REPRODUCTION

REVIEW

Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification

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Abstract

Preservation of female genetics is currently done primarily by means of oocyte and embryo cryopreservation. The field has seen much progress during its four-decade history, progress driven predominantly by research in humans, cows, and mice. Two basic cryopreservation techniques rule the field – controlled-rate freezing, the first to be developed, and vitrification, which, in recent years, has gained a foothold. While much progress has been achieved in human medicine, the cattle industry, and in laboratory animals, this is far from being the case for most other mammals and even less so for other vertebrates. The major strides and obstacles in human and other vertebrate oocyte and embryo cryopreservation will be reviewed here.

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Introduction

Preservation of female genetics can be done through the preservation of germplasm (oocytes and embryos). It can also be done by preservation of ovarian tissue or entire ovary for transplantation, followed by oocyte harvesting or natural fertilization. Germplasm can be collected at different stages in its maturation process using any of the following techniques: 1) following ovulation (natural or chemically induced); 2) by ovum pick up, performed transabdominally, transvaginally, or transrectally. This can be done during natural estrous cycle or following chemical stimulation to achieve superovulation; 3) following ovariectomy, when removing the ovaries due to health issues, as a means of contraception, or post mortem; and 4) after fertilization (natural mating or artificial insemination) at various developmental stages prior to implantation. The collected oocytes can be at any level of maturation including oocytes found in primordial, preantral, or antral follicles, each presenting its own special requirements and sensitivities (Carroll *et al*. 1990*b*, Jewgenow *et al*. 1998). Oocyte harvesting and preserving, however, is practically pointless in the long run if other associated assisted reproductive technologies – *in vitro* maturation (IVM), IVF, *in vitro* culture (IVC), and embryo transfer (ET), are not mastered to support it. Unlike in humans and a handful of domestic and laboratory animals where much progress has been reported, this is far from being the case for the vast majority of vertebrates on Earth. Preservation of females' germplasm poses several difficulties, which will be discussed in the following pages.

Germplasm cryopreservation

Two basic techniques currently rule the field of oocyte and embryo cryopreservation. The first to be developed was the slow freezing technique (Whittingham 1971, Whittingham *et al*. 1972, Wilmut 1972, Willadsen *et al*. 1976, 1978). Following this technique, germplasm is gradually exposed to relatively low concentration of permeating cryoprotectants (CPs). These are usually glycerol or DMSO in the range of 1.0–1.5 M for oocytes or 1.35–1.5 M for embryos, which are added to the culture medium. Other CPs are also in widespread use, alone or in various combinations. These include permeating CPs such as ethylene glycol (EG) and propylene glycol (e.g. Chen *et al*. 2005*b*, Luz *et al*. 2009) and non-permeating ones such as sucrose, glucose, or fructose (e.g. Diez *et al*. 2001, Barcelo-Fimbres & Seidel 2007*b*). The germplasm is then loaded in small volumes into straws and cooled to about −5 to −7 °C where they are kept for several minutes to equilibrate. After equilibration, the solution is seeded to initiate extracellular freezing, and then cooled slowly, at about 0.3–0.5 °C/min, to anywhere between −30 and −65 °C. Once at the desired temperature, the straws are plunged into liquid nitrogen for storage. When following this procedure, seeding of the extracellular solution and a very slow cooling rate ensure that freezing will take place only outside the germplasm, resulting in outward movement of osmotically active water from the germplasm and their gradual dehydration until they reach

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the temperature at which the intracellular matrix vitrifies (Mazur 1963).

The second technique is vitrification. To achieve this, three important factors should be considered:

1) Cooling rate, which is achieved with liquid nitrogen or liquid nitrogen slush. When using liquid nitrogen, the sample is plunged into liquid nitrogen resulting in cooling rates of hundreds to tens of thousands degrees Celsius per min, depending on the container, the volume, the thermal conductivity, the solution composition, etc. (e.g. Yavin & Arav 2007). To achieve liquid nitrogen slush, the liquid nitrogen needs to be cooled close to its freezing point (−210 °C). Slush is generated by the VitMaster (IMT Ltd, Ness Ziona, Israel), a device that reduces the temperature of the LN to between −205 and −210 °C by applying negative pressure. Liquid nitrogen slush is then formed, and the cooling rate is dramatically increased. The cooling rate is especially enhanced in the first stage of cooling (from 20 to −10 °C), when it is two to six times higher than liquid nitrogen (−196 °C) with 0.25 ml straw or any other device such as open-pulled straws (OPS) or electron microscope (EM) grids (Arav & Zeron 1997). It was shown for oocytes and embryos that increasing the cooling rate would improve survival rates by up to 37% (Table 1).
2) Viscosity of the medium in which the embryos are suspended. This is defined by the concentration and behavior of various CPs and other additives during vitrification. The higher the concentration of CPs, the higher the glass transition temperature (Tg), thus lowering the chance of ice nucleation and crystallization. Different CPs and other additives have different toxicity, penetration rate, and Tg. The combination of different CPs is often used to increase viscosity, increase Tg, and reduce the level of toxicity. In the cattle industry, so as to avoid handling of the post-warmed embryos and allow direct transfer, EG is often used as the permeating CP because of its high penetration rate (Saha *et al*. 1996).
3) Volume – the smaller the volume, the higher the probability of vitrification (Arav 1992, Arav *et al*. 2002, Yavin & Arav 2007). Smaller volumes allow better heat transfer, thus facilitating higher cooling rates. Many techniques have been developed to reduce sample volume with an explosion of methods appearing in the literature during the last decade. These techniques can generally be divided into two categories, surface techniques and tubing techniques. The surface techniques (Fig. 1) include EM grid (Steponkus *et al*. 1990, Martino *et al*. 1996), minimum drop size (MDS; Arav 1992, Arav & Zeron 1997, Yavin & Arav 2001), Cryotop (Hamawaki *et al*. 1999, Kuwayama & Kato 2000), Cryoloop (Lane *et al*. 1999*a*, 1999*b*), Hemi-straw (Vanderzwalmen *et al*. 2000), solid surface (Dinnyes *et al*. 2000), nylon mesh (Matsumoto *et al*. 2001), Cryoleaf (Chian *et al*. 2005), direct cover vitrification (Chen *et al*. 2006), fiber plug (Muthukumar *et al*. 2008), vitrification spatula (Tsang & Chow 2009), Cryo-E (Petyim *et al*. 2009), plastic blade (Sugiyama *et al*. 2010), and Vitri-Inga (Almodin *et al*. 2010). To the tubing techniques (Fig. 2) belong the plastic straw (Rall & Fahy 1985), OPS (Vajta *et al*. 1997, 1998), closed pulled straw (CPS; Chen *et al*. 2001), flexipet-denuding pipette (Liebermann *et al*. 2002), superfine OPS (Isachenko *et al*. 2003), CryoTip (Kuwayama *et al*. 2005), pipette tip (Sun *et al*. 2008), high-security vitrification device (Camus *et al*. 2006), sealed pulled straw (Yavin *et al*. 2009), Cryopette (Portmann *et al*. 2010), Rapid-i (Larman & Gardner 2010), and JY Straw (R C Chian, personal communication). Each of these two groups has its specific advantages. In the surface methods, if the size of the drop (∼0.1 μl) can be controlled, high cooling rate can be achieved because these systems are open, and high warming rates are achieved by direct exposure to the warming solution. The tubing systems have the advantage of achieving high cooling rates in closed systems, thus making them safer and easier to handle. Decreasing the vitrified volume and increasing the cooling rate allow a moderate decrease in CP concentration so as to minimize its toxic and osmotic hazardous effects (Yavin *et al*. 2009). Combining these three factors can result in the following general equation for the probability of vitrification:

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

An attempt to compare between slow freezing and vitrification is basically a comparison between a method

Table 1 The effect of cooling rate on survival; comparison between liquid nitrogen and liquid nitrogen slush.

