Vitrification for embryo and oocyte freezing

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In vitro fertilization and embryo transfer (IVF-ET) techniques have improved and more pregnancies have been established associated with higher rates of multiple pregnancies. Multiple pregnancies are associated with various obstetric complications including threatened abortion, threatened premature delivery, fetal malformation, necessity of cervical ligation and caesarean section, increased uterine bleeding at birth, and low birth weight babies. To prevent multiple pregnancies, one of the best approaches is to decrease the number of embryos transferred into the uterus. Consequently, several embryos are left to be cryopreserved for future embryo transfer.

Since Whittingham [1] first succeeded in freezing and thawing mouse embryos, different cryoprotectants, and freezing and thawing techniques have been investigated to improve the method. According to Whittingham's freezing system, 2 to 3 hours in the cryoprotectant are necessary for embryos to be frozen with minimal ice formation in the blastomeres. To avoid the lethal effects of ice formation, Luyte drew attention to the feasibility of using vitrification to cryopreserve biological materials [2]. Rall and Fahy [3] applied the vitrification method to cryopreserve mouse embryos using dimethyl sulfoxide (DMSO), acetamide, propylene glycol and polyethylene glycol. Unfortunately, when embryos were exposed for a long time at room temperature, they were adversely affected by the cryoprotectant at the concentration needed to vitrify embryos. Therefore, to achieve adequate

vitrification, it was necessary a very short period of time to equilibrate embryo with the cryoprotectant. As the initial vitrification solutions were very toxic [3] to embryos, it was essential to formulate nontoxic and more efficient solutions for vitrification. Kasai developed a less toxic cryoprotectant composed of modified phosphate buffer solution (PBS), ethylene glycol, ficoll and sucrose [4]. He showed that the optimal equilibration time in the vitrification solution before rapid cooling varied with the ambient temperature when using the rate of blastocyst formation as a criterion [5]. Since then, other investigators developed more effective modern solutions that contained nonpenetrating additives [6-11], and various vitrification methods have been used for freezing human embryos [12-14]. Vitrification is a short and simple procedure that may be safer and more cost effective than slow cooling for embryo freezing. Kuleshova et al. compared the advantages and disadvantages of slow cooling and vitrification. Their results are shown in Table 1 [15].

Generally, the ultrastructure of oocytes is particularly sensitive to temperature and extracellular osmotic pressure changes during freezing and thawing. Various types of cellular damage are observed during oocyte freezing [16-18]. However, a reasonable number of deliveries have been achieved with the use of vitrified oocytes (Table 2). Although vitrification is still in its early stage of development, it may be more suitable for human oocyte cryopreservation than slow freezing due to its simplicity and

Table 1. Comparison of vitrification and slow-cooling procedures [15]

	Vitrification	Slow cooling
Control of solute penetration	yes	no
Control of dehydration rate	yes	no
Maintenance of physiological temperature during equilibration procedure	yes	no
Duration of time out of incubator	~ 10 min	~ 3 h
Prolonged temperature shock	no	yes
Capture by growing ice crystals	no	possible
Equipment and running costs	inexpensive	expensive

Table 2. Delivery using vitrification for oocyte preservation

Authors	Year	CPA and materials
Kuleshova <i>et al</i> [19]	1999	EG/ sucrose/ open pulled straw
Yoon <i>et al</i> [20]	2000	EG/ sucrose/EM grid
Yoon <i>et al</i> [21]	2003	EG/ sucrose/EM grid

CAP: cryoprotective agent, EG: ethylene glycol, EM: electron microscopy.

capacity to prevent intracellular ice formation. To avoid the formation of intracellular ice, it is necessary to use a high concentration of cryoprotectant and an extremely rapid cooling speed.

Although vitrification has many advantages and has been successfully used for oocyte cryopreservation [19-21], its clinical outcome is still limited. Vitrification results in lower pregnancy and implantation rates in comparison to conventional embryo freezing techniques due to more severe damage caused to oocytes. To improve the quality of oocytes after vitrification, several methods for oocyte cryopreservation have been investigated.

A variety of different types of cryocontainers including electron microscopy grid [22], open pulled straws [23], and cryoloop [24] were developed, thus improving the successful

vitrification rate of human oocytes and embryos.

Since cytoskeletal damage occurs during the vitrification procedure, it is necessary to stabilize the cytoskeletal system. Cytoskeletal stabilizers such as Taxol [25] or cytocharacine B [26] have been used in animal models with good results. However, in order to apply the same method to human oocytes, it is necessary to ascertain the safety of these compounds not only to the post-thaw intracellular cytoskeletal system but also to its postnatal development.

The use of high-speed cooling rate by slush liquid nitrogen (LN) may improve the results of oocyte vitrification. Slush LN offers high speed cooling rate and consequently maintains oocyte quality during the procedure [27].

Another way of improving vitrification is using a sodium-depleted cryoprotectant medium [28]. A sodium-depleted environment prevents the accumulation of excessive intracellular sodium that can induce cell death during thawing. To obtain a sodium-depleted medium it is necessary to add choline. Choline may directly stabilize cell membrane and protect it from the freezing damage.

Takahashi *et al* [29] examined the survival rate of mouse oocytes exposed to vitrification solutions containing various concentrations of ethylene glycol (EG) in relation to the changes in the intracellular calcium concentration. They found that 83% of the oocytes survived a 5

Table 3. Survival rates of mouse oocytes exposed to vitrification solutions containing various concentrations of EG [29]

Vitrification solution	Number of oocytes (n*)	Number of intact oocytes**			
		Time after exposure to EG			
		1 min	2 min	3 min	5 min
0% EG (control)	47 (3)	47 (100) ^a	47 (100) ^a	47 (100) ^a	47 (100) ^a
0% EG + FT	26 (3)	26 (100) ^a	26 (100) ^a	26 (100) ^a	26 (100) ^a
10% EG + FT	23 (3)	23 (100) ^a	23 (100) ^a	22 (96) ^a	22 (96) ^a
20% EG + FT	31 (3)	31 (100) ^a	31 (100) ^a	30 (97) ^a	30 (97) ^a
40% EG + FT	42 (3)	33 (100) ^b	33 (100) ^a	25 (83) ^b	25 (83) ^b

FT: ficoll + trehalose.

Values with different superscript letters within the same column differ significantly. $P < 0.05$.

* Number of replicates.

** percentage in parenthesis.

minutes exposure with 40% EG + ficoll + trehalose. This result implies that nonpermeable cryoprotectants such as ficoll and trehalose contribute to the survival of oocytes during their exposure to the cryoprotectant (Table 3).

Although more reliable and safe oocyte vitrification methods are still necessary to be developed, this technique seems to be effective to preserve human oocytes.

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