Vitrification: An Emerging Technique for Cryopreservation in Assisted Reproduction Programmes

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Abstract: The significance of cryopreservation in assisted reproduction programmes has increased with the introduction of newer technologies. Gametes or surplus human embryos are routinely cryopreserved by a slow freezing protocol, which is a well established procedure. However there are certain disadvantages with these freezing protocols viz. longer time needed for the procedure, intracellular ice crystal formation leading to cellular damage and need for expensive programmable freezing equipment. Vitrification is now regarded as a potential alternative to the conventional slow-freezing method. Vitrification is a process of ultra rapid cooling of a solution containing high concentration of cryoprotectant, inducing a vitrified or glass like state, thereby avoiding the ice crystal formation and cell damage. These protocols involve short equilibration times, fast cooling rates and no expensive equipment.

Vitrification is rapidly emerging as the future of cryopreservation. Till date the results obtained with vitrification, in terms of survival of gametes, fertilization, progression of embryos to blastocysts and pregnancy rates are equal or even better than the conventional slow-freezing protocols. Present review includes basic principles involved in vitrification, equipment needed, applications of vitrification and its limitations.

Key words: Vitrification, slow rate freezing, cryoprotectants and liquid nitrogen

Historically, cryopreservation dates back to 1776, when Lazzaro Spallanzani reported that stallion spermatozoa frozen in snow were able to recover their mobility after thawing. Later in 1866, an Italian physician, Mantegazza introduced the concept of human sperm bank to store semen specimens after his successful experiments with human spermatozoa cooled to -15^{o}C (as quoted by Bunge *et al.*, 1954). In the late 1940's, Chang reported the birth of a litter of rabbits from embryos, stored at 0^{0}C. The discovery of glycerol as a cryoprotectant by Polge and his colleagues in 1949 led to successful freezing of both gametes and embryos from a number of mammalian species including humans. The foundation of modern cryobiology was laid by Lovelock, Polge, Levitt, Luyet, Mazur and others during 1950-60 and the collaborative work of Whittingham, Mazur and Leibo in the 1970's, resulting in successful freezing of mouse embryos with high survival rates following controlled thawing. This paved the way for human oocyte and embryo storage in the 1980's. Protocols for the cryopreservation of spermatozoa, oocytes and embryos have been refined in the last 20 years and gradually adapted as routine procedures in ART programmes. Although the process of cryopreservation has

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evolved over the past 5 decades, it is surprising that even now the technique still remains inefficient for human oocyte cryopreservation.

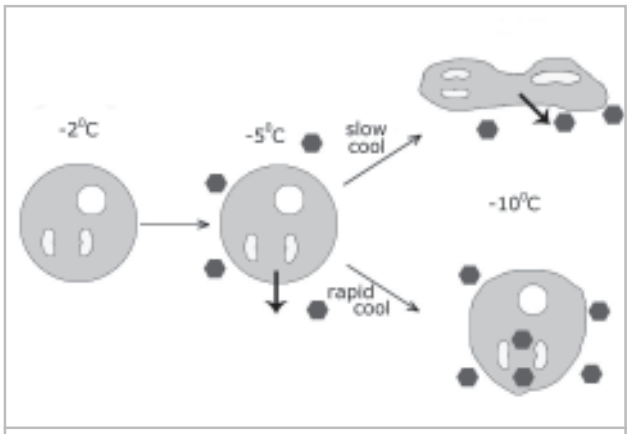
Cryopreservation involves cooling and storage of cells in liquid nitrogen at a temperature of -196° C where all metabolic processes are arrested. It reversibly arrests normal physiological processes and the dynamic cellular events involved in cell division.

There are two methods of cryopreservation viz., slow rate freezing and vitrification. The basic objective of both these methods is the same, i.e. to protect cells from cooling effects (chilling injury), intracellular ice formation, dehydration and toxic effects at both high and low temperatures.

