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Please cite this publication as follows:


Link to official URL (if available):

http://dx.doi.org/10.1016/j.theriogenology.2016.07.018

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Cryopreservation of animal oocytes and embryos: current progress and future prospects

Mandawala AA\textsuperscript{a}, Harvey SC\textsuperscript{a}, Roy TK\textsuperscript{b} and Fowler KE\textsuperscript{a}

\textsuperscript{a}School of Human and Life Sciences, Canterbury Christ Church University, Canterbury, UK

\textsuperscript{b}Genea Biomedx UK Ltd, Discovery Park, Sandwich, UK

Corresponding author:

Dr. Katie Fowler,

School of Human and Life Sciences,

Canterbury Christ Church University,

Canterbury,

CT1 1QU,

UK.

Email: katie.fowler@canterbury.ac.uk

Telephone: +44 1227 781820
Abstract

Cryopreservation describes techniques that permit freezing and subsequent warming of biological samples without loss of viability. The application of cryopreservation in assisted reproductive technology encompasses the freezing of gametes, embryos and primordial germ cells. Whilst some protocols still rely on slow-freezing techniques, most now use vitrification, or ultra-rapid freezing, for both oocytes and embryos due to an associated decreased risk of damage caused by the lack of ice crystal formation, unlike in slow-freezing techniques. Vitrification has demonstrated its use in many applications, not only following in vitro fertilisation (IVF) procedures in human embryology clinics, but also following in vitro production (IVP) of embryos in agriculturally important, or endangered animal species, prior to embryo transfer. Here we review the various cryopreservation and vitrification technologies that are used in both humans and other animals and discuss the most recent innovations in vitrification with a particular emphasis on their applicability to animal embryology.

Keywords – bovine, cryopreservation, embryo, oocyte, porcine, vitrification

Introduction

Over the last few decades, cryopreservation techniques have progressed rapidly. This progress has made a significant impact in many fields, with reproductive medicine possibly the most significant. From initial success in cryopreservation of sperm [1], it is now routinely used for the preservation of oocytes, sperm and embryos within both agricultural systems and in assisted reproductive technology (ART) in humans. Cryopreservation is a process by which biological cells or tissues are preserved at sub-zero temperatures resulting in a radical decrease in the rate of metabolic processes and the ability to store samples for extended periods [2]. However, as would be expected, freezing cells causes
damage and this must be circumvented. The two major causes of cellular damage are the physical
damage caused by the formation of ice crystals and the chemical damage that results from changes in
intracellular solute concentrations. Both of these damage types can be avoided, or at least
ameliorated, by controlling how the temperature is reduced and by modifying the cellular conditions.
For instance, the mechanical damage that results from the piercing action of ice crystals can be
avoided by making the freezing process very rapid and the significant rise in intracellular solute
concentration as the formation of ice crystals increases can be avoided by use of cryoprotectants [3].
Permeating cryoprotectants replace intracellular liquid and decrease ice formation [4]; as such they
need to have low toxicity, be capable of penetrating cells and be able to withstand extremely low
temperatures. Examples of commonly used cryoprotectants include glycerol, ethanediol, dimethyl
sulphoxide (DMSO), ethylene glycol (EG) and propanediol [5]. Whilst most cells cannot survive the
freezing process without use of a cryoprotectant, it is also important to note that simply using such
solutions alone is insufficient for cell survival after freezing (and thawing); survival also depends on
the cell type and its ability to withstand various stresses caused by physical and physiochemical
changes during the process, as well as rates of cooling and warming [5].

