

—Review—

Vitrification: Refined Strategy for the Cryopreservation of Mammalian Embryos

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If cells can be cooled alive to liquid nitrogen temperature, their biological processes are arrested without decreasing viability during storage. Cryopreservation of mammalian embryos, coupled with the embryo transfer technique, can be applied to various aspects in a wide range of species, i.e., for breeding and reproduction of farm animals, preservation of genetic variants in laboratory animals, conservation of wild species and treatment of infertility in human.

Cryopreservation of mammalian embryos became possible in 1972, when Whittingham, Leibo and Mazur [1] reported the successful freezing of mouse embryos in liquid nitrogen, resulting in the production of live young. They suspended embryos in a solution with 1.0 M dimethyl sulfoxide (DMSO), equilibrated there for full permeation, performed ice seeding at a few degrees below freezing point, and cooled the sample very slowly (0.3–0.4°C/min) to –80°C before cooling in liquid nitrogen. The sample was warmed slowly (4–25°C/min) and the solution was diluted stepwise before recovering the embryos in an isotonic solution. In the same year, Wilmut [2] also found that quite a similar method is effective for the cryopreservation of mouse embryos. Although he did not perform embryo transfer to confirm the viability, his group succeeded in getting the first live calf after transferring bovine embryos which had been frozen by a similar method [3]. After these reports, the slow freezing method was shown to be effective for embryos of other species, including the rabbit [4, 5], rat [6], sheep [7], goat [8], horse [9] and man [10, 11].

After freezing mouse embryos by the slow cooling method, about 80% of them are judged to have survived by the ability to cleave further. This method is reliable and still used for freezing mouse embryos in some institutes and laboratories [12, 13], but it has disadvantages in that it takes as long as 2–3 hr for slow

cooling, and that an elaborate machine and liquid nitrogen are required to control the cooling rate. Most attempts to cryopreserve embryos have therefore been directed to simplifying the cooling process. In 1977, Willadsen [14] showed, with sheep embryos, that slow cooling can be terminated at –30––36°C before plunging the sample into liquid nitrogen, on the condition that the sample is thawed rapidly (360°C/min). This method shortened the time required for cooling without reducing embryonic survival, and made it possible to adopt a machine run by electricity. This interrupted slow freezing method is now routinely used for the cryopreservation of mouse [13], bovine [15] and human [16] embryos. In 1980 stepwise methods were reported, which require 10–20 min before cooling in liquid nitrogen [17, 18], but these methods did not become popular, probably because of difficulty in keeping samples at certain subzero temperatures. Then in 1985 Rall and Fahy [19] devised an extremely rapid method called vitrification, in which embryos suspended in a highly concentrated solution are plunged directly into liquid nitrogen from a temperature above 0°C, thus taking only a few seconds for cooling. This method was proven effective by the production of live young after embryo transfer [20, 21]. Vitrification can be defined as the solidification of a solution by an extreme increase in viscosity without crystallization [22]. In an aqueous solution, vitrification therefore means ice-free solidification, while freezing means ice formation.

The vitrification method not only greatly simplifies the cooling process and eliminates the use of elaborate equipment to control the cooling rate, but also eliminates all the physical and chemical injuries caused by extracellular ice, which are major causes of cell injury. A higher rate of survival can therefore be expected in vitrification than in freezing. Since the pioneering work by Rall and Fahy [19], there have been many reports on the vitrification of embryos at various stages in various species. In some cases, very high survival rates have

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been reported [23–25], but the solution and the procedure vary depending on the laboratory, and a standard protocol has not yet been established even for the same type of embryo. In this article, I review various reports on the vitrification of mammalian embryos and oocytes, and evaluate vitrification from the standpoint of the mechanism of cell injury.

