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REVIEW

Current status of vitrification of embryos and oocytes in domestic animals: Ethylene glycol as an emerging cryoprotectant of choice

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Abstract

The cryopreservation of mammalian embryos has become an integral part of methods to control animal reproduction. Numerous vitrification solutions have been formulated with ethylene glycol in combination with macromolecules, sugars and other cryoprotective agents. These indicate that a study of ethylene glycol as a cryoprotectant of choice in vitrification studies would be promising.

To understand the cryobiology of ethylene glycol, several factors have to be studied. These are: cryoprotectant toxicity, osmotic stress and temperature at exposure. Understanding these factors could lead to the formulation of vitrification protocols that would lead to higher viability rates after cooling. First, ethylene glycol must be used as the sole cryoprotectant in a solution without macromolecules and sugars. Second, partial dehydration and permeation prior to cooling to subzero temperatures must be studied to achieve accurate exposure and a one-step dilution method. Third, the toxic effects of ethylene glycol must be overcome without sacrificing its vitrification properties by combining step-wise exposure at appropriate temperatures, low concentration and decreased volume. Fourth, the long-term effects of ethylene glycol on exposed or vitrified embryos must be determined. Lastly, the influence of culture on the viability of vitrified embryos must be studied to improve viability rates after warming.

Key words: cryoprotectant, embryos, ethylene glycol, oocytes, vitrification

Introduction

The cryopreservation of mammalian embryos has become an integral part of methods to control animal reproduction[17]. Cryopreservation is of practical importance to the development of reproductive technologies such as nuclear transfer, cryobanking of animal species and the routine or commercial application of breed improvement like embryo transfer in cattle. Several cryopreservation methods such as conventional (slow), equilibrium freezing, rapid or non-equilibrium freezing (i.e. vitrification) and ultra-rapid freezing have been used to preserve embryos and oocytes of many animal species resulting in the birth of live offspring. Of these methods, vitrification offers a simple, practical and economical approach to the cryopreservation of embryos and oocytes of several animal species.

Vitrification is a thermodynamic process in which the viscosity of a fluid is increased by many orders of magnitude, giving the fluid the mechanical properties of a solid. Since all life proces-

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ses cease when the solution in a biological material is brought to a vitreous (glasslike) state, vitrification is used for the cryopreservation of cells[3]. The probability of vitrification is directly proportional to the cryoprotectant concentration. The choice of the most appropriate cryoprotectant and its concentration is very important for several reasons. The permeating rate of oocytes and embryos by the cryoprotectant is strictly dependent on the type of cryoprotectant used, as well as on the species and on the development stage of the embryos. For a given type of cell, the greater the permeability of a certain cryoprotectant, the smaller is its osmotic effect[2]. Vitrification is a thermodynamic state of metastable equilibrium. When an aqueous solution is vitrified by rapid cooling, ice crystals can form. The probability of ice crystal formation is affected by several thermodynamic parameters, including volume and viscosity[30]. The probability is inversely proportional to viscosity and directly proportional to volume. In typical cryopreservation protocols by vitrification, the probability of ice crystal formation is reduced by increasing viscosity through an increase in the concentration of the cryoprotectants. However, the high concentration of cryoprotectants required in vitrification can be chemically toxic[4].

The use of cryoprotectants in the cryopreservation of animal cells has been popularized by the success achieved in the freezing of semen[28] which resulted in the wide-spread use of artificial insemination in the improvement of domestic animal species. Of the cryoprotectants, glycerol and dimethyl sulphoxide (DMSO) have been used in the cryopreservation of semen and embryos respectively. In the search for less toxic and more effective cryoprotectants, relatively newer cryoprotectants were found to be as equally effective as glycerol or DMSO ; these are ethylene glycol and propylene glycol.