Slow rate freezing: This method involves a brief pre-equilibration of cells in cryoprotectant solutions followed by slow, gradual, controlled cooling at rates optimized for the type of cells being cryopreserved. The whole process is carried out with the use of special programmable cell freezing equipment and requires 3-6 hours to complete.

Cryoprotectants are used to protect the cells from damage due to intracellular ice crystal formation. The temperature of the cells is lowered to a supercooled state and ice crystal growth is initiated within the extra-cellular solution by a process called seeding. As the size of the ice crystals increases, water in the solution is converted from liquid state to solid state. This increases the concentration of solute in the extracellular medium which draws water out of the cell. As a result the cell dehydrates with resultant increase in intracellular solute concentration. This increased intracellular solute concentration further lowers the freezing point of the cell to approximately -35^0 C. The cell is almost devoid of any water at this point and therefore ice crystal formation is negligible when the cell ultimately freezes at this temperature.

The rate at which water leaves the cell depends on the rate of cooling. When the cells are cooled at rapid rate, water present inside the cell will not be able to move out fast enough leading to the formation intracellular ice crystals which are lethal to the cell. If the cells are cooled too slowly, then there will be severe volume shrinkage leading to high intracellular solute concentration, which have deleterious effects on the lipid-protein complexes of cell membranes. In addition the cells that are cooled slowly are potentially affected by chilling injury. Hence the rate of cooling and cryoprotectant concentration employed in the protocol should be optimized to avoid the intracellular ice crystallization and high solute concentration, the two main events involved in cellular injury during cryopreservation.



Principle of cryopreservation. Influence of rate of cooling on intracellular ice formation

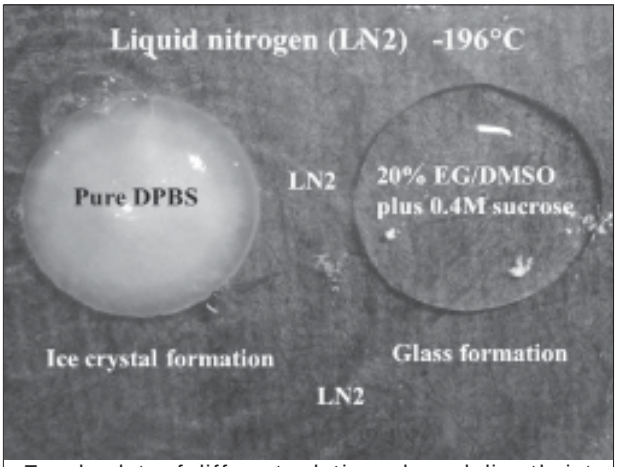
The success of slow cooling depends on achieving this optimal balance between the rate at which water can leave the cell and the rate at which it is cooled before it is converted into ice. Therefore, to achieve this balance, the time to complete slow cooling procedures for human

oocytes and embryos needs a minimum of 90 min. Besides the longer time needed, the slow cooling protocol requires expensive programmable freezing equipment. Apart from these limitations slow cooling protocols are not satisfactory for various types of cells viz, pig embryos, in vitro derived bovine embryos, human MII oocytes and blastocysts which are sensitive to chilling injury.

Vitrification: This method of cryopreservation was developed to overcome the shortcomings of slow freezing protocol. It is the solidification of a solution at low temperature, not by ice crystallization, but by extreme elevation in its viscosity using high cooling rates of 15,000 to $30,000^0$ C/min. The cooling of cells at this ultra high rate of freezing creates a glass like state without intracellular ice formation. Thus, the term vitrification, which means 'turned into glass' was first proposed by Luyet (1937). During vitrification the viscosity of the cytosol becomes greater and greater until the molecules become immobilized and it is no longer a liquid, but rather has the properties of a solid' (Fahy et al., 1986)