Mechanism of Injury in Cryopreserved Cells

During the processing and cooling of cells, and during the recovery of stored cells into a physiological solution, they are at a risk of injury by various factors, including i) toxicity of cryoprotectants, ii) chilling injury, iii) physical injury by extracellular ice, iv) toxicity of electrolytes concentrated with extracellular ice, v) formation and growth of intracellular ice, vi) fracture damage and vii) osmotic swelling and shrinkage. Embryos must circumvent these obstacles in order to survive cryopreservation, but in vitrification, cells do not suffer from injuries caused by extracellular ice (iii, iv); furthermore, the formation and growth of intracellular ice (v), which is a major obstacle in freezing, is less likely to occur because there is no ice outside the cells. In most studies on the freeze preservation of embryos, an empirical approach has been adopted to find the optimal conditions for treatment, because it is not easy to predict the possibility of injuries related to the formation and growth of extracellular ice during cooling (iii, iv, v) [26–28]. But in vitrification it is easier to deduce the optimal conditions because cells are hardly influenced at subzero temperatures except for limited possibilities of intracellular ice (v) and fracture damage (vi).

Toxicity of cryoprotectants

In order to make a solution vitrify in liquid nitrogen, the inclusion of a very high concentration of a permeating agent (cryoprotectant) is necessary, and its concentration can be as high as 8 M or more. The solution can therefore have a strong toxic effect on the cell, and this must be the greatest obstacle to successful vitrification. One strategy to circumvent toxicity is to compose low toxicity solution by i) adopting less toxic additives and by ii) minimizing the concentration of the additives required. The other strategy is to reduce the toxicity during exposure of cells to the solution by iii) minimizing the exposure time and by iv) lowering the exposure temperature. The composition of the vitrification solution and the procedure for exposure are therefore critical for successful vitrification (see below).

Chilling injury

In embryos at certain stages in some species, e.g., pig embryos before the peri-hatching stage [29], bovine embryos before the late morula [30] and *in vitro* produced bovine embryos before blastocoel formation [31], only cooling to below 20–10°C is injurious. In freezing, because it would be difficult to circumvent this injury without special manipulation such as the removal of cytoplasmic lipid from centrifuged embryos [32], the first piglets from cryopreserved embryos were produced after freezing zona-hatched embryos [33]. But in vitrification it may be possible to lessen the chilling injury by passing through the critical temperature zone very rapidly. It has actually been shown that chilling sensitivity is less in vitrification than in freezing in *in vitro* produced bovine compacted morulae [31].

Formation and growth of intracellular ice

The formation and growth of intracellular ice is fatal to the cell. In freezing, cells are at a high risk of intracellular ice formation with seeding by extracellular ice. In order to prevent this, the solution must be seeded and cooled very slowly so that the cells in it become concentrated by gradual dehydration in response to the concentration of extracellular unfrozen fraction through the growth of extracellular ice [26]. When the sample is cooled in liquid nitrogen after sufficient concentration, the cytoplasm of the cells together with the extracellular unfrozen fraction will vitrify without ice formation. On the other hand, in vitrification, the chance of intracellular ice formation is much less because there is no ice outside the cells, but if permeation of a cryoprotectant into the cell and its concentration by dehydration are not enough, intracellular ice can be formed even in vitrification. Upon exposure of embryos to a vitrification solution, they are dehydrated almost instantly, and the cryoprotectant begins to permeate [34, 35], but in some types of embryos, a certain period of exposure will be necessary for permeation of a cryoprotectant required for intra-embryonic vitrification, so that the permeability of the cell membrane to cryoprotectants is an important factor (see below).

Fracture damage

When cryopreserved embryos are recovered from liquid nitrogen, they are occasionally found cracked. This physical injury is thought to be caused by a nonuniform change in the volume of the solution during the rapid phase change between the liquid and the solid, and is called fracture damage [36]. In freezing, more than 50% of embryos can be physically damaged [36–38],

and efforts have been made to reduce it, but the damage has not been eliminated completely [36, 37, 39]. In vitrification, on the other hand, the incidence of physical damage seems to be less than in freezing, probably because of the absence of ice. Its incidences have been reported to be only 1.2% in mouse blastocysts [40] and 3.6% in rabbit morulae [25], but sometimes as much as 20% [41] in mouse 8-cell embryos and 27% in sheep day 6 embryos [42]. It is suggested that flexible containers [23] and inclusion of macromolecules in the solution [41] reduce fracture damage.

