



Evaluation of cryopreservation efficiency on embryo growth after exposing them in intracellular cryoprotectant ethylene glycol or glycerol and replacing them by trehalose

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ABSTRACT

The present study was conducted to see whether toxicity could be reduced by replacing some of the penetrating cryoprotectant (intracellular cryoprotectant) with a disaccharide as well as finding out the optimum equilibration time in a vitrification solution before rapid cooling. Goat ovaries (1,685) were aspirated and 1,761 culturable oocytes (COC) were recovered. The recovered COCs were matured in maturation medium and evaluated on the basis of cumulus expansion. The matured oocytes were coincubated with fresh semen capacitated in TALP media, for 18 h. The inseminated oocytes were further cultured in mSOF for *in vitro* embryo development. The cleavage rate was 11.76% and the development rate of embryos to 4–8 and 8–16 cells morulae was 55.6% and 42.4%, respectively. *In vitro* produced goat embryos (132), 4–16 cell stages, were used for cryopreservation using different protocols. In protocol- 1, 2, 3 and 4, there was no survivability of embryos. In protocol- 5, 6, 7 and 8, the survivability of embryo after freezing was 10, 25, 35.7 and 72.2%, respectively. In protocol- 8, the percentage of live embryos was significantly higher than other protocols. Also it was found that the survivability rate of embryos in protocol 6 and 7 were significantly higher as compared to protocol 1, 2, 3, 4 and 5. However, non-significant differences were found among protocol 1, 2, 3, 4 and 5, and between 6 and 7. From our results, it can be concluded that cryopreservation using 20% ethylene glycol and 0.9 M trehalose for 30 min gives significantly higher post thaw (72.2%) survivability of embryos.

Key words: Caprine, Cryopreservation, Cryoprotectant, Trehalose, Vitrification

Cryopreservation and thawing under controlled conditions have permitted the use of cells and tissues after long-term storage in both commercial and research settings. Embryo cryopreservation has simplified the management of genetic resources in domestic and wild animals, and is essential in commercial embryo transfer technology providing easier and low cost embryo transport, reducing health risk and avoiding loss of animals during transport. In small ruminants, the use of embryo transfer technique (ETT) in breeding schemes is limited as compared with cattle and buffalo (Thibier *et al.* 2000). This is probably due to the excessive cost when compared to the value of animal (Cognie *et al.* 2003). The more satisfactory and commonly used technique of cryopreservation in goats (slow freezing) is unfortunately the more expensive since

it requires an expensive biological freezer, continuous power supply and is labour intensive, whereas vitrification techniques with direct transfer offers a real possibility to reduce the cost of embryo transfer (Vajta *et al.* 2000, Baril *et al.* 2001). Efficient and economical cryopreservation techniques for goat embryos would be advantageous since goats produce diversified products of commercial interest. Moreover, short gestation period (5 months), small size, easy breeding make the goat competitive as compared to cow for the application of biotechnologies. For cryopreservation of cells, cryoprotectant is required. They increase osmolarity of the solution that holds the cells. Thus intracellular cryoprotectant does not cause injury to cell membrane during freezing process but they are also toxic. Higher volume of intracellular cryoprotectants if transferred along with embryo, inhibit embryo implantation. Washing of cryopreserved embryo is therefore required prior to transfer for successful implantation. That is one of the main hurdles for application of embryo transfer technology in the field. The reasons for embryo cryopreservation depend on what the embryos are going to be used for. The vitrification by rapid cooling method decreases the cooling rate reducing the probability of crystallization. On the other

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hand, a balance between this protective layer and the cooling rate needs to be found and it is desirable that the vapour coating does not persist for long and that the volume of the vitrification solution is minimized to ensure that the cells are surrounded by liquid nitrogen increasing the rate of cooling. A high rate of cooling will also allow a reduction in the concentration of cryoprotectant and exposure time. Most efforts to improve vitrification focused on media, cryoprotectants, equilibration times, and dilution. Less attention has been paid to cooling and warming rates during freezing and thawing. Though vitrification protocol do not need sophisticated apparatus but need washing of embryos to eliminate intracellular cryoprotectant which is toxic to cell for proper implantation prior to transfer. It is therefore required to develop a freezing protocol having no or very low level of intracellular cryoprotectant which may be developed by increasing extracellular cryoprotectant after exposing the embryos in intracellular cryoprotectants so that the embryos can be transferred without washing similar to artificial insemination for practical implementation of ETT in the field conditions.