The promise of vitrification

Vitrification eradicates damage caused due to ice crystal formation during the cooling process. The
method involves rapid cooling and liquid solidification due to a substantial rise in viscosity and results
in the formation of a solid glass-like form [6]. This solid ‘glassy’ layer is amorphous; meaning that it
can readjust and take the shape of the cell, hence enabling the cell to maintain its structure and remain
intact, unlike in slow-freezing, where the formation of ice crystals during cooling prevents the cell from
maintaining its structure. See Figure 1 for a schematic comparison of slow-freezing and vitrification.
There have been a number of studies that have compared slow-freezing techniques and vitrification
in human embryology [7][8]; whilst it seems that there is a gradual move towards more widespread
use of vitrification clinically, the literature to date describes no consensus as to which method is the
best. For example, Herrero and colleagues established that cryopreservation of both human oocytes and blastocysts demonstrate competitive pregnancy rates when compared to those obtained through implantation of fresh samples and that vitrification was preferable in terms of minimised cellular damage and higher post-warming survival rates when compared to traditional freezing processes [9][10]. Whilst some still opt for a traditional slow-cooling method via the use of insemination straws, others now use fast-cooling vitrification techniques, employing an array of different vitrification systems including; thin capillaries or straws, Cryotop, Cryoloop, Cryolock, CryoTip, nylon mesh, plastic blades, Vitri-ingá, electron microscopic grid, Gavi and the minimum drop size technique [11]. For example, Kuwayama (2007) discusses the efficiency of the Cryotop method, wherein he states that cryopreservation of blastocysts using the Cryotop method resulted in more live births when compared to any other vitrification system [12]. Moreover, Mukaida and colleagues used clinical results from 725 human blastocysts (of which 80.4% survived vitrification and warming) to establish that the Cryoloop system can be used as an effective method for vitrification of human blastocysts [13]. Additionally, Sugiyama et al. (2010) tested the effect of a plastic blade as a cryopreservation device on survival rates of human embryos and blastocysts following vitrification and warming. Interestingly, this demonstrated that whilst 98.4% of cleavage stage embryos survived vitrification and subsequent warming, all of the blastocysts survived [14]. In a comparative study, Desai and colleagues comparatively assessed three cryo-devices on the impact of vitrification (nylon mesh, micro-capillary tips and an electron microscopy grid), of murine pre-antral follicles and found no significant differences for subsequent in vitro development following vitrification. However, a low survival rate was observed for follicles vitrified using micro-capillary tips, and it was revealed that when a large number of follicles required vitrification, a nylon-mesh was most successful [15]. In 2008, Vitri-ingá, was developed and tested on bovine oocytes; the device showed promising results with an 86% survival rate post warming [16]. The method was subsequently adapted for use with human oocytes and in 2010, Almodin and colleagues evaluated the device’s success, by comparing gestational results achieved via use of frozen-warmed human oocytes vitrified using Vitri-ingá and by those that did not
undergo vitrification. The technology was tested clinically on 125 human patients, of which 79 patients received embryos that were derived from fresh oocytes, while 46 patients were implanted with embryos that were developed using frozen-warmed oocytes vitrified by Vitri-ingá technology; a high survival rate of 84.9% was demonstrated by oocytes that underwent vitrification. Moreover, no significant differences were reported for fertilisation, implantation or pregnancy rates between the patients of the two groups [17]. Successes of vitrification methods have resulted in IVF clinics around the world progressively shifting away from traditional slow-freezing methods for routine use in ART [18][19]. As is evident here, new vitrification techniques are constantly being developed and these can be broadly classified as open or closed – the distinction depending on the degree to which there is, or is not, direct contact between the media and the liquid nitrogen used during the cooling process.

Open and closed vitrification systems: a comparison

In an open system, the oocytes or embryos come into direct contact with liquid nitrogen, whereas in a closed system, they do not. Direct comparisons between these types of systems have been limited; however, the available evidence suggests that the viability of oocytes and embryos post-warming can be similar. For instance, Papatheodorou and colleagues compared open and closed systems by conducting a randomised trial using human sibling oocytes. Whilst survival rates following vitrification using the closed system (82.9%) were slightly lower than that of the open system (91.0%), there was no significant effect on observed, clinical, or ongoing pregnancies between the two groups. Moreover, the closed system group produced higher live birth rates as well as a higher number of healthy babies (27 versus 18) [20]. Researchers in Tokyo demonstrated similar findings, showing no significant difference between blastocyst survival rates using the CryoTip™ (closed system) and the Cryotop (available as either an open or closed carrier system) [21]. Comparisons were also made between slow-freezing and ultra-rapid vitrification of human embryos, which indicated that vitrification was the most reliable; these results are summarised in Table 1. Similarly, comparisons between Rapid-i®
and Cryotop [22], and between Vit Kit Freeze/Thaw (Irvine Scientific, CA) Global Fast Freeze/Thaw Kits (LifeGlobal, Canada) [23] indicate that these systems can produce comparable results.