Model	Survival slush (%)	Survival LN (%)	Sig.	Publication
Bovine MII	48	28	$P<0.05$	Arav & Zeron (1997)
Ovine GV	25	5	$P<0.05$	Isachenko *et al*. (2001)
Porcine blastocysts	83	62	$P<0.05$	Beebe *et al*. (2005)
Bovine MII	48	39	$P<0.05$	Santos *et al*. (2006)
Mouse four-cell embryos with biopsied blastomere	87	50	$P<0.05$	Lee *et al*. (2007)
Rabbit embryos	92	83	NS	Papis *et al*. (2009)
Porcine blastocysts	89	93	NS	Cuello *et al*. (2004)
Mouse MII	>80	>80	NS	Seki & Mazur (2009)
Rabbit oocytes	82	83	NS	Cai *et al*. (2005)

LN, liquid nitrogen; GV, germinal vesicle; Sig., statistical significance; NS, not significant.

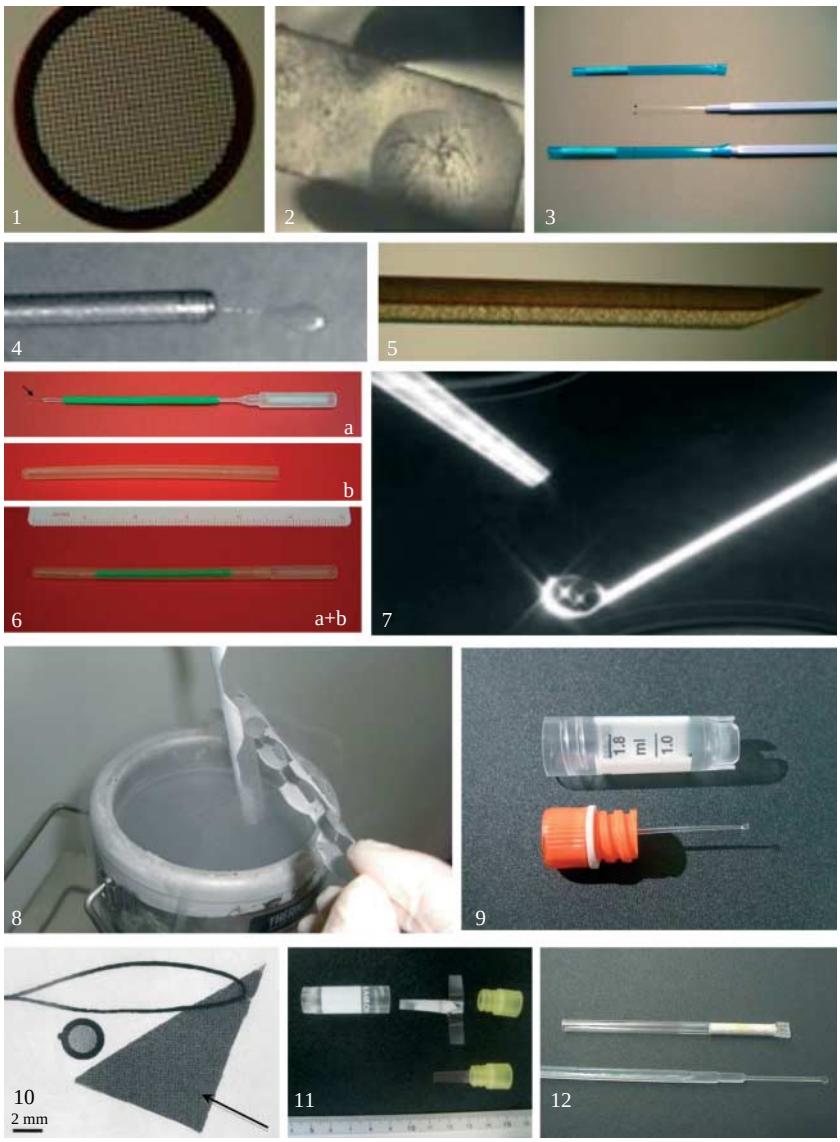

Figure 1 Vitrification surface carrier systems: (1) electron microscope grid, (2) minimum drop size, (3)* Cryotop, (4)* Cryoloop, (5) Hemi-straw, (6)* Cryoleaf, (7)* fiber plug, (8)* direct cover vitrification, (9)* vitrification spatula, (10) nylon mesh; arrow points at the nylon mesh, (11)* plastic blade, (12)* Vitri-Inga. *These photos were kindly provided by Masa Kuwayama (3), Juergen Liebermann (4, 7), Ri-Cheng Chian (6), Shee-Uan Chen (8), King L Chow and Wai Hung Tsang (9), Koji Nakagawa (11), and Carlos Gilberto Almodin (12). Picture reprinted with minor revisions from **Matsumoto H, Jiang JY, Tanaka T, Sasada H & Sato E** 2001 Vitrification of large quantities of immature bovine oocytes using nylon mesh. *Cryobiology* **42** 139–144, with permission from Elsevier. © 2001 Elsevier.

and a physical process. It would be wrong and too simplistic to define the difference between the two by saying that slow freezing is a method in which slow cooling rate and low CP concentrations are used, while in vitrification, high cooling rate and high CP concentration are used. Successful vitrification can occur with a very low cooling rate (Seki & Mazur 2009) and very low concentration of CPs (Arav 1992). Cryopreservation by slow freezing is a process where extracellular water crystallizes, resulting in osmotic gradient that draws water from the intracellular compartment till intracellular vitrification occurs. In cryopreservation by vitrification, both intra and extracellular compartments apparently vitrify after cellular dehydration has already occurred. Owing to these differences, the terms freezing and thawing are relevant to the slow freezing process while cooling and warming are relevant to vitrification. Both slow freezing and vitrification are under the umbrella of cryopreservation. Unlike the controlled-rate freezing method, which requires sophisticated equipment to manage the cooling rate, vitrification can be done relatively cheaply and even under field conditions with no need for special equipment, making it a good alternative for the use in various settings often encountered with wildlife species, such as zoos, poorly equipped locations, and field work in remote sites. However, performing vitrification, and in particular loading the sample properly into or onto the container, does require much experience to be done properly.

Once frozen or vitrified, germplasm can be stored for extended periods of time with no noticeable deterioration. Cryostorage of frozen human embryos for up to 20 years, for instance, was shown recently to have no effect on any of the parameters evaluated – post-thaw survival, and rates of implantation, clinical pregnancy, miscarriage, and live birth (Riggs *et al.* 2010).

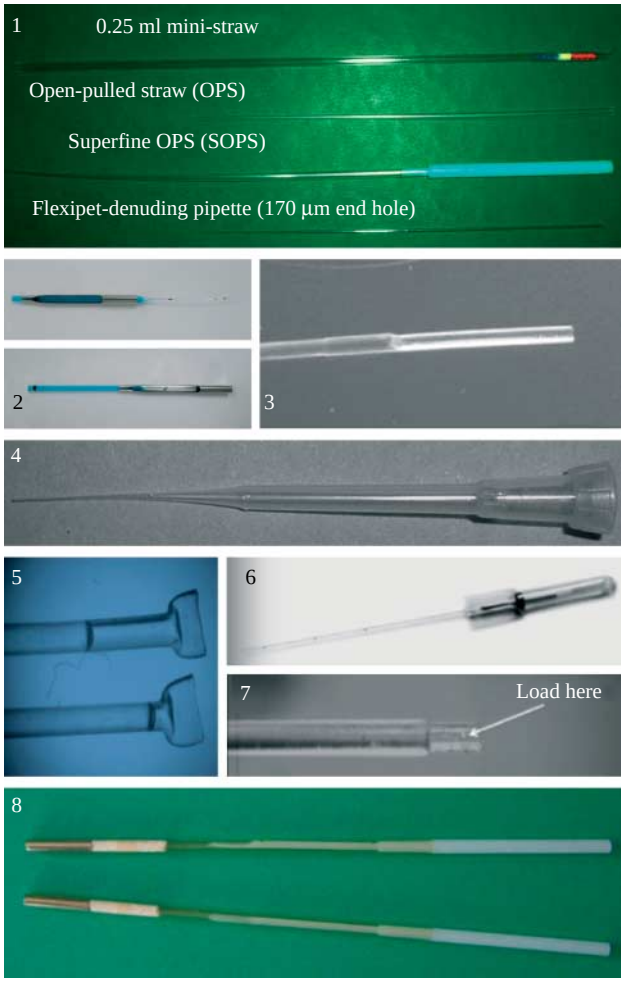

Figure 2 Vitrification tubing carrier systems: (1 top)* plastic straw, (1, 2nd from top)* open-pulled straw, (1, 3rd from top)* superfine open-pulled straw, (1 bottom)* flexipet-denuding pipette, (2)* CryoTip, (3)* high-security vitrification device, (4)* pipette tip, (5) sealed pulled straw, (6)* Cryopette, (7)* Rapid-i, and (8)* JY Straw. *These photos were kindly provided by Juergen Liebermann (1, 3, 6, 7), Masa Kuwayama (2), John Engelhardt (4), and Ri-Cheng Chian (8).

