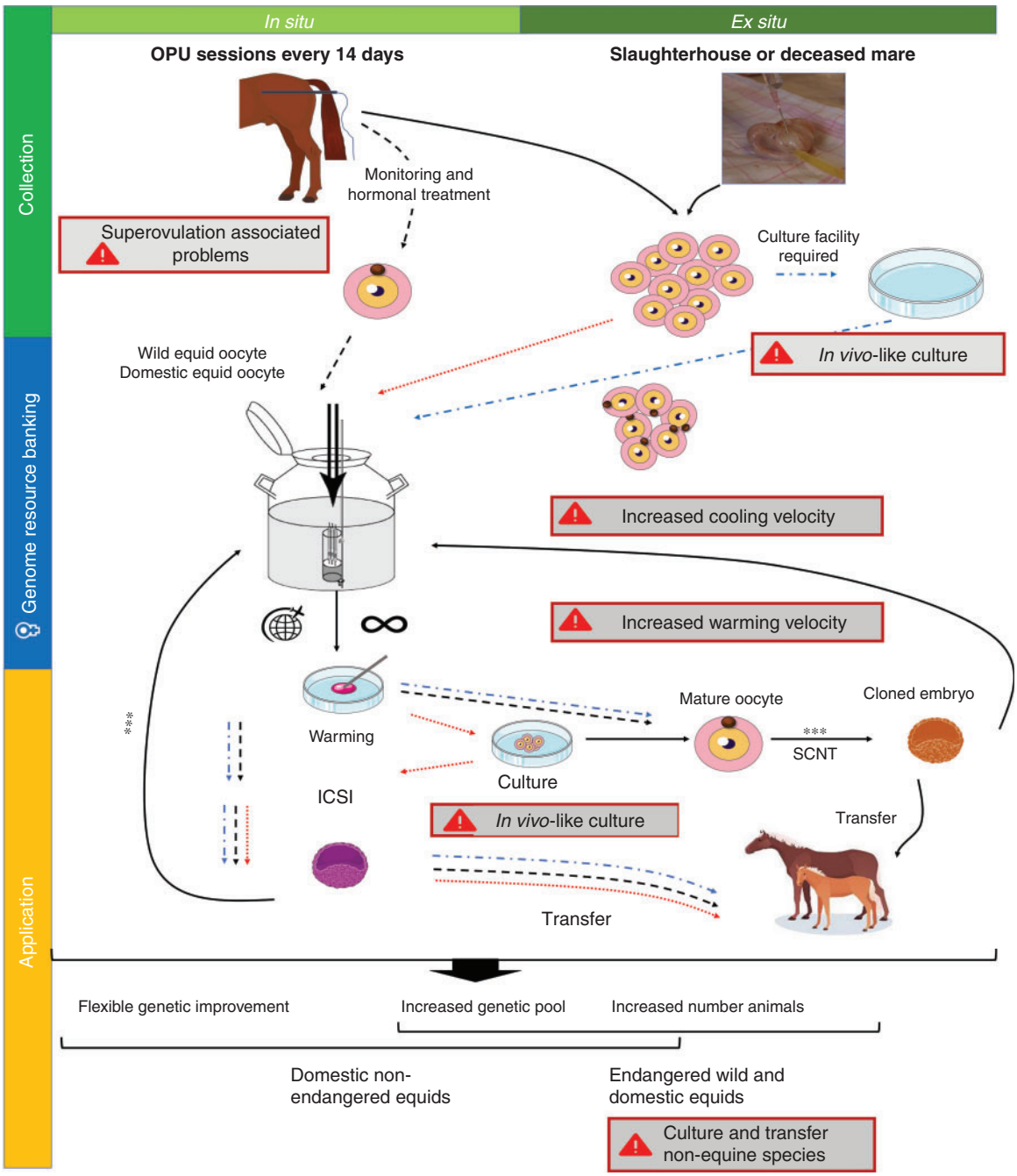

Fig. 1. Equine oocyte cryopreservation framed within equine *in vitro* production. Mature and immature oocytes can be collected from ovaries of living or deceased mares. Because superovulation is problematic in the horse, only one or two mature oocytes can be collected from the ovaries of live mares by ovum pick-up (OPU), whereas 10–20 immature oocytes can be collected and this does not require hormone treatment and monitoring of the mare. Mature oocytes can be stored directly (black dashed arrow; *in vivo* matured), whereas immature oocytes can be stored directly (red dotted arrow) or after *in vitro* culture (blue dash-dotted arrow; IVM). Oocytes stored in the genome resource bank can be transported and warmed at a convenient time and place, after which they are fertilised by intracytoplasmic sperm injection (ICSI) and further processed as in the normal *in vitro* embryo production (IVEP) protocol. Resulting blastocysts are transferred to a recipient mare or can be stored again for later transfer. In addition, vitrified mature slaughterhouse, OPU and ICSI leftover oocytes could serve as a source for the process of somatic cell nuclear transfer (SCNT), allowing horse cloning. The use of cryopreserved oocytes would allow a more flexible genetic improvement of sport horses and non-endangered domestic breeds and increase the gene pool in all equid families with a narrow genetic basis, such as endangered equid species and breeds. ***There are no reports of cloned animals resulting from equine vitrified oocytes or of the cryopreservation of embryos produced from a vitrified oocyte. Warning signs (⚠) indicate non-optimal aspects of the process. Optimisation of one or more of these aspects may increase vitrification efficiency.