Interestingly, conflicting evidence by Paffoni et al. (2011) revealed considerably lower pregnancy rates and a higher ratio of cancelled cycles for vitrification of mature human oocytes using a closed system, as opposed to an open system [24]. Moreover, when embryological parameters were compared for sibling oocytes that were either fresh or vitrified using the closed system, fertilisation rates and cleavage rates were considerably lower for those that underwent vitrification in an open system. The closed system vitrified oocytes also produced embryos of a lower standard, when compared to their fresh sibling oocytes, in terms of both quantity and quality. However, when the same parameters were compared between oocytes that were vitrified using the open system and their fresh sibling counterparts, no significant differences were observed. Though these observations could, most likely, be due to variation in sensitivities between oocytes and blastocysts, this study suggests that an open vitrification system is more reliable than a closed system [24]. As this indicates, the evidence in the literature when comparing open and closed vitrification systems is conflicting. Another important consideration is the potential contamination of samples during the cooling process. Whilst there is currently no clinical evidence of pathogenic contamination during cryopreservation of oocytes and embryos specifically [25], there has been some evidence to show contamination of other types of human tissue, such as bone marrow, through liquid nitrogen during cryopreservation [26]. It has also been noted that slow cooling by the use of static vapour freezers reduces the risk of pathogenic contamination, unlike with use of open vitrification methods in which samples come into direct contact with liquid nitrogen [27]. Closed vitrification however is considered to be aseptic due to the elimination of the potential for pathogen contamination from liquid nitrogen [28] and for this reason closed systems could be regarded as preferable for use in vitrification. Bielanski and Vajta (2009) discuss concerns regarding sterility of liquid nitrogen in ARTs, and it seems that to date, even though methods exist to produce sterile liquid nitrogen, no commercial provider exists [27]. Another,
relatively new vitrification device, Gavi (Genea Biomedx) permits the process to be further standardised and automated. The device comprises of a thin walled pod that allows for rapid vitrification and warming rates; the pod also incorporates a microfluidic design in order to maintain the embryo’s location thereby permitting automated exchange of fluids. Finally, the instrument automatically seals the pods, which, unlike the manual Cryotop method or in other existing open vitrification technologies, eradicates the risk of pathogenic contamination from liquid nitrogen by confirming that the pod is completely closed [10]. Results to date demonstrate that mouse (zygotes, cleavage stage embryos and blastocysts) and human (vitrified-warmed blastocysts) vitrified samples using the Gavi technology produced similar results to those of the control samples which were vitrified using the manual Cryotop method that is currently considered to be the gold standard for vitrification [10].

As is evident from the literature, both open and closed vitrification systems are now used clinically for cryopreservation of human oocytes and embryos; further to this, a number of studies (discussed later) have also been executed in other species.

The mouse as a model

In some cases, the vitrification of non-human oocytes and embryos can be particularly challenging, such as is the case in the pig model; these challenges are discussed later, in addition to a discussion pertaining to oocyte and embryo vitrification of other important agricultural species. The mouse (Mus musculus) is a particularly powerful model for studying mammalian embryo development due to broad morphological similarities [29]. The extensive genome similarities between mouse and human coupled with the experimental tractability of the mouse also provide significant benefits to using this species. Additionally, mouse embryos are also more readily available than those of many agriculturally-important animal species [30]. As such, it is unsurprising that numerous experiments have been conducted on cryopreservation of murine oocytes, embryos and ovaries. Similarly to the
case for humans, comparative studies have demonstrated that vitrification of murine oocytes
and embryos at blastocyst stage [36][37] results in higher post-warming survival rates, fertilisation, and better subsequent in vitro embryonic development than conventional slow-freezing techniques. Similar findings based on vitrification of murine ovarian tissue indicates that cryopreservation of mouse ovaries can be used to preserve fertility, as well as endocrine functions of ovaries, an approach that has been implemented in human models [38][39][40]. Mice have also been used to develop (and test) novel vitrification devices. For example, using murine blastocysts, Kong and colleagues demonstrated that an open pulled straw and a glass micropipette could be used independently as vitrification vessels to obtain high embryo survival rates [41]. Similarly, in a more recent study, a new, simplified technique, ‘needle immersed vitrification’, was developed as a vitrification approach for preservation of ovarian tissue in mice [42]. One of the key benefits of this method is that the technique, which can also be used to vitrify human ovarian tissue, uses a minimal volume of cryoprotectants at a low concentration, thereby resulting in lower toxicity of vitrification solutions, hence resulting in less cellular-damage [42].