Recently, Seki & Mazur (2009) have shown the dominance of warming rate on cooling rate during vitrification. Survival of mouse oocytes after very slow cooling rate (<200 °C/min) with high warming rate (>2000 °C/min) was very high when compared with those cooled very rapidly and warmed slowly. However, we and others (see Table 1) have shown that this is not the case for chilling sensitive oocytes and embryos such as those of bovine, pig, rabbit, and human (Arav & Zeron 1997, Lee *et al*. 2007, Papis *et al*. 2009).

Oocyte freezing and vitrification

Females are born with their life supply of oocytes already in their ovaries. Unlike males, they do not generate new gametes during their reproductive years, or at least so it was generally assumed until recently (e.g. Niikura *et al*. 2009; and reviewed in Tilly *et al*. 2009). At birth, oocytes are dormant at a very early stage of maturation. Once the female reaches puberty, a cohort of oocytes is selected at each estrous cycle to progress in the maturation process and, depending on the species, one or several oocytes are ovulated whereas the rest of the cohort degenerate. To be fertilized, an oocyte needs to reach the metaphase II (MII) stage of maturation, or else the probability of fertilization is very low (Luvoni & Pellizzari 2000). Thus, an IVM procedure should be in hand to handle immature oocytes, and this process is currently developed for only a handful of species and even for these success is often fairly limited (Krisher 2004). Furthermore, collection of immature oocytes following chemical stimulation disrupts the natural maturation process and thus compromises the quality of oocytes even if they were later matured *in vitro* (Moor *et al*. 1998, Takagi *et al*. 2001). During oocyte maturation and follicular growth, oocytes accumulate large quantities of mRNA and proteins needed for continuation of meiosis, fertilization, and embryonic development (Krisher 2004 and citations therein). In the absence of the entire supporting system during IVC, production of some of these needed components is hampered resulting in suboptimal oocytes (Krisher 2004). In some seasonal animals, for example in cats or red deer, oocytes collected out of season often show resistance to IVM and IVF (Spindler *et al*. 2000, Berg & Asher 2003, Comizzoli *et al*. 2003), a problem that can be partially avoided by inclusion of anti-oxidants and FSH in the culture media (Comizzoli *et al*. 2003). Despite numerous studies on the issue, to date, no morphological or other method is able to accurately predict which oocytes have optimal developmental potential (Coticchio *et al*. 2004). Even so, it is clear that oocyte quality is a major determining factor in the success of IVF, early embryonic survival, establishment and maintenance of pregnancy, fetal development, and even adult disease (Coticchio *et al*. 2004, Krisher 2004). Once all these hurdles have been overcome and keeping in mind the importance of oocyte quality, the next major hurdle to overcome is oocyte cryopreservation.

Oocyte cryopreservation: the difficulties

Oocytes are very different from sperm or embryos with respect to cryopreservation. The volume of the mammalian oocyte is in the range of three to four orders of magnitude larger than that of the spermatozoa, thus substantially decreasing the surface-to-volume ratio and making them very sensitive to chilling and highly susceptible to intracellular ice formation (Toner *et al*. 1990, Ruffing *et al*. 1993, Arav *et al*. 1996, Zeron *et al*. 1999). This problem becomes even more pronounced in non-mammalian vertebrates (fish, birds, amphibians, and reptiles) whose oocytes are considerably larger than those of mammals (e.g. Guenther *et al*. 2006, Kleinhans *et al*. 2006). Oocytes of amphibians, for example, are 20–25 times larger than human oocytes. The plasma membrane

of oocytes at the MII stage has a low permeability coefficient, thus making the movement of CPs and water slower (Ruffing *et al*. 1993). They are surrounded by zona pellucida, which acts as an additional barrier to movement of water and CPs into and out of the oocyte. As a result of the freeze–thaw process, premature cortical granule exocytosis may take place, leading to zona pellucida hardening and making sperm penetration and fertilization impossible (Carroll *et al*. 1990*a*, Mavrides & Morroll 2005), a process that can be overcome by the use of ICSI or subzonal sperm insertion. Oocytes also have high cytoplasmic lipid content that increases chilling sensitivity (Ruffing *et al*. 1993). They have less submembranous actin microtubules (Gook *et al*. 1993) making their membrane less robust. Cryopreservation can cause cytoskeleton disorganization, and chromosome and DNA abnormalities (Luvoni 2000). The meiotic spindle, which has been formed by the MII stage, is very sensitive to chilling and may be compromised as well (Ciotti *et al*. 2009). It does, however, tend to recover to some extent after thawing or warming and IVC, recovery that is faster following vitrification than following slow freezing (Ciotti *et al*. 2009). Oocytes are also more susceptible to damaging effects of reactive oxygen species (Gupta *et al*. 2010). Many of these parameters change after fertilization, making embryos less chilling sensitive and easier to cryopreserve (Gook *et al*. 1993, Fabbri *et al*. 2000, Ghetler *et al*. 2005). Despite many advances in the field of cryopreservation, specifically with regards to oocytes (ovulated, mature or immature), their cryopreservation is still not considered an established procedure and thus its current label as experimental technique (Noyes *et al*. 2010). Even in human medicine, fewer than 200 births resulting from cryopreserved oocytes were reported as of 2007 (Edgar & Gook 2007), a number that went up to only 500 by 2009 (Nagy *et al*. 2009). Yet, despite all these difficulties, some success in oocyte cryopreservation has been reported.

Overcoming the difficulties

The first human pregnancy from cryopreserved (by slow freezing) oocyte was reported in 1986 (Chen 1986). This followed success in other (laboratory) species that came a few years earlier, such as the mouse (Whittingham 1977) and rat (Kasai *et al*. 1979). Despite several decades of research since these initial reports, success is still very limited. A meta-analysis on slow freezing of human oocytes showed that clinical pregnancy rate per thawed oocyte was only 2.4% (95/4000) and only 1.9% (76/4000) resulted in live birth (Oktay *et al*. 2006). Vitrification gained a foothold only after 2005, prior to which only ten human pregnancies resulting from vitrified oocytes were reported (Oktay *et al*. 2006). Although high oocyte survival rate is achieved with both methods, fertilization and ET rates are still considerably lower than when fresh oocytes are used (Magli *et al*. 2010). When comparing slow freezing to vitrification, higher oocyte survival rates are achieved by the latter (95%, 899/948 vs 75%, 1275/1683 respectively), but pregnancy rate per thawed/warmed oocyte is still low – in the range of 1.9–8.6% for slow freezing and 3.9–18.8% for vitrification (Chen & Yang 2009). Even among females with repetitive reproductive success, the rate of live birth per oocyte retrieved was reported to be 7.3% (180/2470) among best-prognosis donors and lower than that (5.0%; 52/1044) among standard donors (Martin *et al*. 2010).

Immature oocytes seem to be less prone to damages caused by the chilling (at the nuclear level), freezing, and thawing procedures, and they, too, can be cryopreserved by controlled-rate freezing (Luvoni *et al*. 1997) or vitrification (Arav *et al*. 1993). Preantral oocytes can be preserved inside the follicle, and about 10% seem to be physiologically active after thawing and 1 week of culture (Jewgenow *et al*. 1998, Nayudu *et al*. 2003). In one report, of ∼16 000 small preantral follicles recovered from the ovaries of 25 cats, 66.3% were intact after thawing. Before freezing, 33.9% of the follicles contained viable oocytes. This decreased after thawing to 19.3% if frozen in DMSO and 18.5% if frozen in 1,2-propanediol (Jewgenow *et al*. 1998). However, culture conditions that allow these oocytes to grow and reach full maturation are still largely unknown despite attempts in several species. The only species in which live young were produced from fresh (Eppig & O'Brien 1996) or frozen–thawed (Carroll *et al*. 1990*b*) primary follicles is the mouse. Some, very limited, success was also reported in cats, where following vitrification in 40% EG, 3.7% of the *in vitro* matured oocytes were able to develop to the blastocyst stage following IVF (Murakami *et al*. 2004). The problems associated with maturation of early-stage oocytes *in vitro* are the need to develop the complex endocrine system that supports the development at different stages, other culture conditions that will ensure survival (oxygen pressure for example) and, in many species, the duration of time required to keep the follicles in culture – 6 months or more (Telfer *et al*. 2000). Another option for isolated oocyte freezing is freezing individual primordial follicles and later transplanting them to the ovarian bursa, where they can mature and eventually produce young offspring following natural mating as was shown in mice (Carroll & Gosden 1993). Alternatively, ovarian cortex tissue or the entire ovary can be frozen or vitrified and then, after thawing/warming, transplanted to allow maturation *in vivo* (Candy *et al*. 1995, Revel *et al*. 2001), or else the oocytes can be fertilized and the resulting embryos can then be cryopreserved.