MATERIALS AND METHODS

Goat ovaries were collected from local slaughterhouse located, Bareilly at isothermal condition in normal saline solution (0.85%) fortified with antibiotics (3 µg/ml gentamicin) in a thermos flask at 37–38°C within 2 h of slaughter. Ovary was washed with normal saline and exposed to ethyl alcohol for 30 sec and again washed with saline. The semen samples from adult bucks of animal shed of the institute were collected by AV method, and transported to lab at 38°C within 10 min of collection. All the procedures were carried out in highly sterile condition under laminar air flow cabinet to avoid any bacterial or fungal contamination. All the prepared media excluding OCM were kept for at least 3–4 h in CO₂ incubator at 37°C and 5% CO₂, 90% relative humidity for quenching before use. Goat follicular fluid was collected aseptically and centrifuged, and supernatant was used as follicular fluid in –20°C. The same pool of GFF (goat follicular fluid) was used throughout the study. Following the collection of oocytes in the OCM along with follicular fluid from the goat ovary, the COCs were graded evaluated and selected (Figs 2–5). COC which has undergone 10–15 washing were cultured in 50 µl droplets (15–20 oocytes/droplet) of maturation media (TCM-199 with HEPES media, 10% FBS, 10% FF, 0.5 µg/ml FSH, 10 µg/ml LH, 0.5 µg/ml estradiol) in 35 mm sterile petridish. The droplets were covered with warm non-toxic paraffin oil and cultured at 37.5°C, in an atmosphere of 5% CO₂ and 21% oxygen under humidified air (95%) for 27 h. Fresh semen was used for *in vitro*

fertilization. Sperm capacitation was done using TALP. Semen samples were kept at 37°C and 5% CO₂ in humidified air until co-incubation with matured oocyte. The mature oocytes were transferred to 50 µl drop of fertilization TALP media and 20 µl prepared spermatozoa were added to this. The droplets were covered with warm paraffin oil and kept in CO₂ incubator at 37°C, 5% CO₂ level and 95% relative humidity. After 18 h of sperm co-incubation with oocyte, the presumptive zygotes were washed 10-times in embryo development media and cultured in the same media supplemented with 0.8% BSA. The cleavage rate was observed after 42–44 h of co-incubation.

Cryopreservation of embryos: Good healthy embryos of different stages (Figs 5–9) were separated from the *in vitro* derived embryos and transferred to OCM medium. We used different concentration of various intra- and extracellular cryopreservative for different time period to check the viability of embryo and to make a suitable protocol for cryopreservation of goat embryos.

Experiments 1–4: The embryos were exposed to 20% ethylene glycol prepared in OCM working solution for 5 min. After 5 min, the embryos were transferred to equal volume of 20% ethylene glycol and 0.5 M trehalose prepared in OCM working solution for 5 min. The embryos were transferred to 0.5 M trehalose prepared in OCM working solution for 5 min (experiment-1)/ 10 min (experiment-2)/ 15 min (experiment-3)/ 20 min (experiment-4). Within these 5 min (experiment-1)/ 10 min (experiment-2)/ 15 min (experiment-3)/ 20 min (experiment-4), the embryos were transferred into the 0.25 ml straw having 50 µl OCM in each side and 10 µl of OCM-trehalose solution having embryos separated by 5 µl air at two end of OCM-trehalose column with the help of straw filler (Fig. 1). After loading the embryos into the straw, the open end of the straw was sealed by polyvinyl alcohol powder. The straws were kept at 4°C for equilibration. They were exposed to liquid nitrogen vapour for 30 sec and plunged into liquid nitrogen and kept for 7 days. The loading, sealing and transferring into liquid nitrogen was done within last 5 min (experiment-1)/ 10 min (experiment-2)/ 15 min (experiment-3)/ 20 min (experiment-4).