Principles of vitrification

Vitrification involves exposure of the cell to high concentration of cryoprotectants for a brief period at room temperature followed by rapid cooling in liquid nitrogen. The cells are initially pre-equilibrated in a cryoprotectant solution of lower strength (usually 10 %) resulting in dehydration of the cell and its permeation with cryoprotectant. This is followed by a very short incubation (<30 seconds) in higher concentration of cryoprotectant solution (40%) followed by rapid plunging into liquid nitrogen. The high osmolarity of the cryoprotectants results in complete dehydration of the cell. Since the cells are almost devoid of any water by the time they are immersed in liquid nitrogen, the remaining intracellular water, if any, does not form ice crystals. During warming the entire process of vitrification of the cell is reversed. Cells are exposed in a step wise manner to hypotonic solutions of decreasing strengths of sucrose to remove the cryoprotectant and gradually rehydrate.



Two droplets of different solutions plunged directly into liquid nitrogen: left droplet is pure Dulbecco's phosphate-buffered saline (DPBS) with ice crystallization, in contrast to the right droplet containing an equimolar combination of 20% ethylene glycol and dimethyl sulphoxide with 0.4 mol/l sucrose in DPBS without ice crystallization (glassy, vitrified state) (adapted from Liebermann et al., 2003)

Factors that influence successful vitrification

The success of vitrification depends on the rate of cooling and concentration of cryoprotectant used. The rate of cooling in turn is influenced by the size and volume of the sample vitrification solution respectively (sample carrier systems). Concentration of the cryoprotectants is critical to the success of vitrification as high concentration of cryoprotectant can be toxic to cells. The key elements which influence vitrification are:

- Cryoprotectants
- Carrier systems
- Techniques for better heat transfer

Cryoprotectants

Survival of cells during cryopreservation depends upon the type and concentration of the

cryoprotectants used. There are two important issues related to the use of cryoprotectants (CPA). (1) The addition of a CPA into the cells before vitrification, and (2) The removal of the CPA from the cells after warming. Cells undergo transient shrinkage when a CPA is added and then return to near-normal volume as the CPA permeates. They also undergo transient volume expansion during the removal of the CPA; the degree of volume expansion depends on the rate of removal of cryoprotectant and on the inherent permeability of the cell to water and CPA.

Cryoprotectants facilitate the freezing process by lowering the freezing point, and therefore allow the embryos and freezing medium to be supercooled to a specific sub-zero temperature before seeding (slow rate freezing). Protecting the cell membrane from freeze-related injury. Decreasing the deleterious effects of high salt concentrations as cells dehydrate during the freezing process

Vitrification involves exposure of the cell to high concentration of cryoprotectants for a brief period at room temperature followed by rapid cooling in LN2.

Types of cryoprotectants: They are the permeating types such as eEthylene glycol, Glycerol, Dimethyl sulfoxide (DMSO), Propylene glycol and Acetamide **or** non-permeating such as high molecular weight compounds like Saccharides (Sucrose, Trehalose, Glucose, and Galactose) and macromolecules (Ficoll, Polyvinyl pyrrolidone).

Permeating agents are essential components of the vitrification medium. These compounds are hydrophilic non-electrolytes with strong dehydrating properties. The selection of cryoprotectant is based on their toxicity and permeating properties. Ethylene glycol and glycerol are less toxic than DMSO and propylene glycol, and acetamide are more toxic. Rapidly permeating agents are preferred as exposure time prior to vitrification may be shortened. Further these agents diffuse out of cells more rapidly during warming and prevent osmotic swelling. Ethylene glycol is reported to be the most permeating for mouse morulae, and glycerol is the least permeating for one cell mouse embryos (Kasai, 1996). Emiliani et al (2000) suggested that ethylene glycol is an ideal candidate for human embryo vitrification, as it has a low toxic effect on mouse oocytes, embryos and blastocysts without effecting morphology and developmental functions apart from having rapid diffusion properties through zona and cell membranes.