Embryo freezing and vitrification

For most of the species on Earth, with current knowledge in cryopreservation, probably only male gametes can be preserved, whereas oocytes or embryos at any stage of development cannot. The culprits are in the vast

differences in size, composition, and associated structures. As such, the issue of intracellular ice formation becomes a major concern, even at relatively slow cooling rates. To avoid this from happening, small volume cryopreservation and either high CP concentration coupled with very fast cooling rate to achieve a state of vitrification or lower CP concentration and slow cooling rate to ensure ice formation in the extracellular matrix only (controlled-rate freezing) are utilized. The first reports on successful embryo cryopreservation were published in Whittingham (1971), Whittingham *et al*. (1972) and Wilmut (1972), more than two decades after Polge *et al*. (1949) reported their success in freezing spermatozoa. A modification to cooling rate that came a few years later (Willadsen *et al*. 1976, 1978) resulted in a basic protocol that is still in vast use today. When considered from conservation standpoint, embryo freezing has the advantage of preserving the entire genetic complement of both parents. While in humans and domestic and laboratory animals this is not an issue, for many other species getting both a male and a female together to generate embryos is often a problem, and when such embryos are finally created, one would often opt for letting pregnancy proceed rather than collecting the embryos for storage. Naturally, both male and female embryos should be stored to ensure representation of both sexes and wide genetic diversity. Cryobanking of embryos can thus help in establishing founder populations with the aim of eventual reintroduction into the wild (Ptak *et al*. 2002). However, evolution made each species unique in many respects, one of which is the development of highly specialized reproductive adaptation (Allen 2010), a specialization that is part of the definition of a species (de Queiroz 2005). Thus, what may work for one species does not necessarily work for another. While thousands and thousands of offspring were born following the transfer of frozen–thawed embryos in humans, cattle, sheep, and mice, success is very limited in many other, even closely related species. To date, the number of species in which embryo cryopreservation has been reported stands only at about 40 (humans and domestic and laboratory animals included; Table 2). Obviously, to be successful, the best option is to test and make the necessary adjustments to protocols using embryos of the target species. In wild animals, especially with endangered species, this is often almost impossible, and the opportunity to collect oocytes or embryos is very rare. To overcome this limitation, researchers find it imperative to use laboratory, farm, or companion animals as models during the process of developing the necessary reproductive techniques associated with embryo cryopreservation. In some instances, appropriate model species were found. For example, studies on the domestic cat helped to develop various technologies, which were later applied to non-domestic cats (Dresser *et al*. 1988, Pope *et al*. 1994, Pope 2000) or cattle served as a model for other ungulates (Dixon *et al*. 1991, Loskutoff *et al*. 1995). Unfortunately, for many species (e.g. elephant, rhinoceros), no suitable model can be located, and studies should be conducted with the limited available resources while relying on the already available knowledge from research on other species (Hermes *et al*. 2009). Cryopreservation of embryos in the few mammalian species in which it was attempted shows some, though often very limited, success.

Non-human primates

Whereas non-human primates serve as research models for humans in a wide variety of fields, things work the other way around when it comes to embryo cryopreservation. The first successful non-human primate embryo freezing (baboon; Pope *et al*. 1984) was reported a year after the first reported pregnancy following transfer of a frozen–thawed human embryo (Trounson & Mohr 1983). Things have not changed much since, and many of the advances in embryo cryopreservation (primates and others) were driven by research in human fertility laboratories. Some scattered reports on non-human primate embryo cryopreservation by either controlled-rate freezing or vitrification were published over the years, mostly working on small numbers of animals and showing very limited success (e.g. Hearn & Summers 1986, Cranfield *et al*. 1992, Curnow *et al*. 2002).

Ungulates

A similar situation is found among ungulates. Industry needs pushed frozen–thawed ET in the cattle industry to commercial levels. According to a recent report by the International ET Society, over 300 000 frozen–thawed bovine embryos were transferred in 2008 worldwide (Thibier 2009). This success was driven by at least four important factors – needs of the industry, availability of financial resources to support overwhelming number of studies, the availability of an almost unlimited flow of oocytes from abattoirs that made these studies possible, and the fact that non-surgical collection of embryos is possible in cattle. The situation is so far behind in other ungulates that only a decade ago reviews on assisted reproductive technologies in non-domestic ungulates were to the effect that by that time only one successful embryo cryopreservation has been achieved (Holt 2001). Several factors, in addition to the need, money, and availability mentioned above, are responsible for this disparity between bovine and other ungulates. Non-domestic ungulates usually do not show discernable signs of estrus, and their receptive period is fairly short. This requires a thorough understanding of the estrous cycle, endocrine activity, and methods for monitoring these in each species under study, knowledge that is lacking for almost all ungulates. As in all other wildlife

Table 2 Embryo cryopreservation in mammalian species.