Experiments 5–8: The embryos were exposed to 20% ethylene glycol prepared in OCM working solution for 5 min. After 5 min, the embryos were transferred to equal volume of 20% ethylene glycol and 0.9 M trehalose prepared in OCM working solution for 5 min. The embryos were transferred to 0.9 M trehalose prepared in OCM working solution for 5 min (experiment-5)/10 min (experiment-6)/ 15 min (experiment-7)/ 20 min (experiment-8). Within these 5 min (experiment-5)/10 min (experiment-6)/ 15 min (experiment-7)/ 20 min

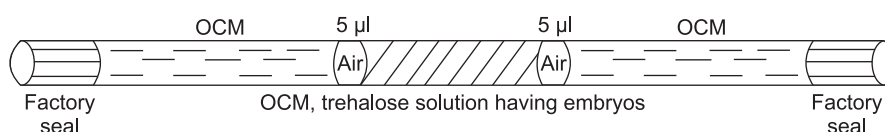


Fig. 1. Straw loading.

(experiment-8), the embryos were transferred into the 0.25 ml straw having 50 µl OCM in each side and 10 µl of OCM-trehalose solution having embryos separated by 5 µl air at two end of OCM trehalose column with the help of straw filler. After loading the embryos into the straw, the open end of the straw was sealed by polyvinyl alcohol powder. After 5 min (experiment-5)/10 min (experiment-6)/15 min (experiment-7)/20 min (experiment-8), they were exposed to liquid nitrogen vapour for 30 sec and then plunged into liquid nitrogen and kept for 7 days. After 7 days, the straws were thawed at 37°C. The embryos (4–16 cells embryos) were separated and washed in OCM. After morphological evaluation, they were exposed to 0.4% trypan blue solution to check viability. The embryos were cultured to see further development *in vitro*.

RESULTS AND DISCUSSION

The present study was carried out to develop a protocol for cryopreservation of embryos by eliminating/decreasing intracellular cryoprotectant so that embryos can be transferred without washing, similar to artificial insemination for practical implementation of the embryo transfer technology in the field conditions.

The number of live (Fig. 11) and dead embryos (Fig. 10), and percentage viability obtained for 4–16 cells embryo for different experiments in given in Table 1.

As in experiment 8 (i.e using 20% ethylene glycol and 0.9 M trehalose, for 30 min), the survivability rate was good, 32 more embryos were cryopreserved by following this protocol. Twenty of them were thawed and out of which 14 were live and were kept in mSOF for 48 h to observe their *in vitro* development. Twelve of these embryos developed to next cell stage in mSOF.