Table 1. Summary of oocyte survival, maturation and development rates in studies that evaluated embryo development

Multistep protocols for cryoprotective agent (CPA) exposure and CPA removal reduce osmotic and volume changes respectively. Different criteria may be used for assessing oocyte survival: intact membrane (e.g. as assessed by dead/live stains), normal spindle and/or chromatin configuration. CCs, cumulus cells; Cp, compact; CR, corona radiata; ED, ethylene glycol (EG) + dimethyl sulfoxide (DMSO); EDS, EG + DMSO + sucrose; EDFS, EG + DMSO + Ficoll + sucrose; EP, EG + polyvinylpyrrolidone; EPS, EG + propylene glycol + sucrose; EPT, EG + propylene glycol + trehalose; Ex, expanded; FBS, fetal bovine serum; GV, germinal vesicle; MII*, *in vivo* matured; MVD, minimum-volume device; NP, not performed; ND, not determined; OPS, open pulled straw; OT, oocyte transfer; PA, parthenogenetic activation

Reference	Meiotic stage	Cumulus cells	Equilibration/ vitrification protocol	Warming protocol	Device	No. intact oocytes (%)	No. MII oocytes (%)	No. cleaved oocytes (%)	Blastocyst rate (%)	No. pregnancies (%)
Maclellan *et al.* (2002)	MII*	CCs	Control			12/12 (100)	NP	NP		10/12 (83) from OT
			ED/EDFS	Three-step sucrose	Nylon Cryoloop	19/26 (73)	NP	NP		3/26 (12) from OT
Tharasanit *et al.* (2006*b*)	GV	Cp and Ex CCs	Control			41/46 (95)–50/54 (96)	84/158 (53)–73/130 (56)	33/69 (48)–35/53 (66)	7/69 (10)–9/53 (17)	NP
	GV		ED/EDS	One-step sucrose	OPS	7/27 (33)–12/26 (63)	95/229 (42)–82/153 (54)	20/73 (27)–28/83 (34)	0–1/83 (1)	
	MII					13/49 (28)–12/38 (32)	70/150 (47)–83/153 (54)	2/52 (4)–8/49 (16)	0	
Maclellan *et al.* (2010)	MII*	Denuded	Commercial Cryotop medium		Cryotop	18/25 (72)	25/29 (86)	15/18 (83)	6/18 (33)	4/6 (66)
Nowak *et al.* (2014)	MII	Denuded	Control			41/56 (73)	53/112 (47)	19/53 (36) PA	ND	NP
			EquiPro-VitKit (Minitube) commercial media or two-step EDS		Rapid-i (Vitrolife)	38/69 (55)–34/54 (63)	49/104 (47)	5/49 (10) PA		
Ortiz-Escribano *et al.* (2017)	GV	CCs and CR	Control			ND	47/88 (53)–50/86 (58)	61/80 (76)	16/80 (20)	NP
			EG/EDS	Three-step sucrose	MVD	ND	32/93 (34)–59/122 (48)	17/56 (30)	0	
			ED/EDS	One-step sucrose	MVD	ND	25/99 (25)–42/122 (34)	30/72 (42)	5/72 (7)	1/5 (20)
Canesin *et al.* (2017)	GV	CR	Control			ND	27/37 (73)	25/27 (93)	5/27 (19)	NP
			Three-step ED/EDS	Three-step sucrose; 1 vs 4 min in first step	Stainless steel mesh	ND	10/35 (29)–12/22 (36)	5/9 (56)–6/9 (67)	0–1/9 (11)	
			Control			ND	49%[A]	86/93 (93)	27/93 (29)	
			Three-step ED/EDS vs EPT vs EPS in M199 or FBS	Three-step sucrose or trehalose	Stainless steel mesh	ND	0–42%[A]	0–3/3 (100)	0–1/10 (10)	
Canesin *et al.* (2018)	GV	CR	Control			ND	33/55 (60)	31/33 (94)	8/33 (24)	NP
			EP/EPT	Four-step trehalose	Stainless steel mesh	ND	1/18 (6)–7/18 (39)	5/7 (75)–2/2 (100)	0	
			Control			ND	52/103 (51)	47/52 (90)	18/52 (35)	
			EP/EPT	No vs one- vs four-step trehalose	Stainless steel mesh	ND	12/63 (19)–14/67 (21)	9/12 (75)–17/20 (85)	0–2/13 (15)	

[A]The number of cryopreserved or control oocytes is not mentioned.

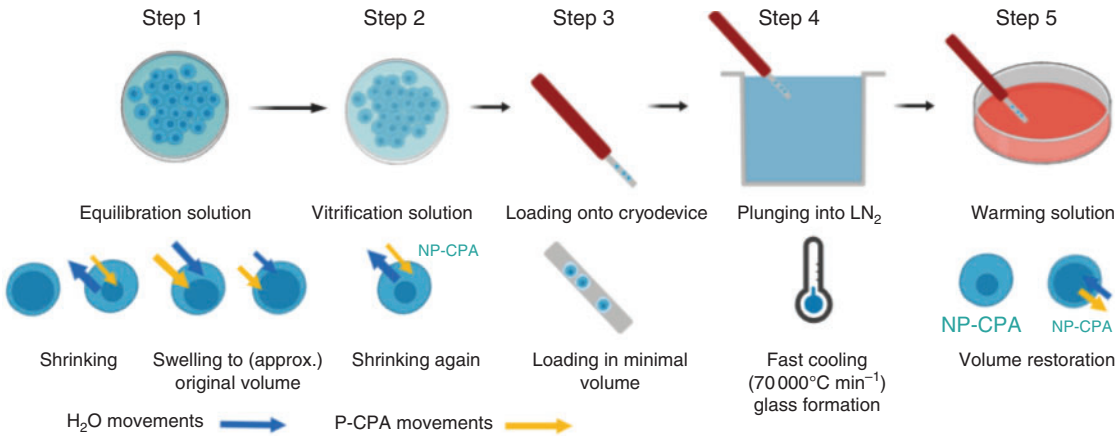

Fig. 2. Schematic representation of the different steps of oocyte vitrification and the associated changes the oocytes must endure. Oocytes shrink and swell as they partially or completely equilibrate with cryoprotective agents (CPAs) in the 'equilibration solution' (Step 1). Oocytes are then briefly held in 'vitrification solution' (Step 2), with very high concentrations of CPAs. The high CPA concentration combined with high rates of cooling (Step 4) and warming (Step 5) can prevent extracellular ice crystallisation. Such high rates can be reached when using Cryodevices (Step 3) with a minimal volume (<1 μL). LN_2, liquid nitrogen; P-CPA, permeating CPA; NP-CPA, non-permeating CPAs. Figure created using BioRender (biorender.com, accessed 9 October 2019).

equine oocytes has practical limitations, such as the requirements for constant monitoring of follicle growth, hormone treatment in the live mare and fixed timing of ICSI. Moreover, there is no reliable, commercially available route to ovarian superstimulation in the mare (Roser and Meyers-Brown 2012). Therefore, equine oocytes are generally recovered by transvaginal aspiration of the immature follicles, which is more efficient for IVEP in the horse (Jacobson *et al.* 2010; Hinrichs 2018). Recovery of immature oocytes requires subsequent IVM before or after oocyte vitrification but, in this case, embryo development and foaling rates are variable and low (0–15% and 20% respectively; Table 1). The current efficiency of equine immature oocyte vitrification precludes clinical application (Hinrichs 2018).