Among the non-permeating agents, mono or disaccharides act by promoting the dehydration of the cells in the vitrification medium and thus decreasing the exposure time of the cells to the toxic effects of cryoprotectants. Addition of saccharides to vitrification medium reduces the amount of permeating cryoprotectant required to achieve the successful cryopreservation. Incorporation of saccharides during warming plays a significant role in the survival of the cells. During warming, water permeates more rapidly into the cells than the cryoprotective additive which diffuses out because of osmotic differences. As a result the excess water inflow causes the cells to expand leading to osmotic injury. However this osmotic injury can be minimized by inclusion of the saccharides which functions as an osmotic buffer and reduces the speed and magnitude of swelling

of the cells, though it cannot totally prevent the cell from swelling (Liebermann & Tucker, 2002).

The polymers like, Polyethylene glycol, PVP and Ficoll modify the vitrification properties of the vitrification solutions. These agents increase the viscosity of the vitrification medium and reduce the concentration of cryoprotectant needed (Liebermann et al., 2003). Macromolecules are less toxic to the cells compared to permeating agents.

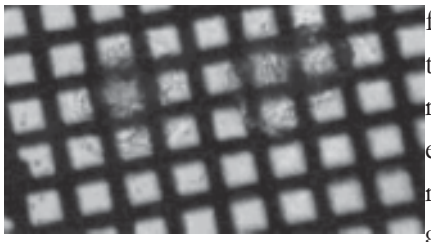
Carrier systems:

Initially, plastic insemination straws and cryovials were used for storage of cells. However these are not ideal tools for vitirification. They have a thick wall and require a large amount of solution for safe loading. Moreover theoretically achievable cooling and warming rates are limited. Hence, it is necessary to consider the rate of cooling and heat transfer while designing the tools for vitrification. The rate of cooling can be increased by using the smallest possible volume of cryoprotectant around the embryo and to create a direct contact (without any thermo insulating layer) between the solution and the liquid nitrogen (ultra rapid vitrification). The use of small volume may have additional advantage as it prevents heterogeneous ice formation (Rall, 1987).

Further when the carrier containing the vitrification solution and cells is immersed in liquid nitrogen, the liquid nitrogen boils and forms a vapour coat around the carrier until the carrier reaches a temperature below the boiling point of liquid nitrogen. The surrounding vapour coat blocks the temperature transfer and this results in reduction of the cooling rates. To make the cell have a direct contact with liquid nitrogen instead of vapour coat, sample size should be minimized so that the duration needed to reach the required temperature where the liquid nitrogen stops boiling is reached faster. So a variety of carriers for vitrification have been developed to meet this requirement.

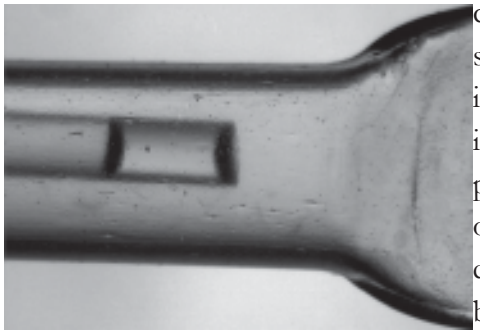
Direct dropping into liquid nitrogen: This is the first developed method to carryout the vitrification (Landa and Tepla 1990; Riha et al., 1994; Yang and Leibo, 1999). However this technique requires large amount of cryoprotectant to form a drop (5 µl) and a thin vapor coat is formed while introducing the drop into liquid nitrogen. This vapor coat functions as insulation layer and decreasing cooling rates.

Electron microscopic grids: The concept of small sample-direct contact approach was



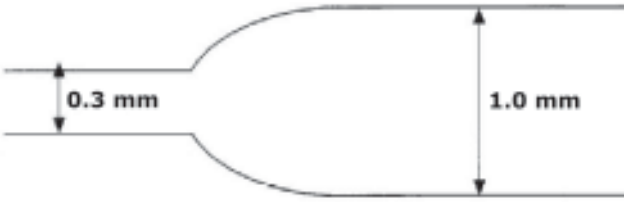
feasible with the development of copper electron microscopic grids. Here the sample is directly loaded into grids and placed in the cryovials before plunging into liquid nitrogen. The size of the drop is very small and the thermo conductive metal grid increases the cooling and warming rates (Martino et al., 1996).