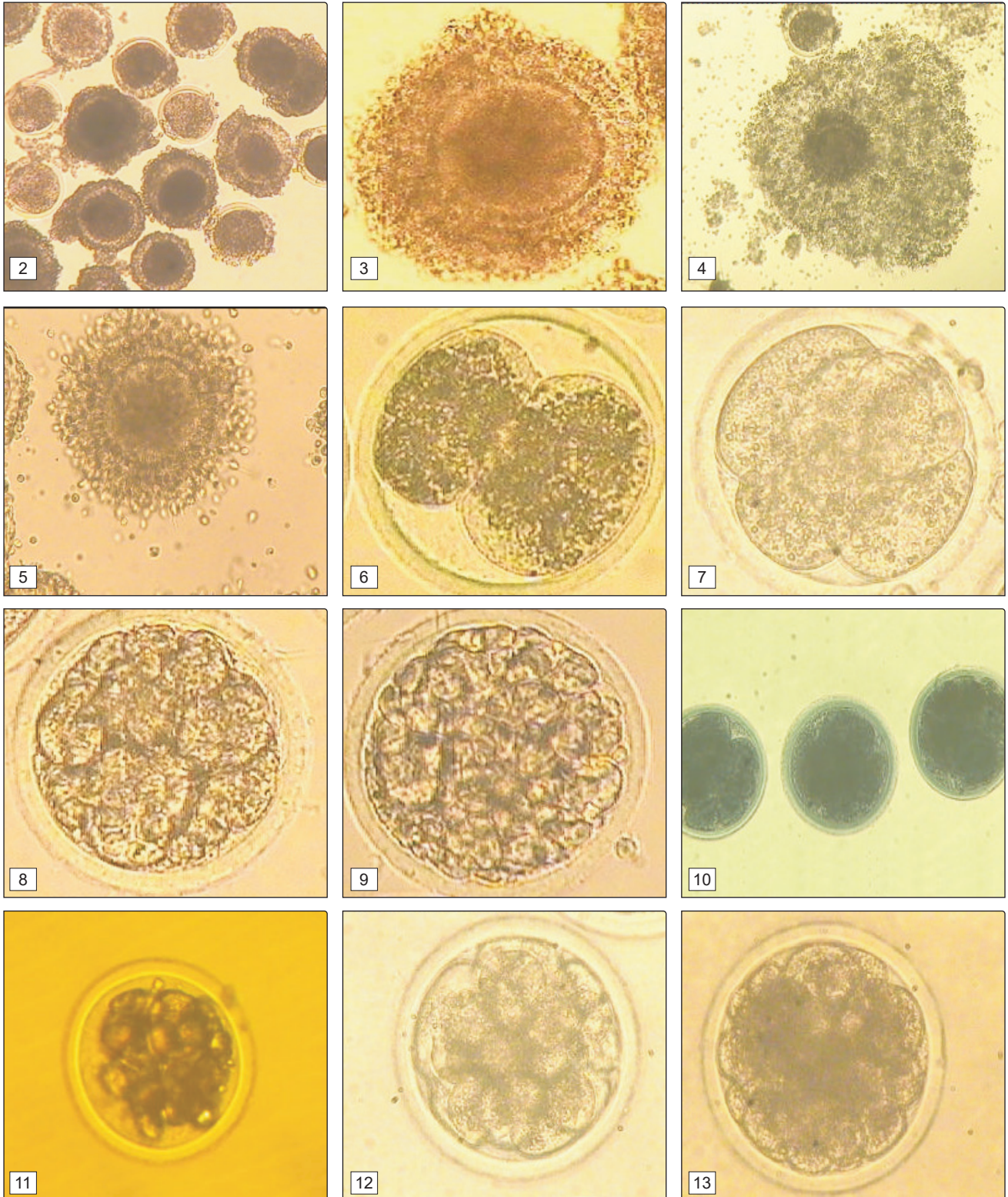
The various types of component and conditions of a cryoprotectant are most essential for embryos to survive during vitrification, because a cryoprotectant is an environment in direct contact with the embryos. The original vitrification solution used by Rall was highly toxic, and needed a 2-step equilibration procedure at different temperatures before cooling. This made the method somewhat complicated. The high concentration of cryoprotectants in a vitrification solution is a relevant factor as it increases the toxicity of the solution. The temperature

and duration of exposure also affects the cryoprotectant toxicity (Rall *et al.* 1985, Kasai *et al.* 1992). The concentration range of cryoprotectants which allows acceptable embryo viability after freezing and thawing is very narrow (Nowshari *et al.* 1995). An appropriate thawing protocol is also essential for embryos frozen by both vitrification and slow freezing (Rall and Wood 1994). The aim of our work was to develop a suitable cryopreservation protocol with low toxicity to embryos to facilitate the direct transfer of embryos without washing of cryoprotectants. Here we studied whether toxicity could be reduced by replacing some of the penetrating cryoprotectant with a disaccharide. We have also investigated the optimum equilibration time in a vitrification solution before rapid cooling. Our vitrification solution consisted of less toxic components, viz. ethylene glycol (Kasai *et al.* 1990) and the natural cryoprotectant trehalose. Trehalose seems to have an important role during freezing, by preventing membrane fusion and aggregation of intramembranous particles and also by stabilizing bilayers (Rudolph and Crowe 1985). The embryos exposed in first group (20% ethylene glycol and 0.5 M trehalose for 15–30 min) had no survivability after the freezing–thawing procedure. This might be owing to the lower concentration of trehalose. In the second group (20% ethylene glycol and 0.9 M trehalose, exposure of embryos for 15–30 min) survivability of embryos was observed. The survivability of embryo in 15, 20, 25 and 30 min exposure was 10, 25, 35.7 and 72.2%. This shows that less time is not sufficient to permit the permeation of cryoprotectants, and it is therefore possible that some ice was able to form. Yokohama *et al.* (1994) demonstrated that ethylene glycol permeates more slowly through the cellular membrane of mouse oocytes than propylene glycol, acetamide or dimethylsulphoxide. The groups exposed for 15, 20, 25 and 30 min had increasing rates of survivability and further development of embryos at later stages but also showed some degenerated embryos. This was probably caused by the toxic effects of the cryoprotectants. The group exposed for 30 min had a good rate of survivability and development of later stage embryos after 24 h of culture. This group also had a very low proportion of degenerated embryos, showing that 30 min was sufficient for the cryoprotectant to permeate and