Strategies to improve oocyte vitrification

Successful outcome of oocyte vitrification is affected by several technical and biological factors, which are shown in Fig. 3 and discussed in the following paragraphs.

Technical aspects

CPAs: for better or worse

Membrane-permeating CPAs reduce osmotic damage during vitrification. As explained above, very high solute concentrations can prevent the formation of ice when an aqueous solution is cooled. This is due, in large part, to the fact that the presence of solutes lowers the freezing point. By increasing the solute concentration to very high levels, the freezing point can be lowered to the glass transition temperature, which makes vitrification possible. Membrane-permeating CPAs are a special case because they can penetrate the cells, which reduces the strong osmotic shrinking of cells and prevents extremely high intracellular electrolyte (salt) concentrations that would occur if only membrane non-permeating solutes were used. In equine oocyte vitrification, glycerol, propylene glycol, ethylene glycol, formamide and DMSO have been used as permeating CPAs, alone or in combination with each other and/or non-permeating CPAs, macromolecules or synthetic polymers (Table 1).

The damage caused by the specific permeating CPAs varies depending on their permeability and chemical and physicochemical properties, as well as on the maturation stage of the oocyte and the temperature at which they are introduced. The permeability of the CPA is important for the osmotic response of the oocyte, which can have implications for potential osmotic damage. In general, high permeability and low intrinsic toxicity are desired. In human mature oocytes, ethylene glycol has approximately half the permeability of propylene glycol and DMSO, but is preferred due to its low toxicity (Best 2015). In the horse, ethylene glycol was mostly used in combination with non-permeating CPAs to preserve equine oocytes. However, it was not until combinations of ethylene glycol–DMSO–sucrose (Canesin *et al.* 2017; Ortiz-Escribano *et al.* 2017) and propylene glycol–ethylene glycol–trehalose (Canesin *et al.* 2017, 2018) were used that blastocyst development was obtained from vitrified equine oocytes (Table 1). This could be due to the fact that a combination of two permeating CPAs can decrease specific CPA toxicity because of the lower concentrations of each CPA used (Vajta and Kuwayama 2006). Only one study directly compared protocols using different CPAs and found the protocol using ethylene glycol–propylene glycol–trehalose to be the most effective, although toxicity was still present (Canesin *et al.* 2017).

The use of extracellular non-permeating CPAs, including sugars (sucrose, trehalose), macromolecules (Ficoll, bovine serum albumin) and synthetic (co)polymers like synthetic ice blockers (polyvinylpyrrolidone, polyvinyl alcohol, SuperCool X-1000 (21st Century Medicine)), may decrease the necessary concentration of toxic permeating CPAs and reduce osmotic damage during warming. Moreover, non-permeating CPAs increase the viscosity of the extracellular solution (Kasai