Species	Scientific name	Achievement	Years	Reference of first report
Primates				
Human	*Homo sapiens*	First pregnancy (IVF eight-cell) then live birth (IVF 8- to 16-cell)	1983	Trounson & Mohr (1983) and Zeilmaker *et al.* (1984)
Baboon	*Papio* sp.	Live birth (i.v. 12-cell)	1984	Pope *et al.* (1984)
Marmoset monkey	*Callithrix jacchus*	Live birth (i.v. four- to eight-cell and morula)	1986	Hearn & Summers (1986)
Cynomolgus monkey	*Macaca fascicularis*	Slow freezing (IVF four- to eight-cell) – pregnancies, vitrification (IVF two- to eight-cell) – *in vitro* evaluation	1986	Balmaceda *et al.* (1986) and Curnow *et al.* (2002)
Rhesus macaque	*Macaca mulatta*	Live birth (slow freezing (IVF three- to six-cell) and vitrification (ICSI blastocysts))	1989	Wolf *et al.* (1989) and Yeoman *et al.* (2001)
Hybrid macaque (pig-tailed and lion-tailed)	*Macaca nemestrina* and *Macaca silenus*	Live birth of a hybrid (IVF two-cell)	1992	Cranfield *et al.* (1992)
Western lowland gorilla	*Gorilla gorilla gorilla*	Freezing outcome not reported (IVF two-cell)	1997	Pope *et al.* (1997)
Ungulates				
Bovine	*Bos taurus*	Live birth (i.v. blastocyst)	1973	Wilmut & Rowson (1973)
Sheep	*Ovis aries*	Pregnancy base on progesterone and later live births (i.v. day 5–8 for both)	1974	Willadsen *et al.* (1974, 1976)
Goat	*Capra aegagrus*	Live births (i.v. day 5–7)	1976	Bilton & Moore (1976)
Horse	*Equus caballus*	Live birth (i.v. day 6)	1982	Yamamoto *et al.* (1982)
African eland antelope	*Taurotragus oryx*	Pregnancy (i.v. blastocyst; palpation at 100 days) and later live birth	1983	Kramer *et al.* (1983) and Dresser *et al.* (1984)
Arabian oryx	*Oryx leucoryx*	Failed transfer (i.v. morula)	1983	Durrant (1983)
Gaur	*Bos gaurus*	Freezing outcome not reported (i.v. blastocysts), then pregnancy by palpation (IVF expanded blastocyst)	1984	Stover & Evans (1984) and Armstrong *et al.* (1995)
Bongo	*Tragelaphus euryceros*	Transfer outcome and embryo stage (i.v.) not reported	1985	Dresser *et al.* (1985)
Scimitar-horned oryx	*Oryx dammah*	Failed transfer (i.v. late morula–blastocyst)	1986	Schiewe *et al.* (1991)
Swine	*Sus domestica*	Live birth (i.v. morula and blastocyst)	1989	Hayashi *et al.* (1989)
Red deer	*Cervus elaphus*	Live birth (i.v. morula and blastocyst)	1991	Dixon *et al.* (1991)
Suni antelope	*Neotragus moschatus zuluensis*	Failed transfer (eight-cell)	1991	Cited in Schiewe (1991)
Wapiti	*Cervus canadensis*	Live birth (stage not reported)	1991	Cited in Rall (2001)
Dromedary camel	*Camelus dromedarius*	First pregnancy (stage not reported) and then first birth (i.v. expanded blastocysts)	1992	Cited and reported in Nowshari *et al.* (2005)
Water buffalo	*Bubalus bubalis*	Live birth (i.v. morula to expanded blastocyst)	1993	Kasiraj *et al.* (1993)
Fallow deer	*Dama dama*	Pregnancy outcome not reported (i.v. morula and blastocyst)	1994	Morrow *et al.* (1994)
European mouflon	*Ovis orientalis musimon*	Live birth (i.v. blastocyst; by vitrification)	2000	Naitana *et al.* (2000)
Llama	*Lama glama*	*In vitro* evaluation then pregnancy (by vitrification) (i.v. hatched blastocysts for both)	2000	Palasz *et al.* (2000) and Aller *et al.* (2002)
Wood bison	*Bison bison athabascae*	Vitrification outcome not evaluated (IVF morula and blastocyst)	2007	Thundathil *et al.* (2007)
Sika deer	*Cervus nippon nippon*	Live birth (IVF blastocysts)	2008	Locatelli *et al.* (2008)
Carnivores				
Domestic cat	*Felis catus*	Live birth (i.v. stage not reported)	1988	Dresser *et al.* (1988)
Blue fox	*Alopex lagopus*	Implantation sites found (stage and source not reported)	2000	Cited in Farstad (2000)
Siberian tiger	*Panthera tigris altaica*	*In vitro* evaluation (IVF, two- to four-cell)	2000	Crichton *et al.* (2000)
African wildcat	*Felis silvestris*	Live birth (IVF, day 5–6 of IVC)	2000	Pope *et al.* (2000)
Ocelot	*Leopardus pardalis*	Live birth (IVF, stage not reported)	2000	Cited in Swanson (2001)
Tigrina	*Leopardus tigrinus*	Freezing outcome not reported (IVF, two- to eight-cell)	2002	Swanson *et al.* (2002)
Bobcat	*Lynx rufus*	Failed transfer (i.v. blastocyst)	2002	Miller *et al.* (2002)
Caracal	*Felis caracal* or *Caracal caracal*	Live birth (stage and source not reported)	2002	Cited in Swanson (2003)
European polecat	*Mustela putorius*	Live birth (i.v. blastocysts by slow freezing and i.v. morula and blastocysts by vitrification)	2003	Lindeberg *et al.* (2003) and Piltti *et al.* (2004)
Geoffroy's cat	*Felis geoffroyi*	Freezing outcome not reported (IVF, stage not reported)	2004	Swanson & Brown (2004)
Serval	*Leptailurus serval*	Failed transfer (IVF, morula)	2005	Pope *et al.* (2005)
Dog	*Canis lupus familiaris*	Live birth (i.v. 8- to 16-cell)	2009	Suzuki *et al.* (2009)
Clouded leopard	*Neofelis nebulosa*	Failed transfer (IVF, morula)	2009	Pope *et al.* (2009)

Table 2 *Continued.*

Species	Scientific name	Achievement	Years	Reference of first report
Glires				
Mouse	*Mus musculus*	Live birth, frozen to −79 °C (i.v. eight-cell and blastocysts)	1971	Whittingham (1971)
European rabbit	*Oryctolagus cuniculus*	Live birth (i.v. eight-cell and morula by slow freezing and i.v. morula by vitrification)	1974	Bank & Maurer (1974), Whittingham & Adams (1974) and Smorag *et al.* (1989)
Rat	*Rattus norvegicus*	Live birth (i.v. two- to eight-cell by slow freezing and i.v. blastocyst by vitrification)	1975	Whittingham (1975) and Kono *et al.* (1988)
Syrian hamster	*Mesocricetus auratus*	Pregnancy (i.v. one-cell to morula) and live birth (i.v. one- to two-cell by vitrification)	1985	Ridha & Dukelow (1985) and Lane *et al.* (1999*a*, 1999*b*)
Mongolian gerbil	*Moriones unguiculatus*	Live birth (i.v. four-cell to blastocyst by vitrification)	1999	Mochida *et al.* (1999)
Marsupials				
Fat-tailed dunnart	*Sminthopsis crassicaudata*	*In vitro* evaluation (i.v. one- to four-cell)	1994	Breed *et al.* (1994)

Achievement is reported as most advanced outcome, followed, in parentheses, by source of embryos (IVF; i.v., *in vivo*-produced embryos; ICSI) and stage of embryos frozen/vitrified. Failed transfer, transferred embryo that failed to lead to clinical pregnancy.

species, what works for one does not necessarily work for another, even closely related species. For example, bovine IVC protocol works well for water buffalo (*Bubalus bubalis*), but when this protocol was used for African buffalo (*Syncerus caffer*), embryos did not develop beyond the morula stage (Loskutoff *et al.* 1995). Whereas hormonal monitoring can be achieved non-invasively through fecal analysis, hormonal administration for synchronization or ovarian stimulation requires stress-inflicting activities such as repeated darting, general anesthesia, or movement restriction by a chute. Thus, progress in this field has been slow. Although ET has produced live births in a number of non-domestic ungulate species, efficiency in *in vitro* technologies (IVM, IVF, and IVC) has been low. For example, in a study on kudu (*Tragelaphus* sp.), of 397 oocytes collected, 79 zygotes cleaved yet only 2 blastocysts were achieved (0.5%; Loskutoff *et al.* 1995). Another example is the Mohor gazelle (*Gazella dama mhorr*) in which embryos produced by IVF with frozen–thawed semen did not develop beyond the six- to eight-cell stage (Berlinguer *et al.* 2008). These studies suggest that before reaching a stage at which embryo cryopreservation is a technology worthwhile pursuing, other associated technologies should reach a level of maturation to support it. To at least partially overcome this limitation, and because they survive the cryopreservation process better, *in vivo* produced embryos were utilized in many of the attempts to freeze embryos from non-domestic ungulates. Still, almost all reported successes were in a few species of some commercial value (such as camels, llamas, and red deer; Table 2).

Carnivores

The order Carnivora includes two suborders – feliformia (cat-like) and caniformia (dog-like). Both suborders have at least one highly accessible member that can act as a model for other species – the domestic cat (*Felis catus*) and dog (*Canis lupus familiaris*) respectively. However, while to date all relevant technologies have been successfully developed in the cat model and applied, with some level of success to other felids, the situation is far behind in the domestic dog, and progress has been slow. Preliminary technologies such as IVM, IVF, and IVC are not yet fully mastered for dogs, and outcome is often unpredictable (Rodrigues & Rodrigues 2006, Mastromonaco & King 2007). In the vast majority of studies, dog zygotes do not progress to advanced embryonic developmental stages (morula and blastocyst; Rodrigues & Rodrigues 2006). Thus, while delivery of the first kittens following transfer of frozen–thawed cat embryos was reported in Dresser *et al.* (1988), the parallel report in dogs was only published two decades later (Suzuki *et al.* 2009, Abe *et al.* 2011). We were unable to find any publication on embryo cryopreservation in a non-domestic canid, other than a reference to an attempt to freeze blue fox (*Alopex lagopus*) embryos, cited as personal communication by Farstad (2000). Attempts were also carried out in another family within caniformia – the mustelids. Some species in this family are of commercial value, primarily in the fur industry, and are thus highly accessible. The European polecat (*Mustela putorius*) was used as a model to develop embryo retrieval, cryopreservation, and transfer technologies to be later applied to endangered species such as the black-footed ferret (*Mustela nigripes*) or the European mink (*Mustela lutreola*). Both controlled-rate freezing and vitrification were attempted, using *in vivo*-produced embryos, with vitrification (Sun *et al.* 2008) producing far better results than the controlled-rate freezing technique (Lindeberg *et al.* 2003).