Very recently the author's group showed that the fracture damage can be prevented completely in vitrification [40]. In order to augment the incidence of the fracture damage, we vitrified and warmed mouse embryos 10 times without taking the embryos out of the straw. When samples were cooled rapidly by direct plunging into liquid nitrogen and were warmed rapidly by soaking them in room temperature water, 75% of the embryos were found to have an injured zona pellucida. But when samples were cooled moderately by suspending them in liquid nitrogen gas (3 min or more) and warmed moderately by keeping them in air at room temperature for 15 sec followed by soaking in water, 100% of the embryos had an intact zona even after 10 cycles of vitrification and warming. Furthermore, the survival was not affected by the repeated vitrification and warming, indicating that vitrification, melting and temperature fluctuation per se do not affect embryonic survival. We also showed that the fracture damage occurs both during cooling and warming but more damage occurs during warming, so that the fracture damage can be prevented by buffering the cooling and warming velocities during passage through the temperature zone where the phase change would occur (-110 – -130°C) [23, 43]. This method, which completely prevents fracture damage, will be especially effective for rabbit embryos, where intact zona is essential for *in vivo* development [37, 44].

Osmotic injury

Just after warming, the cryopreserved cells contain a permeated cryoprotectant. If the cells are directly recovered in an isotonic solution, they are threatened by injury from osmotic swelling, because water permeates far more rapidly than the cryoprotectant diffuses out [45]. Mazur and Schneider [46] showed that fresh mouse and bovine embryos can survive swelling to 200% of their volume in isotonic solution. We have also observed that fresh mouse embryos can survive 30 min of exposure in a 0.3 times isotonic solution at 25°C . But

when vitrified mouse embryos after successful recovery in an isotonic solution were immediately suspended in the hypotonic solution, the survival rate was low, showing that cryopreserved cells are more sensitive to osmotic swelling than fresh ones [47], so that post-warming swelling of embryos should be minimal. At the same time, vitrified embryos need quick dilution of the cryoprotectant after warming because the toxicity of the solution is quite high.

The first strategy to prevent the osmotic damage in freezing was to dilute the sample with an isotonic solution in several steps [1], but embryos will not be able to avoid a certain degree of swelling with this method [48]. Embryos are now most commonly diluted with a sucrose solution to counteract excess water inflow [18, 45, 49]. In a sucrose dilution, however, the molality of the sucrose solution has to be high enough to prevent any swelling upon suspension (for instance, 1.12 M sucrose is required to counteract the osmosis caused by 1.5 M glycerol [48]). An effective method to prevent this swelling with a lower concentration of sucrose is to make cells shrink by pre-suspending them in a solution containing both the cryoprotectant and sucrose before they are transferred to a solution containing sucrose only [18]. The inclusion of sucrose in the freezing solution [16, 50] or the vitrification solution [24, 51] therefore promotes shrinkage and reduces the intracellular amount of a permeating cryoprotectant before dilution, which will help prevent cells from swelling upon direct dilution in a sucrose solution. Although sucrose is almost exclusively used as an osmotic buffer, some other saccharides will also be effective.

To prevent osmotic swelling, rapid permeating cryoprotectants are desirable, since they will diffuse out of the cell rapidly. The permeating properties of cryoprotectants are described below.

After diffusion of the permeated cryoprotectant in the sucrose solution, the cells remain shrunken. This hypertonic shrinkage may also be injurious to the cells, if they are exposed to a high concentration of sucrose for a long time at a higher temperature [52]. As was observed in hypotonic stress, cryopreserved cells are more sensitive to shrinkage than fresh cells [53].

Vitrification Solutions

Table 1 summarizes the composition of cryoprotective additives reported for the vitrification of embryos and oocytes in various mammalian species. All the solutions are composed by adding additives to a physiological solution; in the basic solution, salts were added

Table 1. Composition of cryoprotective additives used for the vitrification of mammalian embryos and oocytes of various species