**Embryology and vitrification progress in agricultural animals**

Human ART procedures are now used clinically worldwide and such methods give families the chance to have healthy offspring, which in many cases would not have been possible before such advances. The ‘one child at a time’ report, published by the Human Fertilisation and Embryology Authority (HFEA) in 2006 [43], aimed to reduce the incidence of multiple births following ART; however, the challenges associated with agricultural animal IVF are somewhat different; procedures developed for humans need to be adapted and scalable for a far larger number of viable embryos to be produced. Additionally, the 100% success rate that is strived for in human IVF is perhaps not such an important consideration in other animal IVF, especially considering the quantity of embryos that are required in comparison. There is increasing interest within agricultural breeding communities to cryopreserve oocytes and embryos of agriculturally important animals such as pig, cow and sheep [44][30]. Both
the domestic pig (*Sus scrofa domesticus*) and cattle (*Bos taurus*) are of significant importance for meat (and milk) production. The United Nations suggests that the world population is predicted to grow to a projected 9.15 billion by 2050 [45]; and it has been indicated that the consumption of meat is expected to rise in order to supply the 20% increase in *per capita* calorific intake. Not only this, but it is current practice to transport live animals that are either genetically ‘superior’ or that are better to matched to particular markets between countries. The transportation of live animals is both expensive and logistically demanding, and hence the prospect of transporting frozen embryos as opposed to live animals is an attractive one [46][47]. Not only would the ability to transport vitrified (and viable) embryos be an approach that would be more practical and cost effective, but it would also minimise the risk of disease transmission both within and between species [48]. In addition, routinely used techniques in human embryology, such as preimplantation genetic diagnosis (PGD) and sex determination techniques, could be usefully adapted for both pig and cow species. This could facilitate in vitro production (IVP) of viable embryos that are predominantly female, which again, would be economically favourable to agricultural breeding companies [49]. Moreover, it is important to note that the challenges faced in animal embryo vitrification and warming (particularly in livestock species) are somewhat different when compared to those faced in human IVF clinics; warming protocols may need to be performed on farm which would inherently increase the risk of potential pathogenic contamination [50]. Furthermore, existing vitrification protocols would need to be adapted for a large number of viable embryos.

In comparison to mice, numerous studies have revealed that freezing oocytes and embryos of agricultural species (especially the pig) is difficult. It has been established for example, that both porcine oocytes and embryos are particularly susceptible to cellular damage by freezing and some trials have reported subsequent blastocyst development rates as low as 5% [51]. Difficulties associated with oocyte and embryo vitrification in the porcine model are primarily due to their high intracellular lipid content [30]; the relative abundance of lipid droplets, particularly in 1-8 cell stage porcine
embryos, makes them exceptionally challenging to work with under a microscope as the lipid bilayers
darken the embryos, thus hindering observation of signs of fertilisation success (such as pronuclei)
during in vitro development. Additionally, studies have indicated that separation of membrane lipids
when freezing impacts post-thaw viability [52]. Hazel (1995) describes a threshold level, defined by
the intracellular lipid composition, below which cell membrane function is weakened due to a phase
transition that occurs in membrane fats [53]. The temperature at which this lipid phase transition
takes place, is inversely proportional to the amount of unsaturated fatty acids within the membrane,
and hence, by altering the composition of their lipid membranes, different organisms have the ability
to adjust this threshold temperature [54]. As such, Hazel & Williams (1990) explain that, the ability of
cells to survive at low temperatures is partly due to the increase in the ratio of unsaturated fatty acids
within the cell membrane [55].

As such, obtaining viable 8- to 16-cell embryos that survive freezing is extremely challenging [30].
However, many studies have been conducted which aim to improve prospects of porcine embryo
cryopreservation and, fortunately, some have had considerable success. For example, the application
of hydrostatic pressure, prior to vitrification has been shown to improve blastocyst survival rates post
warming, to over 10% [51]. Similarly, another study by Li and colleagues showed that embryo
treatment with trypsin or embryo exposure to a solution of high osmolality, could eradicate the lipid
layer in porcine embryos that are produced in vitro [56]. This was significant as the implementation of
this method meant that embryos could be centrifuged without prior micromanipulation (to deplete
the lipid bilayer) with only minimal damage to the zona pellucida [56]. Further studies have
demonstrated that the critical temperature at which damage caused to cells is no longer reversible is
at 15°C [30]. Furthermore, Men et al. (2011) demonstrated that disrupting the lipid bilayers via
micromanipulation and then centrifuging embryos prior to vitrification had a positive outcome on
survival post warm [57]. They also demonstrated that, using this approach, vitrification in a closed-
system was as successful as using open pulled straws, which was a major step forward in porcine
embryo cryopreservation [57]. Furthermore, Berthelot et al., (2000) revealed that an ultra-rapid open pulled straw can be used as a vitrification device for fast cooling of unhatched porcine blastocysts [58], while Galeati et al., (2011) noted low survival rates post thaw for porcine oocytes vitrified using the open Cryotop system [59].