Ethylene glycol has a relatively low molecular weight (62.07) compared to the other cryoprotectants (Glycerol : 92.1, DMSO : 78.13, propylene glycol : 76.1), which enables its rapid influx during equilibration and its rapid efflux during dilution. This property of ethylene glycol has enabled workers to achieve a one-step in-straw dilution and direct transfer of embryos that have resulted in the birth of live offspring[9,40]. The use of ethylene glycol as a cryoprotectant was initiated by Miyamoto and Ishibashi[24,25] in the freezing of mouse and rat embryos by the conventional freezing method. In their first study, ethylene glycol was found to be as equally effective as DMSO for protecting rat embryos during freezing[25]. Of the glycol group, ethylene glycol was also found to be the most protective agent tested for freezing mouse and rat embryos at 1.2 M concentration, an equilibration temperature of 0°C, a cooling rate of 0.5°C/min and a thawing rate of 15°C/min[24]. Ethylene glycol was found to be the cryoprotectant of choice when Kasai *et al.*[14] compared three cryoprotectants (ethylene glycol, glycerol, propylene glycol) in the vitrification of mouse embryos. Several studies later confirmed the effectivity of ethylene glycol as the sole permeable cryoprotectant or in combination with macromolecules, sugars and other cryoprotectants[1,2,11,12,13,19,20,26,35,36,39].

A vitrification solution must provide efficient cryoprotection of all embryonic stages in a wide variety of animal species. Improved methods for the long-term cryopreservation of mammalian embryos by vitrification will depend on the development of less toxic vitrification solutions and procedures that minimize osmotic stresses[30] during equilibration and dilution[29]. The role of ethylene glycol in these protocols deserves careful scrutiny in order to understand its cryoprotective properties. Therefore, this review article was made to present some of the important facts underlying the vitrification process and to identify possible venues for future research on the use of ethylene glycol as the sole cryoprotectant in

vitrification protocols.

Significant achievements in the field of vitrification

Vitrification was first suggested by Luyet[18] in 1937. The real breakthrough came in 1985 with the successful vitrification of mouse 8-cell embryos when Rall and Fahy[29] showed that mouse embryos can be cryopreserved with a solution containing DMSO, acetamide, propylene glycol and polyethylene glycol (VS1). Since 1985, several vitrification solutions have been formulated (Table 1). Kasai *et al.*[14] used 40% ethylene glycol in combination with 30% ficoll (a macromolecule) and 0.5 M sucrose in a solution called EFS (ethylene glycol-ficoll-sucrose) for the vitrification of mouse morulae. Thereafter, Kasai and his group[23,41] succeeded in the vitrification of all embryonic stages of mouse embryos with significantly high development rates. Other workers have successfully used Kasai's method in the vitrification of bovine[19,35] and equine[11] embryos, thus, paving the way for the adoption of a single vitrification method that was effective in all developmental stages of embryonic development in a variety of animal species.

On the other hand, Ishimori *et al.*[13] formulated a vitrification solution containing 25% ethylene glycol and 25% DMSO and succeeded in the vitrification of mouse blastocysts. This method would later be adopted by Vajta *et al.*[36] in the vitrification of bovine morulae and blastocysts.

In 1993, Ali and Shelton[1] vitrified all stages of mouse embryos using three vitrification solutions, namely: VS1 (5.5 M ethylene glycol and 2.5 M glycerol), VS11 (6.0 M ethylene glycol and 1.8 M glycerol), and VS14 (5.5 M ethylene glycol and 1 M sucrose). It is interesting to note that after testing over 6,000 combinations of cryoprotectants, they came up with the above-mentioned vitrification solutions, all containing ethylene glycol. Later, the three vitrification solutions would be used on the successful vitrification of day-6 sheep embryos[2].

In other studies, the combination of ethylene glycol with other cryoprotectants would result in vitrification solutions that prove effective in some developmental stages or in some animal species. Valdez *et al.*[39] used a vitrification solution containing 20% ethylene glycol, 20% DMSO and 10% 1,3 butanediol in the vitrification of mouse blastocysts. Szell and Windsor[34] used a combination of 3.5 or 4.5 M glycerol with either 3.5 or 4.5 M propylene glycol or ethylene glycol in the vitrification of day-6 sheep embryos. Naitana *et al.*[27] used 1.4 M glycerol and 3.6 M ethylene glycol in PBS with 20% fetal calf serum in the vitrification of sheep blastocysts.