Cryoprotective additives			Species examined ⁴⁾		References ⁵⁾
Permeating agents ¹⁾ (v/v)	Non-permeating agents		Embryos	Oocytes	
	Macromolecules ²⁾ (w/v)	Saccharides ³⁾ (molar)			
50 G	—	—	M		54*
40 G	—	—	Rt		55*
42.5 D	—	—		M, Ha	56*, 57
25 G + 25 P	—	—	M, Rb, C, Sh, G		42, 58*, 59–73
30 G + 30 P	—	—	Rb		74*
35 G + 35 P	—	—	Rb		74*
22.5 G + 22.5 P	—	—	C		75*, 76
20 G + 50 P	—	—	Rb		77*
20 E + 20 D	—	—	Rb		78*
25 E + 25 D	—	—	M, Rb, C		79*, 80–84
25 G + 25 E	—	—	M, Sh		79*, 85, 86
13 G + 33 E	—	—	M, Sh		87*, 88
14.2 D + 5.1 A + 22 P	—	—	M, Rt	M, C	89*, 90–98
14.2 D + 10.2 A + 29.3 P	—	—		Rt	99*
20 E + 20 D + 10 B	—	—	M		100*
40.3 P	6 PEG	—	M		23*
47.4 G	6 PEG	—	M, Rt		23*, 101
47.4 G	6 BSA	—	M, Rb, C, Sh, Pg		73, 102*, 103–106
44 E	7.5 PVP	—	M		107*
18.6 D + 13.4 A + 9.6 P	6 PEG	—	M, Rt, C	M, Ha	19*–21, 108–122
16.7 D + 12 A + 8.7 P	5.4 PEG	—	M	M, Hu	19*, 21, 123–127
30 G	—	1.0 S	M,		51*, 128–130
30 E	—	1.0 S	M, Sh, Hu		87*, 88, 131, 132
45 E	—	0.5 S		M	133*
19.5 D + 20.2 P	—	1.0 S	M, Rt	M	134*, 135–140
10 G + 20 P	—	1.0 S	Rb		141*
20 E + 20 G	—	0.75 (S + Gc)	C		142*
40 E	18 Ficoll	0.3 S	M, Rb, C, Ho, Ma	Ho	24*, 40, 143–156
40 G	18 Ficoll	0.3 S	M, C		145*, 152, 157, 158
50 G	15 Ficoll	0.25 S	M		152*
40 E	18 Ficoll	0.3 T	Pg, Hu		159*, 160
30 E	21 Ficoll	0.35 S	M		147*, 158
40 E	20 PVP	0.3 T	C		161*, 162
38.6 P	2.3 BSA	0.25 T		C	163*
40 E	5 PEG	0.5 S	Rt		164*
50 G	50 Percoll+	0.75 S	M		41*
40 E	7.5 PVP	0.25 Ga	C		31*

1) D: dimethylsulfoxide, A: acetamide, P: propylene glycol, G: glycerol, E: ethylene glycol, B: 1,3-butanediol. 2) PEG: polyethylene glycol, BSA: bovine serum albumin, PVP: polyvinylpyrrolidone. Serum and – 0.3%BSA are not shown. +: Percentage in v/v. 3) S: sucrose, T: trehalose, Gc: glucose, Ga: galactose. 4) M: mouse, Rt: rat, Rb: rabbit, Ha: hamster, C: cow, Sh: sheep, G: goat, Ho: horse, Pg: pig, Ma: a marsupial, Hu: human. 5) Those with an asterisk are the first reports for each solution.

to the molar base (per volume) in the first solution [19], but in most of the other solutions salts were added to the molal base (per water). Additives can be classified into 3 categories, that is, permeating agents, macromolecules and small saccharides.

Permeating cryoprotectants

To prevent the formation of intracellular ice in large cells, such as mammalian embryos, permeation of a cryoprotectant is essential. A permeating agent is therefore included in all vitrification solutions. In the first solution, Rall and Fahy [19] used DMSO, acetamide

and propylene glycol as the permeating components. After their pioneering report, however, numerous solutions have been devised with not only the 3 agents but also glycerol and ethylene glycol, in combination or as a single permeating component. So far the 5 agents have almost exclusively been used for the vitrification of mammalian embryos and oocytes (Table 1). The mechanism of the protective action of various permeating agents is considered to be the same, but the toxicity and the permeating properties of the agents are quite different, and have significant effects on the survival of vitrified embryos.

From the results of the toxicity test of some agents in the 3 categories on mouse morulae, it is clear that the permeating agent is responsible for the toxicity of the vitrification solution [144, 145, 147]. Among the 5 major permeating agents, ethylene glycol is the least toxic, followed by glycerol, and acetamide is the most toxic, at least to mouse morulae [165]. In bovine blastocysts, ethylene glycol and glycerol are less toxic than propylene glycol [145], but the relative toxicity of various agents may not be the same in other stages of oocytes/embryos and in other species.