Whilst not as sensitive as pig embryos, cow embryos are also sensitive to cooling injury at very low temperatures and initially only a few studies revealed that 8- and 16-cell embryos survived vitrification at 0°C [30]. Polge and Willadsen also demonstrated that blastocysts were better adapted to withstand cooling when compared to 8- and 16-cell embryos or even morulae, which has led scientists to focus most of their attention to vitrifying embryos at only the blastocyst stage [30]. It is important to note that there has been some evidence in the literature that flushed cattle embryos produced in vivo exhibit differences in their cryopreservation properties when compared to their in vitro fertilised counterparts (oocytes aspirated either from ovaries obtained from a slaughter house or by ovum pick up following super stimulation). Specifically, Stachecki and Wiemer (2007) note that, in comparison to their in vivo produced counterparts, bovine embryos that are developed in vitro are more sensitive to cooling [60]. The definitive cause for this is still unclear, however one reason for may be due to the metabolic differences prevalent during preimplantation development (from oocyte to hatched blastocyst) of bovine embryos based on how they were developed (in vivo or in vitro) [61].

Methods of vitrification of bovine embryos have significantly progressed in recent years; specifically, a study by Park and colleagues showed that an electron microscopy grid could be used as an effective vitrification container (instead of traditional straws), to achieve high embryo survival rates when vitrifying bovine blastocysts [62]. In another study, Mucci and colleagues compared embryo survival (defined by blastocyst hatching rate) for bovine embryos that were either vitrified or slow-frozen, and found a significant positive skew towards vitrification (43% survival rate for vitrified embryos, compared to 12% for slow-frozen embryos) [63]. Similarly, another comparative study noted that,
whilst there might be slight differences between using fresh and vitrified oocytes for IVF, for nuclear
transfer experiments with cultured fibroblast cells, use of vitrified oocytes resulted in better
embryonic development [64]. Additionally, a review by Dalvit and colleagues indicated that
vitrification of in vitro matured bovine oocytes and embryos was successful, with no significant
differences being observed between vitrified-warmed and fresh oocytes, with respect to both survival
and embryonic development [65]. Additionally, no significant oocyte morphological differences were
found following oocyte vitrification-warming using the Cryoptop method and open-pulled straws [66].

In comparison to bovine embryos, studies suggest that cryopreservation of ovine (sheep) embryos is
relatively easier, primarily because the stage of development at which embryos are frozen is not
detrimental to post thaw success rates; embryos at the 1-cell, 2-cell and blastocyst stages have all
been viable after cooling at temperatures as low as 0°C [30]. However, findings demonstrated that
comparatively, slow-freezing with ethylene glycol was still more successful than vitrification [67]. In
the hope of developing vitrification techniques for ovine oocytes, Mullen and Fahy addressed some
issues associated with the approach, which included changes in levels of messenger RNA and injury to
the cytoskeleton of cells [51]. Whilst these are significant issues, some scientists have been successful
in obtaining blastocyst development rates of at least 10% post-warm [51] and better success rates
with using open pulled straws, for vitrification of sheep embryos, has been demonstrated more
recently [68][69]. Additionally, the first successful vitrification (using a cryoloop) of sheep oocytes at
the germinal vesicle stage was reported in 2013 by Moawad and colleagues, and since then,
techniques for ovine oocyte vitrification have been evolving [70]. One particular challenge pertaining
to mammalian oocyte cryopreservation is their extremely high cellular volume when compared to
other cell types; this makes them extremely sensitive and even more susceptible to intracellular ice
formation during the process of cryopreservation due to a lower surface-to-volume ratio [71]. Other
factors that make oocyte cryopreservation more challenging include the presence of the zona
pellucida and the decreased permeability of oocyte plasma membranes, both of which can hinder the
movement of water and cryoprotectants in and out of the oocyte [72]. Fertilisation however, alters many of these parameters and hence, embryos are generally less challenging to cryopreserve [73].