The attempt to use ethylene glycol as a single cryoprotectant in a vitrification solution was initiated by Kasai *et al.*[14] who did not pursue further research after obtaining poor results with 40% ethylene glycol alone in the vitrification solution. However, Saha *et al.*[32] compared three vitrification solutions containing 40% ethylene glycol, 40% ethylene glycol+0.3 M trehalose and 40% ethylene glycol+0.3 M trehalose+20% polyvinylpyrollidone (PVP) in the vitrification of bovine blastocysts. The results that they obtained indicated that 40% ethylene glycol alone yielded lower rates of viability of bovine blastocysts compared to 40% ethylene glycol+0.3 M trehalose, and 40% ethylene glycol+0.3 M trehalose+20% PVP. Furthermore, Hotamisligil *et al.*[12] studied the effects of ethylene glycol on the membrane integrity, microfilament organization and developmental potential of mouse oocytes using 0 to 8 M ethylene glycol. During exposure to 0.5 to 2 M ethylene glycol, oocytes showed maximum shrinkage to 55.5% of the isotonic volume within the first min and re-expanded to their initial volume within 15 min. The transfer of these oocytes to higher (4 to 8 M) concentrations of ethylene glycol for 1 to 5 min after 15 min of equilibration in 2 M ethylene glycol

Table 1. List of selected vitrification studies with ethylene glycol from 1985–1997

Year	Author / s	Species	Stage	Cryoprotectant	Exposure method
1985	Rall & Fahy[29]	Mouse	8 cell	20.5% DMSO, 15.5% acetamide, 10% propylene glycol, 6% polyethylene glycol (VS1)	4 steps
1990	Kasai *et al.*[14]	Mouse	morulae	40% ethylene glycol (EG), 30% Ficoll, 0.5 M sucrose (EFS)	1 step
1992	Valdez *et al.*[37]	Mouse	blastocysts	20% EG, 20% DMSO, 10% 1,3 butanediol	2 steps
1992	Ishimori *et al.*[13]	Mouse	blastocysts	25% EG, 25% DMSO	2 steps
1993	Miyake *et al.*[23]	Mouse	all stages	EFS	1 step
1993	Ali & Shelton[1]	Mouse	all stages	5.5 M EG+ 2.5 M glycerol, 6 M EG +1.8 M glycerol, 5.5 M EG+1 M sucrose	1 step
1993	Ali & Shelton[2]	Sheep	Day 6	5.5 M EG+ 2.5 M glycerol, 6 M EG+1.8 M glycerol, 5.5 M EG+ 1 M sucrose	1 step
1993	Zhu *et al.*[41]	Mouse	expanded blastocysts	EFS	1-2 steps
1993	Tachikawa *et al.*[35]	Bovine	blastocysts	EFS	1 step
1993	Mahmoudzadeh *et al.*[19]	Bovine	compact morulae, early blastocysts	VS1, 7.15 M EG 2.5 M ficoll 0.3 M sucrose	1 step
1994	Hochi *et al.*[11]	Equine	Days 5-7	EFS	2 steps
1994	Szell & Windsor[34]	Sheep	Day 6	3.5 M or 4.5 M each of glycerol+ propylene glycol or EG	2 steps
1994	Darvelid *et al.*[7]	Bovine	morulae, blastocysts	40% EG+ 0.5 M sucrose and 35% EFS	3 steps
1995	Mahmoudzadeh *et al.*[20]	Bovine	compact morulae to expanded blastocysts	EFS	2 steps
1995	Naitana *et al.*[27]	Sheep	blastocysts	1.4 M glycerol, 3.6 M EG, in PBS with 20% FCS	2 steps
1996	Hotamisligil *et al.*[12]	Mouse	oocytes	0-8 M EG in Hepes-HTF (Human tubal fluid) with 0.5 M sucrose	2 steps
1997	Bautista *et al.*[5]	Mouse	8-cell	2 M and 7 M EG in PBS with 10% calf serum	2 steps

was tolerated well. However, bleb formation was observed on the surface of the cells and microfilament distribution was disturbed when exposure was prolonged (>5 min) at higher (>4 M) concentrations of ethylene glycol. Bleb formation was suppressed when 0.5 M sucrose was added to the solution. On the other hand, Bautista *et al.*[5] showed that ethylene glycol can be used as the sole cryoprotectant in the vitrification solution containing 7 M ethylene glycol in PBS with 10% calf serum. Mouse 8-cell embryos were successfully vitrified using this solution leading to high proportions of the embryos developing to the blastocyst stage in culture.