Open-pulled straw (OPS): The concept of OPS is based on capillary activity principle. French mini-straws are used to prepare the OPS. Straws are heated for a brief period over a hot plate, and pulled manually until the inner diameter and the wall thickness of the central part decreases from 1.7 mm to approximately 0.8 mm, and from 0.15 mm to approximately 0.07 mm, then a cut is made at the narrowest point with a razor blade. Loading of cells is carried out by capillary effect; by simply touching a 1- to 2-µl droplet containing the oocytes or embryos with the narrow end of the straw. This end is then immediately submerged into liquid nitrogen. Despite some turbulence, the liquid



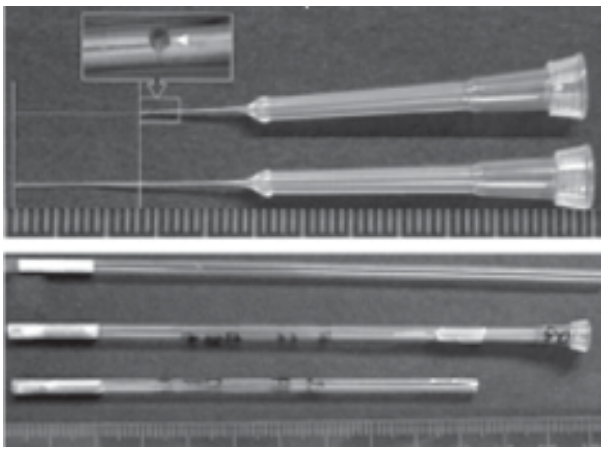
column solidifies immediately, instead of pouring out or getting dispersed before solidification, which are common phenomena if original-sized open straws are used. Warming is done by placing the end of the straw directly into the holding medium. The vitrified medium became liquid as soon as the holding medium entered the straw. Immediately, by means of the sedimentation, the oocytes or embryos float out of the straw into the holding medium. Since the volume of the solution occupied in the column is decreased from 5 µl to 1 µl, there will be a 10 fold increase in the achievable cooling rates and a 30% decrease in the cryoprotectant concentration. However floating of the straws is a problem. This can be avoided by inserting a standard plastic plug into the wide end (Vajta et al., 1998a) .

Glass micropipettes: Glass micropipettes are prepared from capillary glass pipette (outer/inner diameter: 1.0/0.8 mm). These pipettes are pulled with a pipette puller until the outer diameter of the central part is decreased from 1.0 mm to approximately 0.3 mm. The glass micropipette capillaries are cooled in air and then broken at the narrowest point. The weights of open pulled straw and glass micropipette capillary straws are approximately 0.070 vs 0.0984 g, respectively, and the volume loaded into a narrow column was 2.68 vs 0.14 mm^3 per straw, respectively. Glass micropipettes have several advantages compared to open pulled straws. Since glass has a higher density than plastic, glass micropipettes do not float. Open pulled straws can be pulled up to 0.8mm outer diameter where as with glass micropipettes it is possible to achieve 0.3 mm outer diameter, which in turn makes it possible to reach high cooling and warming rates compared to opened pulled straws. Although the narrower glass micropipettes capillary tend to cool faster than the opened pulled straw, the loading of oocytes



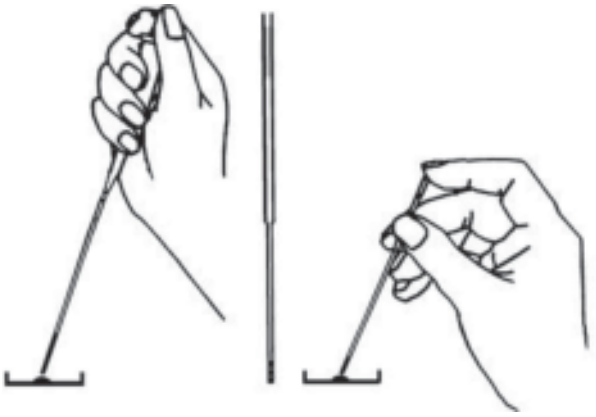
or embryos and vitrification solution into the glass micropipettes is more difficult due to increased capillary action. Care must be taken during loading to restrict oocytes or embryos and vitrification solution to the narrow portion of glass micropipettes (Kong et al., 2000).