As discussed previously, apart from the use of vitrification in achieving successful IVP in breeding livestock, cryopreservation also has potential to save the fate of certain endangered species and ingenious animal breeds [74]. In order to achieve this, it is important to be able to successfully preserve primordial follicles (the main source of female gametes) of these species by slow-freezing or fast-cooling. Gathering sufficient ovarian tissue (whole ovaries, ovarian cortical tissue or isolated follicles) from endangered species can however be a significantly limiting factor and hence, many farm and domestic animals are used as models to optimise cryopreservation techniques [75]. Specifically, domestic livestock, cats, dogs and capuchin monkeys are commonly used as models to enhance our current understanding of reproductive physiology and species-specific differences in non-domestic ungulates, wild felids, rare canid breeds, and new world primates respectively [74]. Thus, Leibo and Songsasen discuss how using these species as models to study cryopreservation and hence preserve their genetic material, could pave the way to potentially eliminate the risk of extinction in endangered species [76].

**Embryology and vitrification progress in aquaculture**

Many of the same challenges around the need to increase levels of production and to improve stock that are found in agriculture also exist in both fish and invertebrate aquaculture. For many aquaculture systems, there is also the additional problem that production depends on the harvest of broodstock or seed from wild populations [77]. Cryopreservation of sperm is relatively common within aquaculture, but widespread implementation of cryopreservation within the industry has however only occurred in a limited number of species, particularly salmon and turbot [77]. Work that has been done indicates that vitrification also works well in fish as an alternative to conventional
cryopreservation (e.g. [78]), but this approach has not been well explored in invertebrate systems, which represents a major opportunity for future research [79].

Unfortunately, successful cryopreservation of intact fish embryos has yet to be achieved. Some success has been reported (e.g. [80]), but protocols have proved difficult to replicate (e.g. see [81]). The limiting factors for fish embryo cryopreservation include their large size, their multi-compartmental nature, their high chilling sensitivity and their low membrane permeability [82]. Given these challenges, without a significant breakthrough, the successful cryopreservation of fish embryos at a commercial scale looks to be unlikely. The cryopreservation of primordial germ cells is however possible (e.g. [83][84]) and this represents a viable approach for the conservation of genetic resources and research purposes, but not for large-scale production.

**Future prospects**

Whilst there has been considerable success with vitrification of oocytes, embryos and even ovaries or ovarian tissue (particularly in humans and the mouse model), advantages of using closed systems instead of open systems, is a topic that is still widely debated in the literature; additionally, more research is required to produce more data, and importantly, more reliable results especially in agricultural animals. The development of automated devices for vitrification is potentially a huge leap forward in this regard; however, adaptation of such technology for use in agriculturally important animals such as pig and cow, whilst taking into consideration the scale of production that would be required for use in the agricultural industry, is yet to be attempted. The successful implementation of a closed, automated vitrification method could potentially revolutionise the field and would in no doubt be in the interest of agricultural breeding companies worldwide.
Acknowledgements

We thank Canterbury Christ Church University for supporting AM, SH and KF.

Conflict of interest

Tammie Roy is an employee of Genea Biomedx, the company that manufactures the vitrification device Gavi.

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<td>Development into blastocysts</td>
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<td>4-cell embryos</td>
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<td>Sample</td>
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<td>CryoTip™ (closed) (%)</td>
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<td>Blastocysts</td>
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<td>Deliveries</td>
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Table 1: Summary of results obtained from [21]; comparison of slow-freezing versus vitrification, and the CryoTip™ (closed) versus Cryotop (open) systems.
Figure 1: Comparison of rates of ice crystal formation between slow-freezing (left) and vitrification (right): figure illustrates that during slow-freezing, water flows out of cells due to extracellular ice crystal formation, thus causing mechanical damage to the cell structure. In contrast, during vitrification, cells are inserted into vitrification medium of high viscosity, which prevents extracellular ice crystallisation and hence, cells remain intact. In both instances, cryoprotectants prevent intracellular ice crystallisation.
Initial cell
Before cooling

SLOW-FREEZING

Cell inserted into freezing medium
-5°C

Water flows out of cell due to extracellular ice crystal formation

Damage to cell, extracellular ice crystals form
-196°C

VITRIFICATION

Cell inserted into vitrification medium
-5°C

Cell remains intact, “glassy” layer forms, no ice crystals
-196°C

Figure 1