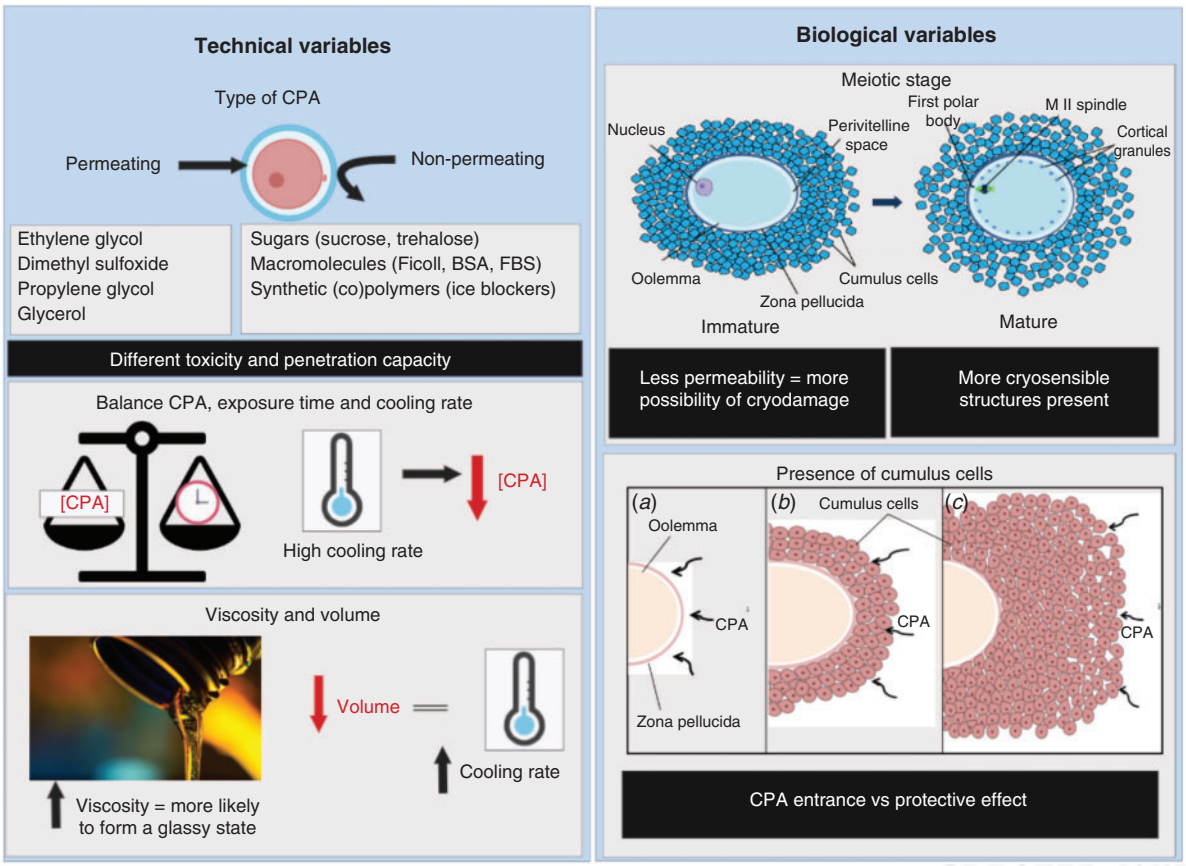

Fig. 3. Technical and biological variables in oocyte vitrification. Technical variables include the characteristics of cryoprotective agents (CPAs), the balance between CPA concentration and exposure time (affecting both cryoprotection and CPA toxicity) and high cooling rate (enabling a lower CPA concentration). The lower left panel shows how medium viscosity and sample volume can affect the chance of successful vitrification. Biological variables include the characteristics of the oocyte at different meiotic stages and the presence of cumulus cells ((a) absent in denuded oocytes, (b) few in corona-radiata oocytes and (c) many in comlete cumulus-oocyte complexes), which may protect the oocyte from toxic CPA effects but may also hamper CPA penetration. Figure created using BioRender (biorender.com, accessed 9 October 2019).

1997; Orief *et al.* 2005), which may affect the ice nucleation behaviour of the solution and thereby increasing the chances of vitrification at a reduced permeating CPA concentration. In equine, sucrose is most commonly used as a non-permeating CPA (Table 1). Similar pronuclear formation was found for horse oocytes vitrified in a DMSO–ethylene glycol medium containing sucrose or trehalose after 24 h of IVM (Maclellan *et al.* 2001). However, the highest vitrification efficiency for immature horse oocytes was reached when a vitrification medium containing trehalose was used (42% maturation (10/24), 80% cleavage (8/10) and 10% blastocysts (1/10)), whereas no blastocysts were obtained from sucrose-containing media (Canesin *et al.* 2017). In humans and mice, oocyte vitrification in trehalose-containing medium is also associated with higher blastocyst rates than vitrification with sucrose (Coello *et al.* 2016), but the difference is not always statistically significant (Zhang *et al.* 2017; Lestari *et al.* 2018), probably due to variations in the protocols used.

Injection of sugars into the oocyte can avoid osmotic stress associated with the use of non-permeating CPAs (Woelders *et al.* 2018). Moreover, the assumed stabilising effect of sugars on membrane stability (Crowe *et al.* 1992) would then also apply to the intracellular membrane surfaces, including these of organelles. In fact, this is a strategy copied from nature, where metabolically produced extra- and intracellular sugars allow a broad variety of organisms to cope with below-zero temperatures (Crowe *et al.* 1992). Sugars appear to have a low intrinsic toxicity and can increase the glass transition temperature. Therefore, several routes of intracellular administration in oocytes have been tried (Eroglu *et al.* 2003; Rao *et al.* 2015). Microinjection seems the most efficient technique to bring sugars into the oocyte and reduces the amount of permeating CPA needed while improving cryosurvival and embryo development in mice and humans (Eroglu *et al.* 2002, 2003, 2009). In fact, in murine mature oocytes this approach resulted in equal blastocyst rates after vitrification–warming compared with blastocyst rates obtained with fresh oocytes, and resulted in healthy offspring after embryo transfer into recipients (Eroglu *et al.* 2009). Such a strategy has not been evaluated in horse oocytes, but may reduce or even avoid CPA toxicity. Similarly, synthetic ice blockers are copied from naturally occurring glycoproteins or proteins and are believed to mitigate

heterogeneous nucleation by recognition of ice nucleators or perhaps ice itself (Wowk *et al.* 2000). Their use resulted in improved survival rates in vitrified murine embryos (Fahy *et al.* 2004; Badrzadeh *et al.* 2010) and survival and cleavage rates in porcine oocytes (Macedo *et al.* 2006; Santos *et al.* 2017). In equine oocytes, the addition of synthetic ice blockers in the vitrification media has also been tried, such as Supercool X-1000, a copolymer of polyvinyl alcohol, which improved maturation rates of vitrified–warmed oocytes (Curcio *et al.* 2014*b*). However, embryo development and intrinsic toxicity has not yet been assessed (de Leon *et al.* 2012; Curcio *et al.* 2014*a*, 2014*b*).

The removal of CPAs after warming is a critical step because the highest osmotic damage may occur during this phase (Wang *et al.* 2011). If the oocytes in vitrification medium are suddenly washed in physiological saline, water enters the cell more rapidly than an intracellular CPA can leave it, which may increase cell volume to a critical level at which the cell may burst (Oda *et al.* 1992). Therefore, to minimise this risk, in general stepwise warming in solutions of decreasing osmolality is performed to remove CPAs. However, a recent study on equine oocyte vitrification found that a single-step warming in base medium without added sugar was as effective as a protocol that included warming the oocytes in base medium with 0.4 M trehalose, followed by stepwise lowering of the trehalose concentration (Canesin *et al.* 2018). The single-step method may improve oocyte recovery and simplify practical aspects of the vitrification process (Canesin *et al.* 2018).