Gel-loading tips: Tominaga and Hamada (2001) reported on the gel loading tips as a container for vitrification. This tip is modified by cutting off 10 to 15 mm of the tip and connecting it to a 0.1- 2.0 µl micropipette. The tip part of the gel loading tip holding the cells is directly plunged into liquid nitrogen. The tip is released from the micro pipette and stored in LN2 by means of 0.5 ml straws. Authors claim that the volume of the cryoprotectant can be best handled with these tips connected to micro pipette compared to OPS and glass micropipettes, apart from



achieving comparable high cooling rates.

Closed-pulled straw (CPS): The CPS are made like the OPS. They differ from OPS in terms of

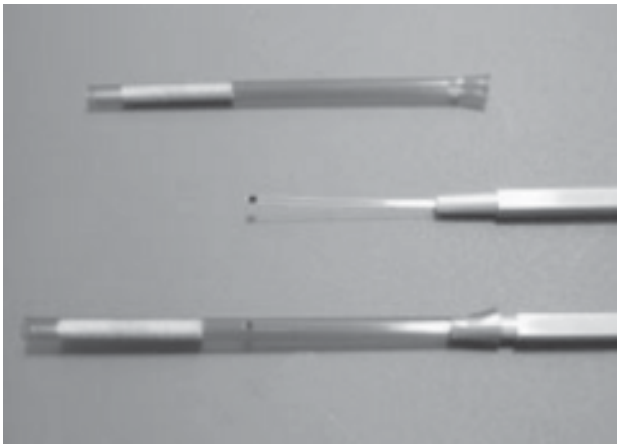


loading the cells. The tip of the pulled straw is loaded with 2 mm of vitrification medium, 2 mm of air, 2 mm of vitrification medium containing cells, 2 mm of air, and 2 mm of vitrification medium using a syringe. The straws are then directly plunged into liquid nitrogen.Through this closed loading system of CPS, the cells do not have direct contact with liquid nitrogen thereby limiting the morphological injury, which is otherwise possible with OPS and electron microscopic grids (Chen *et al*., 2001).

Cryotip: Cryotip consists of a plastic straw with a narrow end (250μm inner diameter, 20-μm wall

thickness and 3-cm length) connected to a wider end (2000-μm inner diameter and 150-μm wall thickness, 4.5-cm length) protected with a movable metal sleeve. Cells are loaded into narrow part of the straw (holding approximately 1μl) with a connecting syringe. The straw is then heat sealed at both the ends, a protective sleeve is pulled over the narrow part and the straw is plunged into liquid nitrogen. During warming, contents in the straw are released by making a cut at the ends and expelling them with the syringe connected to the wider end of the straw. Here, cells are not injured because they do not come into direct contact with the liquid nitrogen. However cooling rates achieved are lower when compared to other methods (Kuwayama et al., 2005).

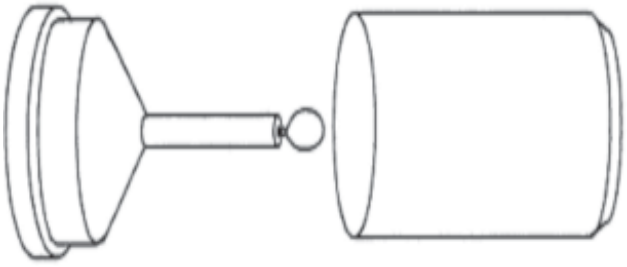
Cryotop: Cryotop consists of a 0.4 mm wide × 20 mm long × 0.1 mm thick polypropylene strips



attached to a plastic handle and provided with a cover straw (Kuwayama et al., 2005). Individual oocytes or embryos are collected in an extremely small volume (<0.1μl) of vitrification medium, placed on the strip, and submerged into liquid nitrogen. Then, the plastic cover straw is placed over the strip to provide protection during storage. During warming, the protective cover straw is removed in liquid nitrogen and the end of the polypropylene strip is immersed directly into the warming medium. Here the cells have direct contact with liquid nitrogen (Kuwayama et al., 2005).