Glires

Embryo cryopreservation in mice was the first to be reported among all mammals (Whittingham 1971,

Whittingham *et al*. 1972, Wilmut 1972), and work on Glires (rodents and lagomorphs) has concentrated on species of laboratory importance – mice, rats, rabbits, gerbils, and hamsters. Using *in vivo*- or *in vitro*-produced embryos, both controlled-rate freezing and vitrification were attempted, the latter generally giving better results. Still, the rate of live birth per cryopreserved embryo is mostly low, around 10% and often even less. The exception was an early report on the vitrification of *in vivo*-produced rat embryos in which ~30% of the vitrified embryos resulted in young pups (Kono *et al*. 1988).

Marsupials

The last mammalian group in which attempts at embryo cryopreservation were reported is the marsupials. In comparison to other mammals, marsupial oocytes are much larger in size (~200–250 μm), their zona pellucida does not form corona radiata and is already shed off at ovulation, and a large yolk compartment takes up much of their cytoplasm (Rodger *et al*. 1992, Breed *et al*. 1994). All these characteristics make cryopreserving their oocytes far more difficult than cryopreserving the already hard-to-freeze eutherian mammals' oocytes, so the alternative is to cryopreserve embryos. In the only published attempt to do that, *in vivo*-produced fat-tailed dunnart (*Sminthopsis crassicaudata*) embryos were cryopreserved using both controlled-rate freezing and vitrification. Post-thaw/warming cleavage rate was low – 17, 0, or 18% when cryopreserved by controlled-rate freezing, or vitrified with DMSO or with EG as CPs respectively (Breed *et al*. 1994). Although under the light microscope as many as 80% of the thawed and warmed embryos looked morphologically normal, most had multiple damages to intracellular organelles when evaluated with the aid of electron microscopy.

Other vertebrates

The situation is much less advanced in all other vertebrates (fish, birds, reptiles, and amphibians) where noticeably less efforts have been invested and the challenges are often considerably more complex. In comparison to mammals, embryos in all these classes are usually substantially larger in volume resulting in a lower surface area-to-volume ratio, and thus poorer water and CP movement across cellular membrane during chilling, freezing, and thawing. These embryos have large yolk compartment, enclosed in the yolk syncytial layer (YSL). The behavior of the yolk during cryopreservation differs from that of other embryonic compartments, making cryopreservation very complex. Embryos in these vertebrates have at least three membrane structures – YSL, plasma membrane of the developing embryo, and the chorion membrane, which surrounds the perivitelline space (Kalicharan *et al*. 1998, Rawson *et al*. 2000). Each of these membranes has a different permeability coefficient to water and CPs, resulting, for example, in water permeability in the range of one order of magnitude lower in fish embryos than in other animals (0.022–0.1 μm× min per atm for zebrafish (Hagedorn *et al*. 1997*a*) compared with 0.722 in *Drosophila* (Lin *et al*. 1989) or 0.43 in mice (Leibo 1980)). As if to complicate things even further, the different embryonic compartments have different water content and different osmotically inactive water content (Hagedorn *et al*. 1997*b*). All these make embryos in these classes highly susceptible to chilling injury and, with the currently available knowledge and techniques, make their cryopreservation extremely complicated and often practically impossible (Zhang & Rawson 1996, Robles *et al*. 2003, Cabrita *et al*. 2006, Edashige *et al*. 2006, Hagedorn 2006). Attempts to overcome these hurdles and freeze embryos in these classes were made, but to date successful and reproducible embryo cryopreservation in any member of these vertebrates has never been described, and embryo cryopreservation in any of these groups seems far off.

Points for improving survival of cryopreserved oocytes and embryos

In an attempt to improve survival of the cryopreserved germplasm, several possible manipulations have been proposed so as to strengthen the weak links in these biological systems. These relatively sensitive aspects include the cellular membrane, the cytoskeleton, intracellular lipids, intracellular water, and manipulations to IVC conditions. Other aspects that have been recognized as having an effect on survival through the cryopreservation process are age of oocyte donor and, at least in some species, season of collection.

Optimal embryonic stage for cryopreservation

Despite many advances in the field of embryo cryopreservation, there is still no consensus as to the optimal developmental stage for embryo cryopreservation. A study on human embryos comparing the outcome of IVF–ET for embryos frozen at the pronuclear (day 1), cleavage (day 3), or blastocyst stage (Moragianni *et al*. 2010) found no difference between the three in rates of implantation, clinical pregnancy, multiple pregnancy, twin pregnancy, and the male/female ratio. The only difference found was in post-thaw survival rate where day 3 embryos had lower survival than day 1 or blastocyst. Interestingly, calculations based on the data in this study revealed an overall sex ratio (0.4689) and sex ratio of day 1 ETs (0.3427), both significantly lower than the current US national birth sex ratio of 0.5122 ($P=0.024$ and $P=0.000004$ respectively; binomial exact, cumulative probability, one-tailed). Day 3 and blastocyst sex ratios did not differ from 0.5122. In another study, embryos

were frozen at the zygote, day 2, and day 3 stages and transferred after thawing (Salumets *et al*. 2003). Here, too, there were no differences between groups in rates of clinical pregnancies, implantation, delivery, and birth. Miscarriage rate was higher in the day 3 group (45%) compared with the zygote group (21.3%) and day 2 embryos (18.3%). Efficiency (birth rate per thawed embryo) was low (overall, 7.3%; zygotes, 7.1%; day 2, 7.6%; and day 3, 4.2%). Yet others suggest that day 5 and 6 blastocysts are superior as, following vitrification, survival rate was 96.3% and implantation rate was 29.4% (Liebermann 2009). Clinical pregnancy rate in this study, calculated as a percentage of vitrified embryos or a percentage of warmed-transferred embryos, was 21.1 and 42.8% respectively.

Cellular membrane

The cellular plasma membrane is very sensitive to chilling and is often damaged during cryopreservation (Zeron *et al*. 2002). Cholesterol is present in the plasma membrane, and its level and the ratio between cholesterol and the membranes' phospholipids determine to a great extent the membrane fluidity and thus its chilling sensitivity (Darin-Bennett & White 1977, Horvath & Seidel 2006). Enriching the plasma membrane with cholesterol or unsaturated fatty acids can be done by incubating the cells with cholesterol-loaded methyl-β-cyclodextrin, or cholesterol- or unsaturated fatty acids-loaded liposomes. While the addition of cholesterol to the cryopreservation media had no effect (positive or negative) on cryopreserved *in vitro*-produced bovine blastocysts (Pugh *et al*. 1998), it seems to have benefited vitrified oocytes whose cleavage to the eight-cell stage after warming and IVF was slightly improved (55 vs 41% for the control, $P<0.05$; Horvath & Seidel 2006). The addition of unsaturated fatty acids to bovine oocytes by electrofusion of liposome with their plasma membrane decreased their sensitivity to chilling (Zeron *et al*. 2002).

Cytoskeleton

One of the cellular components often damaged during cryopreservation is the cytoskeleton (Dobrinsky *et al*. 2000). Its stabilization can thus be expected to improve cryosurvival. This was attempted by the addition of cytoskeleton stabilizing components such as cytochalasin B or D or colchicine to the culture media prior to cryopreservation. Pig embryos cultured with these components survived vitrification but survival seemed to be stage dependent. The addition benefited expanding and hatching blastocysts but not embryos at the morula or early blastocyst stages (Dobrinsky *et al*. 2000). The treatment with cytochalasin B, however, seems to cause irreversible actin depolymerization which may compromise embryonic survival (Tharasanit *et al*. 2005).

Intracellular lipids

The role of intracellular lipids is not fully understood. Some suggest that they are needed as an energy source for the oocyte and developing embryo (Sturmey *et al*. 2009). Others suggest that they are needed as a lipid source for cell division (Yoneda *et al*. 2004). Lipid content depends on the stage of embryo development, significantly decreasing after the morula stage (Romek *et al*. 2009), thus making early stages more susceptible to low temperatures. Following the observation that embryos with high intracellular lipid content are more prone to cryoinjury, at least three methods to manipulate these lipid droplets have been attempted.