Another important variable is the exposure time to CPAs in vitrification solutions, which will further determine the delicate balance between cryoprotection and CPA toxicity. Vitrification protocols for embryos and oocytes usually make use of a two-step increase in CPA concentrations (e.g. see Rall and Fahy 1985). In the first step, the cells or tissue are incubated for a relatively long time (10–15 min) in the 'equilibration solution', which has approximately half the CPA concentration deemed necessary for vitrification to allow equilibration regarding intra- and extracellular CPA concentrations and osmotic conditions. This means that cells shrink due to (rapid) water efflux, but then slowly regain their original volume as CPAs more slowly enter the cells. After the equilibration step, cells or tissues are then placed in the vitrification solution, which has the full strength needed for vitrification. The cells rapidly lose water but are then directly plunged into liquid nitrogen, not allowing them to regain their original volume. More recent successful applications of such two-step protocols include mature human oocytes (Kuwayama *et al.* 2005). However, for horse oocytes, despite good maturation rates (Rosati *et al.* 2012; de Leon *et al.* 2012) and some blastocyst development from equine vitrified oocytes, the relatively long exposure to CPAs was associated with CPA toxicity, affecting subsequent embryo development (Canesin *et al.* 2017). Tharasanit *et al.* (2006*a*, 2006*b*) tried vitrification of equine oocytes after only 30 s exposure to the equilibration solution followed by 25 s in the vitrification solution. Exposure to CPAs alone resulted in little damage, but only one of 257 sperm-injected immature and mature vitrified oocytes resulted in a blastocyst (Tharasanit *et al.* 2006*b*). Perhaps this could have been caused by vitrification in open pulled straws (OPS), which results in relatively lower cooling and warming rates (Li *et al.* 2012). Regardless, later studies showed that shorter CPA exposure times can actually yield better results. For example, Ortiz-Escribano *et al.* (2017) obtained blastocyst development and were the first to report the birth of a healthy foal after IVM and ICSI of equine oocytes vitrified in the immature stage, followed by embryo culture and transfer of blastocysts into a recipient mare. In addition, Canesin *et al.* (2018) reported that a shorter total exposure time (105 s) was beneficial for blastocyst development, although it did not prevent CPA toxicity. Although the short exposure is not sufficient for equilibration in the equilibration solution (i.e. it results in only partial intracellular loading of CPAs), simulations on the basis of a mathematical model of oocyte membrane transport showed that the entry of CPAs into the oocyte is still significant and, due to the very rapid efflux of water, the intracellular osmolality reached becomes equal to that of the extracellular vitrification solution within a few seconds, making conditions sufficient for vitrification (Woelders *et al.* 2018). However, the rate of CPA influx may still be a factor of importance, because superior results regarding the vitrification of equine immature oocytes were seen after partial removal of the cumulus cells, leaving only corona radiata-surrounded oocytes, which may have reduced resistance for the entry of CPAs into the oocytes (Ortiz-Escribano *et al.* 2017). More research on exposure times to CPA, CPA concentrations and possible CPA combinations is needed in equine, because most of the research has been extrapolated from other species in which oocytes have a different membrane permeability and cytoplasm composition. Further improvement of the vitrification of equine oocytes may be obtained by increasing the cooling and warming rates in order to allow the use of lower CPA concentrations.

Speed of vitrification and warming

Fast cooling and warming rates are crucial for the process of vitrification because they enable vitrification at lower total solute (CPA) concentrations. As explained above, metastable vitrification can be obtained at lower CPA concentrations by using very high cooling rates to reach glass transition before ice nucleation can occur. In addition, at high cooling rates, chilling injury (hypothermia-induced cellular changes) may be outrun due to a fast passage through the critical temperature zone (Martino *et al.* 1996; Orief *et al.* 2005). The use of very high warming rates (Mazur 1984) is also crucial in preventing the growth of intracellular ice primordia and the formation of damaging intracellular ice during warming. Indeed, a series of studies by the group of the late Peter Mazur showed that high warming rates are very important in the vitrification of mouse oocytes and embryos, in fact more important than rapid cooling (Mazur and Paredes 2016). Whether this translates to oocytes of other species, including horses, is not clear, but it should be kept in mind during the process (Gallardo *et al.* 2016).

The speed of cooling and warming depends on the volume and surface area of the sample and the heat transfer to or from the cooling or warming system (Orief *et al.* 2005). Although initial vitrification of equine oocytes was done using conventional straws (i.e. 200 µL phosphate-buffered saline (PBS) with ~45 µL vitrification solution; Hochi *et al.* 1996), a great many

minimum-volume cryodevices (from 1.5 to <0.1 μL) have since been developed in order to achieve rapid cooling rates of, for example, 70 000°C min^{-1} (Li *et al.* 2012; Paredes and Mazur 2013), which improved the vitrification of bovine and porcine oocytes compared with protocols using larger-volume devices (Liu *et al.* 2008; Morató *et al.* 2008). Increased cooling rates also coincided with improved development of vitrified equine oocytes (Table 1).

Cryodevices include open (Cryoloop, open pulled straw, Cryotop, solid surface vitrification, stainless steel mesh) and closed (Rapid I (Vitrolife), VitriSafe (Cryo Bio System)) systems. Open devices theoretically allow faster cooling and warming rates, resulting from both lower specimen volume and direct contact between specimen and liquid nitrogen, better preserving the oocyte ultrastructure (Bonetti *et al.* 2011). In human medicine, direct comparison between open and closed devices did not result in different development and pregnancy rates (Cai *et al.* 2018). A direct comparison between the two systems for equine oocytes is missing, but embryo development has only been evaluated with open devices (Table 1). Although some open devices, like the stainless steel mesh, additionally allow for simultaneous vitrification of a large number of oocytes (Canesin *et al.* 2018), closed systems may have the ability to protect the oocytes against contamination and transmission of pathogens in the liquid nitrogen (Bielanski and Vajta 2009; Criado *et al.* 2011; Vajta *et al.* 2015). Nonetheless, some open devices can be sealed after being plunged into liquid nitrogen, which prevents possible exchange of pathogens between samples (e.g. Cryolock (Biotech Inc.)). Although export of equine semen and embryos has been regulated for venereal and vertically transmitted diseases, there is not enough information available to estimate the possible risk for disease transmission after vitrification of equine oocytes.

Increasing the cooling rate even more is possible by using 'slush' nitrogen, liquid helium or solid surface vitrification (Orief *et al.* 2005). These techniques improve the heat transfer by reducing the insulating sheath of nitrogen gas around the oocyte (Leidenfrost effect), although this effect may be very small in minimum volume cryodevices. An additional factor is the lower temperature of 'slush' nitrogen, which is nitrogen at the melting (−210°C) instead of boiling (−196°C) point. Similarly, the use of liquid helium (−269°C) would increase the rate of heat transfer. All three techniques have been used successfully with oocytes from other species (Cha *et al.* 2011; Yu *et al.* 2016; Guo *et al.* 2017) and may be promising for horse oocytes. Only one study on equine oocytes reports vitrification using solid surface vitrification (de Leon *et al.* 2012), a technique characterised by direct contact of the droplets containing the oocytes with the precooled carrier surface. In that study, no difference was found in maturation rate and membrane integrity compared with oocytes vitrified in OPS (de Leon *et al.* 2012), but blastocyst development was not assessed.