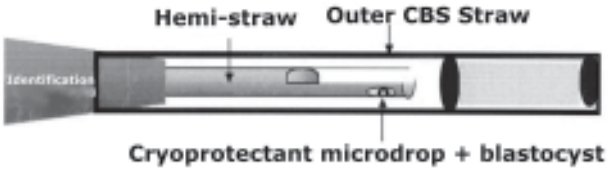
Cryoloop: Cryoloop approach is based on small volume-direct contact principle. Cryoloop consists of a nylon loop (20mm wide; 0.5– 0.7 mm in diameter) mounted on a stainless steel pipe inserted into the lid of a cryovial. A metal insert on the lid enables the use of a handle with a small magnet for manipulation of the loop if required. After cells are treated in vitrification medium, the cryoloop is immersed into cryoprotectant solution to create a thin film on

the loop. The cells are then transferred from vitrification medium onto the film of cryoprotectant on the cryoloop. The cryoloop containing the cells is then plunged into the cryovial, which is previously submerged and filled with liquid nitrogen. The vials are stored



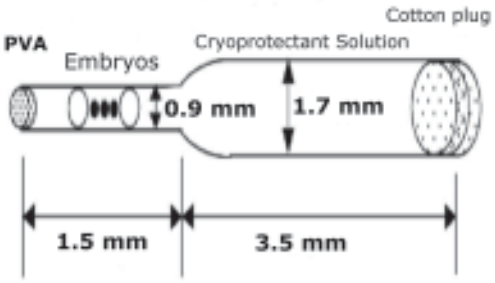
in standard canes. During warming, the vial is opened and the loop containing cells is removed from the liquid nitrogen and immersed directly into a well containing warming medium. The cells immediately drop from the loop into the warming solution. As the cells have direct contact with liquid nitrogen and also the final volume of the cryoprotectant surrounding the cell (as thin film of cryoprotectant is formed around the cells) is extremely small, it is possible to achieve a very high cooling rate.

Hemi straw system: In this system the end of a 0.25 ml straw is cut with a sharp scalpel



(Vanderzwalmen et al. 2000). After pre-equilibration in vitrification medium the cells are loaded in as small a volume as possible (< 1.0 µl) on to the inner surface of the open edge of the 0.25 ml straw. The straw is then plunged vertically into liquid nitrogen, and placed into a 0.5 ml cryostraw held under liquid nitrogen. The cryostraw is then plugged with a plastic colour-coded plug before cryostorage.

Sealed opened-pulled straws (SOPS): Sealed opened pulled straw method is a modification of opened pulled straw method. SOPS are prepared by pulling the central part of heat soften

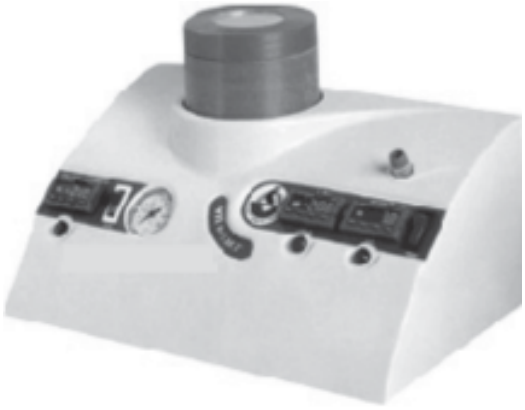


standard straws (0.25 ml) until its inner diameter and wall thickness decrease from 1.7 mm to 0.9 mm, and from 0.15 mm to 0.07 mm. The straws are cut at the narrow part and the half with cotton plug is used for vitrification. During vitrification the cells are aspirated into a column located between two columns of cryoprotectant solution separated by air bubbles. The other end of the straw is sealed with polyvinyl-alcohol sealing powder before plunging it into liquid nitrogen (Lopez-Bejar and Lopez-Gatius, 2002)