Table 1. Cryopreservation of embryo

Experiment No	No of embryos	No of live embryos	% of live embryos	No of dead embryos	% of dead embryos	No of lost embryos
1	10	0	0 ^a	9	100 ^c	1
2	13	0	0 ^a	10	100 ^{bc}	3
3	11	0	0 ^a	9	100 ^c	2
4	12	0	0 ^a	9	100 ^{bc}	3
5	10	1	10 ^a	7	90 ^{bc}	2
6	12	3	25 ^b	7	75 ^{bc}	2
7	14	5	35.7 ^b	6	64.5 ^{ab}	3
8	18	13	72.7 ^c	3	27.5 ^a	2

^{a,b,c}Different subscripts in a column differs significantly (P<0.05).



Figs 2–13. **2.** COC after collection (4×); **3.** Good/Excellent COC (10×); **4.** Mature goat oocyte (4×); **5.** Mature goat oocyte (10×); **6.** 2 cell embryo (20×); **7.** 4 cell embryo (20×); **8.** 8–16 cell embryo (20×); **9.** Morula (20×); **10.** Dead embryo (20×); **11.** Live embryo (20×); **12.** 4 cell embryo develop into 8 cell in mSOF after freezing and thawing; **13.** 8 cell embryo develop into 16 cell in mSOF after freezing and thawing.

equilibrate effectively, but with low toxic effects. In this study, we chose to evaluate the efficacy of ethylene glycol (EG) solutions for cryopreservation of goat embryos because EG is one of the least toxic of the known

cryoprotectants. It very readily permeates cell membranes (Songsasen *et al.* 1995), which reduces the problems caused by osmotic shock and can allow direct dilution or transfer of embryos after traditional slow-cooling protocols. Mouse

embryos treated with 30% EG showed significantly higher survival rates than those exposed to equivalent concentrations of Me₂SO, propylene glycol, glycerol, 2, 3-butandiol, or 1, 3-butandiol (Valdez *et al.* 1992). These advantages compensate for the fact that to form vitrifying solutions it needs to be used at higher concentrations than those of other penetrating cryoprotectants such as propylene glycol (Boutron *et al.* 1979). Earlier EG was used at high concentrations in rapid-cooling and vitrification solutions for oocytes and embryos. The reported efficacy and toxicity varied significantly, depending on the composition of the cryoprotectant solution, number and length of equilibration steps, temperature, and species and stage of embryo development. EG is used in combination with sucrose or trehalose, but these solutions cause a slight reduction in the developmental potential of both oocytes and embryos even after only a very brief exposure. EG was reported to provide considerable protection when used as the component of vitrification solutions because it seemed the least harmful of 6 common cryoprotectants examined for mouse blastocysts (Valdez *et al.* 1992). EG has also been successfully used for vitrification of human oocytes (Chung *et al.* 2000, Kuleshova *et al.* 1999), human blastocysts (Lane *et al.* 1999), bovine blastocysts (Steponkus *et al.* 1990, Kasai *et al.* 1990), as a solution containing 40% v/v EG, 10% wt/v sucrose, and 18% wt/v Ficoll 70,000 MW. The combination proved very versatile and relatively non-toxic as it is effective for embryos of many, but not all, species. In most instances, embryos can be inserted directly into Kasai's solution, but in some instances, e.g., mouse expanded blastocyst-stage embryos, a pre-equilibration step in a low concentration of EG is required (Zhu *et al.* 1993). Pre-equilibration in a solution with a low concentration of EG in combination with vitrification in this EG-based solution was also effective for early stage human embryos (Mukaida *et al.* 1998). Other cryoprotectant combinations that have recently become more popular include the combination of EG and Me₂SO (dimethyl sulphoxide) developed by Ishimori and colleagues (Ishimori *et al.* 1993) and the combination of EG (20% v/v), Me₂SO (20% v/v), and 17 wt% sucrose (Vajta *et al.* 1997). These solutions, in combination with specific pre-equilibration steps and a high rate of cooling, have proved particularly effective for cow embryos (Ishimori *et al.* 1993, Vajta *et al.* 1997) and oocytes (Vajta *et al.* 1998). All these combinations of EG-based solutions have one feature in common, a high (30% or more) total concentration of penetrating cryoprotectant. This study demonstrated that the concentration of penetrating cryoprotectant can be reduced to as little as 20 volume % and still allow effective cryopreservation of 2-, 4-, and 8- to 16-cell goat embryos. Excellent *in vitro* development was achieved after cryopreservation with solution of 20% ethylene glycol and 0.9 M trehalose in this study. That may suggest that it is safer to use solutions for cryopreservation of embryos. Our concern stems from a previous study in which severe chromosomal anomalies and fetal malformations were induced by rapid cooling in solutions