Biological aspects

Maturation stage

Oocytes can be cryopreserved in the immature or mature stage. Mature oocytes are in the middle of the second meiotic division, the MII stage, characterised by the presence of chromosomes organised by the spindle apparatus. The latter is sensitive to several types of cryodamage, which may lead to several chromosome and DNA abnormalities in cryopreserved oocytes and can cause subsequent developmental arrest (Sterzik *et al.* 1992; Ishida *et al.* 1997; Berthelot-Ricou *et al.* 2011).

In contrast, in immature oocytes, microtubules are not organised in the MII spindle. Therefore, in principle, the cryopreservation of immature germinal vesicle stage oocytes bypasses the risk of chromosome aberrations because, at this stage, the chromatin is decondensed and protected by a nuclear envelope (Hinrichs *et al.* 2005). As such, the immature stage was believed to be more suitable for cryopreservation because the chance of chromosomal aberrations would be smaller (Brambillasca *et al.* 2013). Immature bovine oocytes were also found to have a somewhat lower permeability for water and some CPAs (DMSO and ethylene glycol) compared with mature oocytes, suggesting that different procedures for loading and the removal of CPAs may be required for different oocyte developmental stages (Agca *et al.* 1998, 2000).

Generally, better results are obtained following the vitrification of mature than immature oocytes. In human, oocyte cryopreservation is generally performed at the *in vivo* mature stage after hormone stimulation. When hormone treatment is not desirable, IVM oocytes can be vitrified, although implantation and pregnancy rates are lower (Chian and Cao 2014). So far, the vitrification of human immature oocytes has resulted in only one live birth and few pregnancies (Tucker *et al.* 1998; Wu *et al.* 2001; Kan *et al.* 2004). Bovine oocytes are mostly cryopreserved at the mature stage (Mogas 2019), although calves have also been born after the vitrification of immature oocytes (Zhou *et al.* 2010). In porcine, immature oocytes seem to be more sensitive to cooling than IVM oocytes (Rojas *et al.* 2004), although vitrified–warmed immature oocytes could also reach the blastocyst stage (Gupta *et al.* 2007).

In the horse, the ideal meiotic stage for cryopreservation remains poorly studied, but most research has been done in immature oocytes. This is because the vitrification of immature oocytes allows a more flexible application as the neighbourhood of culture facilities to *in vitro* mature oocytes is not required and a higher number of oocytes can be preserved (Hinrichs 2018). Only two studies have compared vitrification of immature and IVM horse oocytes, demonstrating better spindle quality and chromosome configuration in oocytes after IVM when compact cumulus–oocyte complexes were vitrified at the immature than mature stage (Tharasanit *et al.* 2006*a*, 2006*b*). However, cumulus morphology seems to affect these results because no differences were perceived between vitrified mature and immature oocytes when cumulus cells were expanded at recovery time. The only blastocyst reported resulted from vitrification of an immature oocyte with compact cumulus cells (Tharasanit *et al.* 2006*b*).

Cryopreservation of *in vivo*-matured equine oocytes has never been compared directly with IVM or immature oocytes but, as mentioned before, the highest efficiency (number of blastocysts or pregnancies over the initial number of vitrified oocytes) has been obtained when equine oocytes were vitrified after *in vivo* maturation (Maclellan *et al.* 2002, 2010). Hence,

optimisation of *in vivo*-like maturation and culture systems during IVEP could be a key factor to improving outcomes starting from both fresh and vitrified oocytes. These *in vivo*-like optimisations can include the supplementation of signalling or supporting molecules present *in vivo* in the genital tract (e.g. extracellular vesicles) or three-dimensional follicle or oviduct culture systems (da Silveira *et al.* 2012, 2017; Ferraz *et al.* 2018). However, in addition to the maturation conditions, the origin of the oocyte needs to be taken into account. *In vivo*-matured oocytes collected from preovulatory follicles represent a rather uniform population with high developmental potential. Immature oocytes can originate from juvenile, growing or atretic follicles, implying a more variable and generally lower developmental competence (Hinrichs 2010; Torner and Alm 2018).

Presence of the cumulus layer

The role of cumulus cells during the cryopreservation of oocytes seems to be species specific and dependent on the maturation stage (Fujihira *et al.* 2005; reviewed in Ortiz-Escribano 2017). Cumulus cells communicate with the oocyte through transzonal projections, which pass through the zona pellucida and connect to the oolemma by gap junctions. During *in vitro* culture, the presence of cumulus cells is crucial for the acquisition of meiotic competence (Tanghe *et al.* 2003). In equine studies it was found that although vitrification causes damage to the outer layers of the cumulus, it does not affect communication through gap junctions or the ability of the oocyte to mature (Tharasanit *et al.* 2009). In fact, multiple layers of cumulus cells are believed to protect the oocyte chromosomes against cryodamage in both mature and immature oocytes, which was strengthened by the observation that denuded oocytes had reduced spindle quality after vitrification (Tharasanit *et al.* 2006*a*, 2009). Conversely, excessive layers of cumulus cells negatively affect vitrification outcome, because the survival of vitrified IVM bovine oocytes surrounded by multiple layers of cumulus cells was significantly lower than that of vitrified denuded mature oocytes (Ortiz-Escribano *et al.* 2016). Similarly, equine immature oocytes surrounded by multiple layers of cumulus cells had poorer IVM rates after vitrification than oocytes surrounded only by corona radiata cells. Furthermore, vitrified immature corona radiata oocytes resulted in a blastocyst rate of 7% (5/72 injected oocytes) and the birth of a foal (Ortiz-Escribano *et al.* 2017). Therefore, partial denudation of immature oocytes, leaving only corona radiata cells, seems to be a key point in immature equine oocyte vitrification. The presence of several layers of cumulus cells may impair exchange of water and CPAs, leading to a lower intracellular concentration of CPA and, as such, reduced protection against cryodamage. Moreover, a reduction in the cumulus layers and the number of oocytes loaded on the cryodevice also reduces the sample volume, and thus increases the cooling and warming rates (Ortiz-Escribano *et al.* 2017).