Phenazine ethosulfate (PES) is a regulator of cell metabolism. It increases glucose metabolism, glycolysis, oxidation of NADPH to NADP, production of CO_2, and utilization of the pentose phosphate pathway (PPP), which is important in the process leading to oocyte maturation (Downs *et al*. 1998, De La Torre-Sanchez *et al*. 2006, Gajda 2009). Activity of the PPP is embryonic developmental stage dependent, peaking at the two-cell and morula stages and being lowest at the blastocyst stage. PES can increase the PPP activity sixfold, indicating that embryos can potentially reach high levels of PPP activity (O'Fallon & Wright 1986). When IVP bovine zygotes were cultured in the presence of PES, the resulting blastocysts contained lower number of medium (2–6 μm) and large (>6 μm) lipid droplets than control or zygotes cultured in the presence of FCS (Barcelo-Fimbres & Seidel 2007*a*). While the presence of PES brought a reduction in lipid droplets, the number of these was even lower in *in vivo*-produced same-stage embryos (De La Torre-Sanchez *et al*. 2006). This may explain, at least in part, why *in vivo*-produced embryos survive cryopreservation better than *in vitro*-produced ones (Rizos *et al*. 2002). Post-cryopreservation survival of blastocysts averaged over vitrification and slow freezing (between which there was no difference) was 91.9, 84.9, and 60.2% of unfrozen controls ($P<0.01$) for PES, control, and FCS groups respectively (Barcelo-Fimbres & Seidel 2007*b*). The effects of PES on *in vivo* embryonic and fetal development, however, are still unclear (Barcelo-Fimbres *et al*. 2009).

Microsurgical removal of lipids (delipidation), following high-force (>10 000 ***g***) centrifugation, was done in pigs (Nagashima *et al*. 1994) whose early-stage embryos have high lipid content and thus do not survive cryopreservation well. Delipidated two- to eight-cell embryos developed normally to blastocysts *in vitro* and produced normal progeny following ET. Similar procedure was conducted on bovine IVM/IVF one-cell embryos, which were then cultured to the 8- to 16-cell stage before being frozen (Ushijima *et al*. 1999). The development to blastocyst of these embryos was similar to control (20/126 compared with 35/176 respectively), but after freezing, more delipidated embryos developed

to blastocyst than control or sham-operated embryos (12/53, 2/43, and 5/59 respectively). Similarly, vitrified porcine-advanced blastocysts derived from delipidated two-cell embryos had similar post-warming survival to the control (not vitrified) blastocysts (72 vs 92%; Kawakami *et al*. 2008). The developing blastocysts, however, seem to be affected by delipidation. In a study on IVM/IVF porcine embryos, it was found that cell number per blastocyst was lower than in the control (19.8 vs 24.2 respectively; $P<0.05$; Yoneda *et al*. 2004). This method was also proved beneficial to pig oocytes that developed well up to morula stage following IVF and IVC (Nagashima *et al*. 1996). The beneficial effect of delipidation was also demonstrated on embryos exposed to chilling without freezing. Delipidated porcine embryos at the one-cell or two- to four-cell stages developed *in vitro* significantly better than non- or partially delipidated chilled embryos and similar to the non-chilled intact controls (Nagashima *et al*. 1994). The development of delipidated embryos was also demonstrated *in vivo*. Porcine delipidated embryos at the two- to four-cell stage, which were frozen–thawed and then transferred, developed normally to term (Nagashima *et al*. 1995). This procedure, however, is very time consuming so it is not practical when large numbers are involved.

An alternative procedure that has been tested is polarization of the lipids by centrifugation without their removal by micromanipulation (Esaki *et al*. 2004). In this study, parthenogenetic porcine IVP morulae were vitrified immediately after centrifugation and compared with morulae that were delipidated by micromanipulation and non-treated morulae with and without vitrification. Development to blastocyst stage was for centrifugation, delipidation by micromanipulation, and non-vitrified control (82.5, 82.1, and 84.6% respectively), while only 8.6% of non-treated vitrified morulae developed to blastocysts.

Blastocoelic fluid depletion

Blastocysts present special challenge to cryopreservation because of the large number of cells in multiple layers they contain and the blastocele that presumably does not dehydrate sufficiently during freezing or prior to vitrification (Kader *et al*. 2010). Excessive water in the blastocele may lead to ice formation, which is damaging to cellular structures. To minimize this risk, removal of some of this blastocoelic fluid has been attempted. Removal of these fluids can be done by perforating the blastocele and letting the fluid flow passively out or by microsuction. In mice, microsuction of blastocoelic fluid was done before vitrification (Chen *et al*. 2005*a*). Blastocysts that underwent microsuction showed, after warming, better survival rate (92 vs 80%, higher rate of expanded blastocysts (89 vs 59%) and live young from transferred warmed expanded blastocysts (34 vs 9%)). In horses, where blastocysts can reach sizes of 500–600 μm, making them highly vulnerable to chilling injury and cryodamage, blastocele collapse was achieved with the aid of Piezo drill (Choi *et al*. 2009). Of ten vitrified–warmed treated expanded blastocysts, three were used for *in vitro* evaluations and seven were transferred, resulting in five pregnancies (71%). A recent study on mouse blastocysts compared fresh control with vitrified non-hatched or assisted hatched non-expanded blastocysts (Kader *et al*. 2010). While survival was the same for all (100%), the assisted hatched group showed higher DNA integrity compared with the non-hatched group (94.63 vs 84.36%; $P<0.01$) and similar to the control. When blastocele aspiration was used in expanded blastocysts before vitrification, it showed similar survival and DNA integrity (100 and 90.08%) to spontaneously hatched expanded blastocysts (100 and 88.45%) and the control (fresh, 100 and 95.47%) but higher than vitrified expanded blastocysts with no intervention (90.9 and 77.61%).

Manipulations to the zona pellucida

The zona pellucida that surrounds embryos acts as a natural barrier, hindering the free movement of water and CPs between the intra- and extracellular compartments. To overcome this barrier, several possibilities are available. Extending the IVC of blastocysts can eventually lead to natural hatching, or hatching can be achieved artificially by acid perforation of the zona pellucida. Alternatively, the zona pellucida can be perforated using laser or micromanipulations or it can be removed by exposing the embryos to acidic solution. *In vivo*-produced mouse morulae that were cultured till the embryos hatched through the zona pellucida and then vitrified achieved a survival rate of 77% (79/103) after warming (Zhu *et al*. 1996). Denuded (by acidic treatment) and then vitrified rabbit blastocysts resulted in 91% survival after warming (Cervera & Garcia-Ximenez 2003). When assisted hatching, using diode laser, was conducted before freezing of human blastocysts, 75.4% post-thaw survival and 31.4% clinical pregnancies were achieved (Kung *et al*. 2003). In this study, however, there was no frozen–thawed control group for comparison. Vitrified human blastocysts that underwent natural hatching (full or partial) were compared with blastocysts that did not hatch. Post-warming survival rates were 82% (31/38), 72% (72/100), and 64% (25/39) for fully hatched, partially hatched, and intact zona pellucida groups respectively (Zech *et al*. 2005). Using assisted hatching by blowing acid on the zona pellucida in mouse six- to eight-cell embryos before freezing improved post-thaw survival (100 vs 81.25%; $P<0.01$) and development to blastocyst stage (39.38 vs 18.46%; $P<0.01$; Hershlag & Feng 2005).

IVC conditions

FCS

In some studies, FCS in the culture medium seems to enhance embryo survival through cryopreservation. Porcine embryos cultured with FCS to the blastocyst stage survived vitrification better than those that were not ($P<0.05$, 42.9 vs 28.6% respectively; Men *et al*. 2005). In most other studies, however, FCS was shown to have negative effect on embryos. The presence of FCS resulted in lower post-thaw survival of bovine blastocysts as compared with the control (60.2 vs 84.9%; $P<0.01$; Barcelo-Fimbres & Seidel 2007*b*) or bovine blastocysts and late morulae compared with the control (50.48 vs 68.01; $P<0.01$; Pugh *et al*. 1998). It was also reported to retard embryo development (Abe & Hoshi 2003) and to alter the levels of various mRNA relevant for embryonic development and recognition (Rizos *et al*. 2003), resulting in lower embryonic quality. It was suggested that lipids from the serum find their way into the embryos, thus negatively affecting their cryosurvival (Pugh *et al*. 1998). Culture of bovine IVP zygotes in the presence of FCS resulted in higher number of large (>6 μm) lipid droplets in the resulting blastocysts as compared with the control (Barcelo-Fimbres & Seidel 2007*a*). However, this is not the sole explanation as fatty acid-free serum still caused elevated lipids compared with *in vivo* controls (De La Torre-Sanchez *et al*. 2006). To overcome this, alternative sources of proteins such as Ficoll, polyvinyl alcohol, polyvinylpyrrolidone, or hyaluronic acid are used to substitute FCS (Gajda 2009 and citations therein).