The major achievements in the field of vitrification evidently, were obtained with the use of ethylene glycol in combination with other cryoprotectants, macromolecules or sugars. However, the use of ethylene glycol as the sole cryoprotectant in the vitrification solution need to be studied further.

Problems and prospects of vitrification with ethylene glycol

Whether indeed ethylene glycol can be used as the sole cryoprotectant in a vitrification solution without macromolecules or sugars is a subject for future research. Further studies have to focus on a variety of factors relevant to the success of vitrification. To understand the mechanism of cryoprotection of ethylene glycol, the following factors have to be considered:

A. Cryoprotectant toxicity

Cryoprotective agents are organic solutes that are used to protect intracellular organelles during long-term storage in liquid nitrogen. Permeating cryoprotectants intracellularly interact to influence microfilament and microtubule dynamics[8]. At higher concentrations required in vitrification, cryoprotectants become toxic to the cells[3].

To overcome cryoprotectant toxicity, several techniques have been adopted. A common approach is to provide a mixture of cryoprotectants in the vitrification solution. Since the concentration of each cryoprotectant in the solution is low, the specific toxicity of the cryoprotectant is not observed[22]. The addition of macromolecules and sugars also results in the reduction of the chemical toxicity of the cryoprotectant[14]. Macromolecules and sugars assist in the formation of stable glass at low temperatures, control the rate of permeation by the cryoprotectant resulting in the prevention of excessive swelling before (equilibration) and after (dilution) warming, and increase the viscosity of the solution while lowering the concentration of the permeable cryoprotectant, thereby lowering the coefficient of toxicity.

The use of small volumes of cryoprotectants to reduce chemical toxicity is another approach. An example of this method is called the Minimum Drop Size (MDS) technique[3]. With a small volume of the vitrification solution (about 5 μl), this method has been used in the successful vitrification of previously unfreezable pig embryos and oocytes. In the MDS technique, the probability for ice crystal formation is decreased by reducing the volume of the cryopreserved sample, rather than through an increase in the concentration of cryoprotectants[4].

B. Osmotic stress

Cellular osmotic injury is brought about by excessive shrinkage before cooling during one-step exposure to slow or non-penetrating cryoprotectant. It can also result after warming by the excessive swelling during dilution of the intracellular cryoprotectant[3]. The vitrification approach requires cells to be placed in solutions containing hyperosmotic concentrations of both permeating and non-permeating solutes as a consequence of which they become partially dehydrated before the onset of cooling to subzero temperatures[22]. Partial dehydration can be achieved by placing the cells in hyperosmotic

non-permeating sucrose or by a combination of high concentrations of the permeating solutes, short exposure time and temperatures near 0°C[22,30]. A step-wise addition of the vitrification solution can reduce osmotic stress[13,20]. However, the role of step-wise addition of the cryoprotectant needs greater investigation, as the study of Mahmoudzadeh *et al.*[20] would indicate. Bautista *et al.*[5] exposed mouse 8-cell embryos in 2 and 7 M ethylene glycol in PBS with 10% calf serum in two-steps. They found that mouse 8-cell embryos can tolerate exposure to these concentrations of ethylene glycol without appreciable loss of viability *in vitro* (Table 2). If indeed ethylene glycol's toxicity can be overcome by a step-wise addition and sucrose dilution can be avoided, the process of vitrification would be much simpler, paving the way for in-straw dilution and direct transfer of vitrified embryos.

C. Temperature at exposure

The temperature at exposure to the cryoprotectant also has an effect on the viability of vitrified embryos as the study of Bautista *et al.*[5] indicated. The temperature affects the permeation of the cryoprotectant into the cells and thus, may lead to osmotic stresses. Bautista *et al.*[5] have found that when mouse 8-cell embryos were exposed at temperatures over 24°C to 2 and 7 M ethylene glycol in PBS with 10% calf serum before vitrification, the *in vitro* viability of the vitrified-warmed embryos were low (Table 3). However, when the embryos were exposed to the vitrification solution at 18 to 22°C, very high viability rates *in vitro* were obtained (Table 4).