containing equally low concentrations of cryoprotectant (11 and 22vol% Me₂SO + 0.25 M sucrose). Unlike the 20% EG solution used in this study, the solutions used in the other study (Shaw *et al.* 1991) formed ice on cooling. The cryopreservation solutions used in this study contained relatively little EG (20%) and large amounts of non-penetrating additives (trehalose). However, the high total solute concentration of these solutions did permit vitrification during cooling, with ice formation during warming, and resulted in embryos and fetuses with completely normal developmental potential. Meryman *et al.* (1966) first proposed a role in the prevention of osmotic injury during the rapid removal of extracellular water, together with the ability to coat the cells and protect the cell membrane from denaturation. This was extended to embryos (Titterington *et al.* 1995). It has been demonstrated that large molecular compounds such as dextran or Ficoll in the protectant serve as bulking agents; and the inclusion of a macromolecule in solutions facilitates vitrification by increasing the tendency of the solutions to supercool. The presence of high concentrations of endogenous macromolecules in the dehydrated cytoplasm of embryos allows the formation of intracellular glass (Rall *et al.* 1987). Increase in the concentration of high-molecular-weight solutes in a solution will raise the viscosity; it can also raise the glass transition temperature. Thus, at some moderate concentration, the solution may become too viscous to support ice crystal growth. Whereas BSA, serum, and other products of biological origin (such as egg yolk) can be valuable in reduction of cryoinjury, they are potential sources of infectious agents (viruses and fungus) and may alter the freezing characteristics of the solution. Considerable effort has therefore been spent in the development of vitrification solutions that do not contain any components of animal origin. Youngs *et al.* (2011) cryopreserved embryos of cattle, sheep, and goats using glycerol placed into a 1 M solution of sucrose for 10 min for removal of the CPA before transfer to a recipient (surrogate) female and cryopreserved in EG may be directly transferred to the uterus of a recipient. Gibbons *et al.* (2011) cryopreserved the morulae and blastula of sheep and goat using vitrification solution, two-step equilibrium thawing and observed highest pregnancy rate in goat. Fan *et al.* (2015) developed a robust open pulled straw (OPS) vitrification technique for cryopreserving hamster embryos and observed high blastocyst rates from embryos vitrified at 2-cell, 4-cell, 8-cell, or morula stage. The solution containing trehalose is viscous. Rapid mixing should be avoided because embryo damage might result. It is strongly recommended that the embryo be inserted into the straw with the pre-prepared large column of such solutions. Equilibrations of embryos are completed in 20% EG. In the final step of the protocol, carbohydrate-based solution acts as a soft capsule for additional dehydration of embryo. Such a procedure assists in preventing ice formation inside the embryo by increasing the total concentration of solute and cell contents (macromolecules) inside the cells.

The need to use carbohydrate-based solutions will arise through the understanding that traditional penetrating solutions have unavoidable high toxicity. It seems sensible to use these glass-like solidifying solutions with low concentrations of penetrating agents in embryology for a range of species. When toxicity information for a wide range of macromolecules have been studied systematically and become well known, the use of carbohydrate-based solutions as an alternative to penetrating cryoprotectant mixtures may prove to be applicable to other cell types and tissues.

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