Oolemma, zona pellucida, cytoplasm and organelles

Membranes containing lipid bilayers, like the oolemma and mitochondria, are especially sensitive to cryopreservation. An ultrastructural evaluation in vitrified–warmed oocytes revealed no changes in the zona pellucida (Hochi *et al.* 1996). However, the oolemma and mitochondria are affected, similar to that of other species. This is why the integrity of the membrane is one of the principal survival criteria. Lipid bilayers may suffer from cold shock and chilling injury, from CPA toxicity and/or dehydration associated with high CPA concentrations, or other hazardous conditions found during vitrification–warming. Membrane damage may include an altered configuration of membrane lipids and proteins, and reduced metabolic function and membrane permeability (Best 2015). In the mitochondria, composed of mitochondrial matrix enclosed by an inner and outer membrane, swelling and reduced matrix density have been observed in equine oocytes after vitrification (Hochi *et al.* 1996; Curcio *et al.* 2014*a*). Mitochondrial damage alters the synthesis of ATP, which is essential for maturation and embryo development; ATP contributes to microtubule assembly and disassembly, and is critical to meiotic spindle formation, sperm aster formation and syngamy (Eichenlaub-Ritter *et al.* 2004). Moreover, damage to the mitochondria and vitrification itself may promote the formation of reactive oxygen species, which, in turn, initiate damage to lipids, proteins, enzymes and nucleic acid structures due to lipid peroxidase and apoptosis. The susceptibility of the equine oocyte to cryopreservation-induced damage is thought be related to the large amount of lipids present in the membranes and cytoplasm of equine oocytes, which may increase oxidative damage or lower membrane permeability, especially after lipid phase transition (Hochi *et al.* 1996; Ambruosi *et al.* 2009). Culturing the oocytes in media that minimise the production of lipids and the addition of several antioxidants in culture and vitrification media yielded promising results in other species and may improve equine oocyte vitrification (Abe *et al.* 2002; Gupta *et al.* 2007; Chankitisakul *et al.* 2013; Yashiro *et al.* 2015).

Vitrification may also affect communication mechanisms with the extracellular environment through gap junctions and hemichannels. In the oolemma, communication between the oocyte and the cumulus cells, crucial to the maturation of vitrified immature oocytes, can take place through the gap junctions. Adversely, these gap junctions may induce the so-called 'bystander death effect' by allowing cell death messengers to pass from apoptotic cumulus cells into the oocyte (Decrock *et al.* 2009). Although early reports suggested that gap junctions are affected during equine oocyte cryopreservation (Hochi *et al.* 1994, 1996), Tharasanit *et al.* (2009) did not find structural or functional differences between the gap junctions of vitrified and fresh oocytes. The specific induction of bystander cell death messengers from cumulus cells to the oocyte by vitrification, as well as the effect of vitrification on the hemichannels, has not been assessed yet in the horse. In addition to constituting gap junctions, hemichannels exist as free hemichannels in the oolemma of the oocyte (Sáez *et al.* 2003). These are typically closed but, when opened under stress conditions, they could allow entry or escape of essential ions and damaging molecules, which may threaten the normal physiology and vitality of the oocyte. Blocking vitrification-induced opening of hemichannels was promising in feline and bovine oocytes (Snoeck *et al.* 2018; Szymańska *et al.* 2018).