As mentioned above, the permeating properties of cryoprotectants are closely related to intracellular ice formation and post-warming osmotic swelling. Rapidly permeating agents are generally favorable, because exposure time before cooling can be shortened, and because they are more likely to diffuse out of the cell rapidly, which helps prevent osmotic swelling. Leibo [34] and Jackowski *et al.* [35] analyzed the permeability of mouse 1-cell zygotes and oocytes from the volume change during suspension in a glycerol solution. Upon suspension, they shrink quickly by losing water in response to the extracellular osmolality, because water diffuses out far more rapidly than glycerol permeates. And as the cryoprotectant permeates, water re-enters the cell to maintain intracellular osmolality and the cell regains its volume slowly, so that in a more permeating cryoprotectant, cells will regain the volume more quickly. Of the five cryoprotectants, ethylene glycol is the most permeating for mouse [158] and bovine [166] morulae and blastocysts, but the permeating properties are different in oocytes/embryos at other stages. For instance, in mouse 1-cell zygotes, ethylene glycol is less permeating than propylene glycol, and glycerol is as permeating as DMSO and propylene glycol in mouse morulae, but in 1-cell zygotes, it is much less permeating than other cryoprotectants [158]. Because this is also true for *in vitro* produced bovine embryos [166], suitable cryoprotectants may be different for oocytes and em-

bryos at different developmental stages. The membrane properties must also differ according to the species, but although information about the permeating property of various cryoprotectants is still limited, it seems that the difference in the permeability of the cell membrane among oocytes/embryos of various species is much less than the difference among oocytes/embryos at various developmental stages.

At least for morulae and blastocysts, ethylene glycol seems to be a good candidate for the permeating component of a vitrification solution, considering the toxicity and the permeating properties. Actually ethylene glycol is now in wide use for embryos of various species (Table 1). But ethylene glycol may not always be the best agent even for blastocysts, because a glycerol-based solution is more effective than an ethylene glycol-based solution for zona-hatched mouse blastocysts [157]; probably because ethylene glycol permeates too fast, thus being toxic. For oocytes, on the other hand, solutions with DMSO, propylene glycol or acetamide have been more frequently used than those with ethylene glycol or glycerol (Table 1). Ethylene glycol is less permeating to oocytes than some other agents, and glycerol is almost nonpermeating to oocytes of the mouse [158] and cattle [166].

Macromolecules

It is reported that because the incorporation of a macromolecule promotes vitrification of a solution [22], by incorporating a macromolecule, it is possible to reduce the toxicity of the solution by decreasing the concentration of a permeating agent(s) required for vitrification of the solution. Among macromolecules used for vitrification of mammalian oocytes/embryos are polyethylene glycol (PEG) [19], Ficoll 70 [24], bovine serum albumin [102], polyvinylpyrrolidone [107] and Percoll [41] (Table 1). Macromolecules are nonpermeating to the cell and much less toxic than permeating agents. On macromolecules few comparative studies have been done, but in a toxicity test, it is found that Ficoll 70 is less toxic than PEG when it is mixed with 40% (v/v) ethylene glycol [165].

Some solutions contain serum as a physiological component. A lot of macromolecules are contained in serum, and these will promote extracellular vitrification. On the other hand, because macromolecules in the cytoplasm will promote intracellular vitrification, the number of macromolecules in the blastocoelic cavity of blastocysts is much smaller, and therefore, for successful vitrification of blastocysts, permeation of a permeating agent and its concentration, especially in the blastocoel, will be important.

Small saccharides

Macromolecules contribute to the osmolality only a little, while mono- and disaccharides exert considerable osmotic effects as non-permeating agents. Incorporation of a small saccharide therefore promotes dehydration and thus intracellular vitrification, and it reduces the amount of intracellular cryoprotectant, which will help reduce the toxic effect of the cryoprotectant and prevent over-swelling of the cell during removal of the permeated cryoprotectant.