The interplay of these factors, therefore,

Table 2. Viability of 8-cell embryos exposed to 2 and 7 M ethylene glycol (EG) in PBS with 10% calf serum

Group	Step 1 Exposure to 2M EG (min)	Step 2 Exposure to 7M EG (min)	Number exposed	Number cultured	Number & (%) developed to blastocyst
Control	—	—	—	48	48(100)
Treated:					
	2	2	30	30	30(100)
	5	2	30	30	29(96.7)
	10	2	30	30	28(93.3)
	2	5	30	30	28(93.3)
	5	5	30	30	29(96.7)
	10	5	30	30	28(93.3)

Data were pooled from 2 replicates.
From Bautista *et al.*[5]

Table 3. Viability of 8-cell embryos vitrified in 2 and 7 M ethvlene glycol (EG) in PBS with 10% calf serum at temperatures over 24°C

Group	Step 1 Exposure to 2M EG (min)	Step 2 Exposure to 7M EG (min)	Number vitrified/ warmed	Number cultured	% developed to blastocyst (Mean±SEM)
Control	—	—	—	50	100±0.0[a]
Treated:					
	2	2	50	50	32.0±14.6[b]
	5	2	50	50	30.0±12.2[b]
	10	2	50	50	18.0± 9.7[bc]
	2	5	50	50	0.0± 0.0[c]
	5	5	50	50	0.0± 0.0[c]
	10	5	50	50	0.0± 0.0[c]

Values are mean percentages of 5 replicates.
a), b), c) Values with different superscripts differ significantly ($P<0.05$).
From Bautista *et al.*[5]

Table 4. Viability of 8-cell embryos vitrified in 2 and 7 M ethylene glycol (EG) in PBS with 10% calf serum at 18 to 22°C temperature

Group	Step 1 Exposure to 2M EG (min)	Step 2 Exposure to 7M EG (min)	Number vitrified/ warmed	Number cultured	% developed to blastocyst (Mean±SEM)
Control	—	—	—	30	100±0.0[a]
Treated:					
	2	2	50	50	94.4±4.0[a]
	5	2	50	50	88.0±4.9[a]
	10	2	50	50	84.0±7.5[a]
	2	5	50	50	24.0±2.4[b]
	5	5	50	50	20.0±8.9[b]
	10	5	50	50	14.0±7.5[b]

Values are mean percentages of 5 replicates.
a), b) Values with different superscripts differ significantly ($P<0.0001$).
From Bautista *et al.*[5]

has to be considered in the formulation of new vitrification protocols using ethylene glycol. As the study of Mahmoudzadeh *et al.*[20] and Bautista *et al.*[5] would indicate, toxicity and osmotic stress can be minimized leading to the formulation of vitrification procedures that are effective and yielding high viability rates after warming. Furthermore, Bautista *et al.*[5] showed that it is possible to use ethylene glycol as the sole cryoprotectant in the vitrification solution.

In future researches, the role of ethylene glycol in vitrification procedures must be studied exhaustively to understand its physico-chemical properties. For one, further studies are needed in the use of ethylene glycol as the sole cryoprotectant in the vitrification solution. Likewise, the influence of other factors must be studied. Leibo and Loskutoff[16] have stated that the culture medium not only influences the development of vitrified-warmed embryos but also exerts a significant effect on their survival after cryopreservation. Understanding the mechanism by which ethylene glycol affects the development of embryos in culture is an important step towards the formulation of future vitrification solutions. Furthermore, the long-term effect of ethylene glycol on vitrified embryos must be studied *in vitro* or *in vivo*.

Conclusion

Ethylene glycol has been proven to be an effective cryoprotectant with low toxicity and efficient permeability. Its compatibility with the other cryoprotectants has enabled the formulation of many successful protocols in the vitrification of oocytes and embryos in most domestic animal species. To understand its versatility as a cryoprotectant of choice, the singular cryoprotective properties of ethylene glycol must be studied exhaustively. First, ethylene glycol must be used as the sole cryoprotectant in a solution without macromolecules and sugars. Second, partial dehydration and permeation prior to cooling to subzero temperatures must be studied to achieve accurate exposure and a one-step dilution method. Third, the toxic effects of ethylene glycol must be overcome without sacrificing its vitrification properties by combining step-wise exposure at appropriate temperatures, low concentration and decreased volume. Fourth, the long-term effects of ethylene glycol on exposed or vitrified embryos must be determined. Lastly, the influence of culture on the viability of vitrified embryos must be studied to improve viability rates after warming.

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