High hydrostatic pressure

The application of high hydrostatic pressure to gametes and embryos at a level of 20–90 MPa (200–900 times the atmospheric pressure) seems to benefit their cryosurvival. The level of pressure and its duration depend on the species and the type of gamete or embryonic developmental stage. For example, porcine oocytes optimally withstand pressure of only 20 MPa, whereas mouse blastocysts can survive pressure as high as 90 MPa for 30 min and then recover to the same level as the control (Pribenszky *et al*. 2005, Du *et al*. 2008). Porcine oocytes do not survive a much lower pressure of 60 MPa (Pribenszky *et al*. 2008). Such improved survival was demonstrated, for example, in pig and bovine oocytes (Du *et al*. 2008, Pribenszky *et al*. 2008), mouse blastocysts (Pribenszky *et al*. 2005), and boar spermatozoa (Pribenszky *et al*. 2006). This technique was initially demonstrated by Pribenszky *et al*. (2005) and Du *et al*. (2008) who suggested that the pressure put the cells under stressful conditions that lead them to produce and accumulate chaperone proteins such as heat shock proteins. These proteins seem to be beneficial to the cells during cryopreservation, which is also a stress-inducing procedure. In one study, for example on porcine IVM oocytes, 20 MPa was compared with 40 MPa, showing that the 20 MPa was superior to the 40 MPa and both groups were significantly better than the control (vitrification without pressure treatment) (13.1, 5.3 vs 0% respectively; $P<0.01$; Du *et al*. 2008). At 20 MPa, more blastocysts were produced when the pressure before vitrification was applied at 37 °C compared with 25 °C (14.1 vs 5.3%; $P<0.01$).

EG and calcium

EG is often used as the sole CP or along with others in freezing and vitrification of gametes and embryos. Its effect seems to be beyond being a permeable CP because some interaction between EG and calcium seems to take place. When rat oocytes were vitrified in vitrification solution of 15% EG, 15% DMSO, and 0.5 M sucrose and 20% FCS, survival and cleavage rate after activation of vitrified warmed oocytes was 98.3 and 78.4% respectively, but zona pellucida sperm penetration rate was very low (3.6%, 6/168) and a high level of cortical granule exocytosis was noted (Fujiwara *et al*. 2010). When the oocytes were vitrified in EG-supplemented calcium-free media without DMSO, they had 79.4% survival, 72.8% cleavage after activation, 63.9% zona pellucida penetration, and 23.1% of the oocytes developed to blastocyst. When vitrified with DMSO without EG, survival was only 23.6% but was 90.7% when both EG and DMSO were present in calcium-free solution. The cause behind these results might be in the fact that EG and DMSO were showed to cause rise in mouse oocyte intracellular calcium (Larman *et al*. 2006), and this induces cortical granule exocytosis and the hardening of the zona pellucida (Ben-Yosef *et al*. 1995, Larman *et al*. 2006). The removal of the calcium from the vitrification solution can thus alleviate this effect, making sperm penetration through the zona pellucida possible.

Age

Age is a factor both from the donor and from the recipient perspectives. In a recent study on human patients attending fertility treatments, age-related differences were found in both fresh and frozen IVF embryos (Zhou *et al*. 2009). Analysis of their results based on age groups (<35 vs ≧35) by two-tailed *z*-test indicates that in the fresh ET group, there were differences in rates of high-quality embryos (72.7 vs 61.7%, $z=5.559$, $P<0.01$), implantation (32.4 vs 20.6%, $z=6.016$, $P<0.01$), and clinical pregnancy (50.2 vs 38.3%, $z=3.76$, $P<0.01$), whereas in the frozen–thawed embryo, group differences were found only in the rate of high-quality embryos (70.3 vs 44.8%, $z=3.85$, $P<0.01$). In another study on human cryopreserved embryos, three age groups were compared: – 22–33, 34–37, and 38–45 years (Goto *et al*. 2011). Post-thaw comparison found that age affected the proportion of good-quality blastocysts (62.3, 56.3, and 41.1%

respectively), and a tendency was found to a decrease in clinical pregnancy rate, viable pregnancy rate, and delivery rate with increasing age. In cattle, a comparison between heifers and cows identified differences in pregnancy rate for both surgical and non-surgical fresh ET, but no difference was identified when frozen embryos were used (Hasler 2001). However, in cows, non-surgical frozen ET resulted in lower pregnancy rate compared with surgical transfer (38.5 vs 71.1%). In a study on mouse oocytes, age-related decrease in the number of oocytes retrieved following superovulation was noted (Yan *et al.* 2010). Oocytes retrieved from older females had lower survival and cleavage rate after vitrification, rate of development to blastocyst went down with maternal age when oocytes were vitrified but not in the control (no vitrification), and blastocyst quality (total cell number and ratio of inner cell mass to trophectoderm) was lower in the older age groups in both vitrified and control treatments.

Season

While time of the year can be expected to have its influence on seasonal animals, several studies have shown that the season has its effect on oocytes and embryos of continuous breeders as well. In a study on zebu (*Bos indicus*) *in vivo*-produced embryos, the season (dry or wet) had significant effect on embryo quality measured by the TUNEL assay. Embryos collected during the rainy season had a lower number of apoptotic cells, both following IVC and after freezing (Marquez *et al.* 2005). In cattle, a clear seasonality was found between summer and winter oocytes in a wide variety of measures, including conception rate, number of 2–8 mm follicles per ovary, percentage of ovaries with fewer than ten follicles, number of oocytes recovered per ovary, and cleavage rate following chemical activation all the way through to the blastocyst stage (Zeron *et al.* 2001). The authors suggested that these differences were related to differences found in membrane phospholipids' composition being richer in saturated fatty acids in the summer and in mono- and polyunsaturated fatty acids in the winter. This difference results in a more fluid membrane and a phase transition temperature six degrees lower in the winter compared with the summer. This higher fluidity and lower phase transition temperature can influence the tolerance of the oocytes to chilling and cryopreservation (Zeron *et al.* 2002). Season, however, seems to have no effect on recipients of transferred embryos in continuous breeders. In one study, conducted on cattle in the USA (Pennsylvania and California) and Holland, no effect was found for the season on transfer of either fresh or frozen–thawed embryos (Hasler 2001). In a study on human frozen–thawed ET, no seasonality was noted with respect to the time of transfer (Dunphy *et al.* 1995); however, the number of cycles analyzed was small (321) resulting in low power for the study.

Conclusions

The co-evolution of reproductive technology and cryobiology has accelerated extensively in the last century. Generally speaking, embryos 'like' to be either in the uterus or in LN. Likewise, oocytes would rather be in the follicle, fertilized or in LN. Anywhere else is potentially damaging. The major damaging factors, which occur during cryopreservation, are associated with chilling injury, osmotic stress, CP toxicity, and ice crystallization (Mazur *et al.* 1972, Quinn 1985, Saragusty *et al.* 2009). In general, we are trying to reduce these damages by increasing cooling and warming rates using vitrification. In the past, vitrification was based on the combination of a high cooling rate and high concentration of CPs, which caused chemical toxicity and osmotic stress. The major breakthrough in the field of vitrification came when sample volume was reduced to a level that permitted lowering the CP concentration. We believe that commercialization of vitrification solutions and containers will contribute to accelerate the development of the field of oocyte and embryo cryopreservation. Success has been reported in a handful of mammalian species, but differences between species make cryopreservation techniques' dissemination difficult. Current improvements alone will not suffice to overcome the hurdles on the way to successful oocyte and embryo cryopreservation in all vertebrates other than mammals. Those are waiting for other breakthroughs in the field of cryobiology that will facilitate cryopreservation of their germplasm. Some attempts to improve cryopreservation outcome through manipulations to germplasm have been reported, but more studies are needed to identify the more promising ones, which will be incorporated into routine oocyte and embryo cryopreservation protocols.

Declaration of interest

The author J Saragusty declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported; A Arav has interest in IMT, Ltd Israel.

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