As a small saccharide, sucrose is sometimes incorporated in the freezing solution [16, 50]. In vitrification solutions, sucrose is more frequently used, because full permeation of the permeating agent is not necessary but toxic. Actually, it was found that incorporation of sucrose reduces the toxicity of a vitrification solution significantly [24]. Because such an effect is not specific to sucrose, other saccharides, such as trehalose [159], glucose [142] and galactose [31] have been used (Table 1). It has been shown that sucrose and other saccharides such as glucose, galactose and fructose are virtually non-toxic when used at a refrigerated temperature [167], although they can be harmful at higher temperatures [52, 53].

Vitrification solutions

As shown in Table 1, various vitrification solutions have been composed. In some of these solutions, probably including those which contain only 30% permeating agent(s), ice may be formed during warming, which is called devitrification. Although these solutions may not be called true vitrification solutions, devitrification is not always harmful, if the ice is not formed inside the cell.

As shown in Table 1, there are 4 categories of solutions: those with only a permeating agent(s), those with a permeating agent(s) + a macromolecule, those with a permeating agent + a saccharide(s) and those with all 3 components. Considering that each category of agents plays a role, the last group seems to be the most reasonable, although the optimization of the composition is necessary.

Procedures for Exposing Embryos to a Vitrification Solution

One step exposure

The simplest way for vitrifying embryos is to transfer them from a physiological solution to a vitrification solution directly, and then to plunge the sample into liquid nitrogen. Some types of embryos can survive quite well by this one step exposure, but the time and tempera-

ture of the exposure have critical effects.

To prevent intracellular ice, a cryoprotectant must permeate the embryo and be concentrated during the exposure so that longer exposure is desirable. On the other hand, they must circumvent the toxic effects of the solution, in which case shorter exposure is desirable. For these reasons the optimal exposure time for successful vitrification must be a compromise between preventing toxic injury and preventing intracellular ice formation. Nakagata [89, 90] uses a solution containing DMSO, acetamide and propylene glycol, and this solution is highly toxic. His strategy for circumventing the toxicity is to reduce the exposure time to only 5–10 sec. By this method he succeeded in the vitrification of not only embryos but also oocytes, which are more difficult to cryopreserve than embryos.

The optimal time is dependent on the exposure temperature, because both permeation of a cryoprotectant and its toxicity are largely influenced by the temperature. The optimal time is longer at a lower temperature, and shorter at a higher temperature. For instance, in the vitrification of mouse morulae in an ethylene glycol-based solution, very high survival was obtained with exposure periods of 2–10 min at 5°C, 30 sec–5 min at 20°C and 30 sec–1 min at 25°C [165]. In expanded mouse blastocysts, on the other hand, high survival was not obtained with any exposure time at 20°C. The survival was improved not by lowering the temperature of exposure [147] but by raising it [165]. Therefore, for one step vitrification of cells where longer exposure is required for permeation, it may be preferable to adopt more permeating conditions with a shorter exposure time, rather than less toxic conditions with a longer exposure time.

Stepwise exposure

In some cases, e.g., blastocysts with a large blastocoel, high survival may not be obtained with the one-step exposure, because cells are injured by the toxicity before enough permeation is attained. In this case, a stepwise method will be useful, where embryos are first suspended in a solution containing a lower concentration of a cryoprotectant at room temperature for permeation without causing toxic injury, and then exposed to a vitrification solution for a short period at room temperature. If the second exposure is at a refrigerated temperature, embryos can be exposed for a longer time, but embryo manipulation at room temperature would be more convenient than at a refrigerated temperature. In the first report of Rall and Fahy [19], they exposed embryos in 3 steps including 2 steps at 4°C, but the second step would not be necessary.

Conclusions

So far, numerous vitrification solutions have been developed, and successful vitrification has been reported for mouse [21, 24, 58, 89], bovine [59, 118, 145], rat [113, 164], rabbit [25, 74], sheep [69, 102], goat [67], horse [150] and human [160] embryos. Vitrification is a simple and refined strategy. Because cells do not suffer from injuries caused by extracellular ice, it would be possible to deduce the optimal treatment by characterizing each type of embryo and oocyte, such as by shape, size, permeability to cryoprotectants, sensitivity to cryoprotectant toxicity and tolerance to osmotic swelling and shrinkage. Because these characteristics are different for embryos/oocytes at different stages and species, it may seem reasonable to define the optimal condition for each type of cell, but reported data suggest that a single protocol is more likely to be applicable to cells of other species, if the stage of development is equivalent.

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