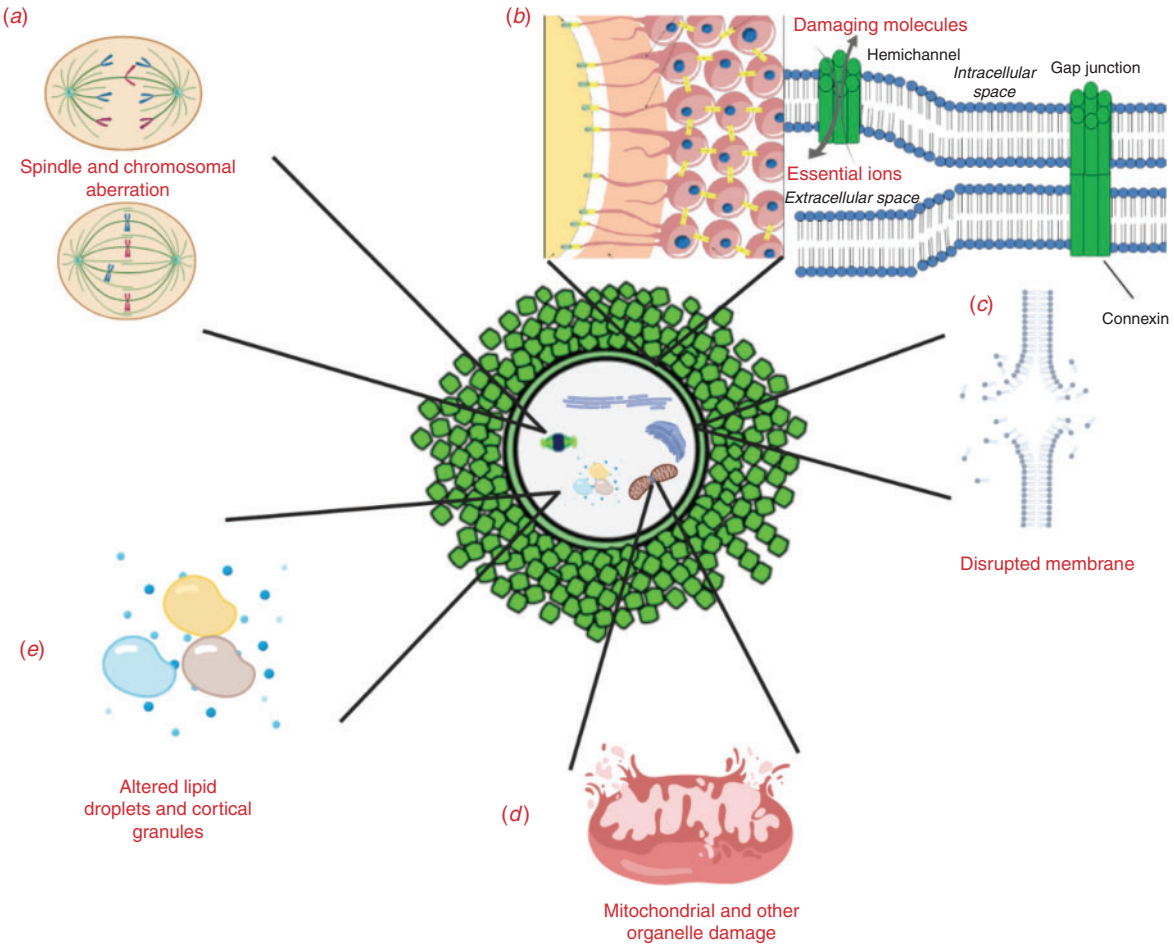

Fig. 4. Schematic representation of some alterations observed in oocytes after vitrification–warming. (*a*) Disruption of the actin network, depolymerisation of microtubules and altered length and volume of the spindle, impairing correct chromosome alignment, have been described in vitrified–warmed mature oocytes and in immature equine oocytes that were matured *in vitro* after warming (Tharasanit *et al.* 2006*a*, 2006*b*, 2009; Ducheyne *et al.* 2019). (*b*) Open hemichannels (which are normally closed) have been found in bovine and feline oocytes after vitrification, possibly causing a release of essential ions and allowing the entry of damaging molecules (Snoeck *et al.* 2018; Szymańska *et al.* 2018). (*c*) Fractures and less conspicuous disruption of lipid bilayer membranes affecting the developmental capacity of the oocyte, (*d*) swelling and changes in the shape and density of organelles such as mitochondria, Golgi apparatus and smooth endoplasmic reticulum and (*e*) lipid droplets and extruded cortical granules (in vitrified matured oocytes) with debris in the perivitelline space have often been noticed (Hochi *et al.* 1994, 1996; Curcio *et al.* 2014*a*). Figure created using BioRender (biorender.com, accessed 15 June 2019).

It is clear that the functionality of the oocyte is threatened by several types of damage to the oolemma, its gap junctions and hemichannels and to critical organelles, although it is not clear how strong these separate effects may be. Apart from damage that can be visualised microscopically, the competence of the oocytes to sustain embryo development after ICSI and to yield healthy blastocysts is determined by several cytoplasmic factors. Most likely the functionality of several organelles or cell cytoplasmic factors involved in maturation and further development may be affected by vitrification. Strengthened by the observation that after warming many presumed mature oocytes seem not able to develop further, it has been suggested that cytoplasmic maturation, including accumulation of mRNA, proteins, substrates and nutrients, is negatively affected by vitrification (Tharasanit *et al.* 2006*b*).

Spindle, cytoskeleton, chromosomal integrity and epigenetic alteration

The most extensively studied structures in equine oocytes after cryopreservation are probably the actin cytoskeleton and meiotic spindle (Tharasanit *et al.* 2006*a*, 2006*b*, 2009; Ducheyne *et al.* 2019), which have been characterised by the disruption of the subcortical actin network, depolymerisation of microtubules, a reduced length and volume of the spindle and an increased metaphase plate thickness. Such changes may impair correct chromosome alignment and cause cytokinesis failure, leading to chromosomal aberrations and subsequent developmental failure. In theory, freezing at the immature stage should be less damaging to the as yet unformed spindle, but changes have been reported in both mature and immature equine oocytes (Tharasanit *et al.* 2006*a*, 2006*b*, 2009; Ortiz-Escribano *et al.*

2017; Ducheyne *et al.* 2019). Although repolarisation of the spindle is seen after warming in some human oocytes vitrified at the mature stage (Minasi *et al.* 2012), a similar effect does not seem to occur in vitrified equine oocytes (Tharasanit *et al.* 2006*a*). However, spindle disassembly and potential repolarisation may also depend on the temperature at which the vitrification process occurs, because processing below a physiological temperature causes rapid spindle disassembly, without a chance of repolarisation (Larman *et al.* 2007). Therefore, spindle damage is an important negative predictor when novel strategies are tested. Molecules directly targeting the spindle can be added to the culture and vitrification or warming media. Those 'spindle stabilisers', like cytochalasin B, paclitaxel and docetaxel, were previously used in immature and mature oocytes from different species to preserve the integrity of the spindle, but have not been tested yet in horses. A schematic representation of changes and damaging processes found in oocytes after vitrification and warming is shown in Fig. 4.

Recent studies on bovine and mouse oocytes have focused on unravelling the effect of vitrification on epigenetics and gene expression (Yan *et al.* 2014; Chen *et al.* 2016). It is clear that the more handling procedures to which germplasm is subjected, the more chances there are that an epigenetic effect or changes in gene expression levels will occur. Moreover, there is an increased incidence of epigenetic alterations when artificial reproductive technologies are used involving human oocytes (Vermeiden and Bernardus 2013; Lazaraviciute *et al.* 2014), and a link between artificial reproductive technologies and epigenetic disorders in cattle has been suspected (Urrego *et al.* 2014). Epigenetics and gene expression studies on horse oocytes could provide clues about which developmental pathways are altered in vitrified equine oocytes and which genes may serve as potential markers for oocyte damage. Although transcriptomic and (epi)genetic alterations have the potential to impair long-term health, no associations with increased adverse obstetric and perinatal risks, altered embryonic metabolome or congenital anomalies or aneuploidies were found for vitrified human oocytes (Levi Setti *et al.* 2013; Cobo *et al.* 2014).

Conclusion

The vitrification of equine immature oocytes would improve the flexibility of horse assisted reproductive techniques and open the door towards the conservation and worldwide distribution of valuable female genetics. Equine oocyte vitrification has resulted in embryo development and live foals, but low clinical efficiency urges more research on this topic. This research should be focused on optimising technical aspects of vitrification, as well as on limiting damage to the oocyte, where the spindle, oolemma and mitochondria seem especially vulnerable. In addition, chromosomal instability may be the cause of the current low results. At present, mostly immature oocytes are used for vitrification, but it may be interesting to focus on IVM oocytes, matured under *in vivo*-like conditions, because *in vivo*-matured oocytes have yielded the best results so far. Blastocyst development and foaling rate are the best outcome parameters to estimate the true value of optimised vitrification procedures.

Conflicts of interest

The authors declare no conflicts of interest.

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