Techniques for better heat transfer

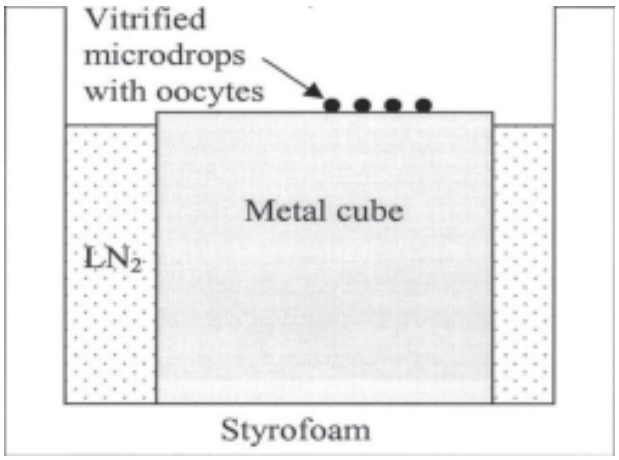
As the results of vitrification are directly related to the rate of heat transfer and cooling rates, a few techniques have been developed to meet this requirement.

Vitmaster: The Vit-Master is a new device which reduces the temperature of liquid nitrogen



temperature to as low as –205 to –210°C by applying negative pressure (Arav et al., 2002). The super-cooled liquid nitrogen facilitates better heat transmission between liquid nitrogen and the vitrification medium. Before starting the vitrification, cryovials and liquid nitrogen are placed into the Vit-Master apparatus. Negative pressure (–0.9 bar) is applied to decrease the liquid nitrogen temperature, Cryoloops containing cells are plunged directly into the super-cooled liquid nitrogen, and then screwed into cryovials using a magnetic holding rod.

Solid surface vitrification: Metal surfaces cooled with liquid nitrogen provide a more efficient



method of heat transfer and further increase the cooling rates. Based on this concept 'solid surface vitrification (SSV) device' was developed (Dinnyes et al., 2000). Cells are loaded into small drops which are placed on the surface of a steel cube that is covered with aluminum foil and cooled to around -150^0 C to -180^0 C by partial immersion into liquid nitrogen. The sizes of the micro drops vary between 1 and 2 µl, and are instantaneously vitrified. The droplets are moved with a nitrogen-cooled forceps into 1-ml cryovials for long-term storage. This method has the advantage of container less vitrification in microdrops and increased heat exchange.

Applications of vitrification in reproductive medicine:

Vitrification of spermatozoa: The preservation of male fertility after radiotherapy and/or chemotherapy became a reality with the introduction of cryopreservation of sperm. However indications of sperm cryopreservation have been broadened to other categories of patients (viz, severe oligozoospermia, cryptozoospermia) with the advent of intracytoplasmic sperm injection (ICSI) which requires only few viable spermatozoa.

Commonly used methods for cryopreservation of spermatozoa involve exposure of straws to liquid nitrogen vapour followed by direct plunging into liquid nitrogen. This results in differences in cooling rates among different straws causing a significant fall in survival and motility after thawing. This procedure has been gradually replaced by automated slow freezing process. However, even with the automated cryobiological freezers the motility of spermatozoa after freezing and thawing is only 50-60%. This decrease in motility could be due to unusual response of spermatozoa to different cooling rates. There is little difference in survival rates with cooling rates of 1^0 C or 100^0 C /min (Henry et al., 1993) unlike other cells which have optimum cooling rates.

This kind of insensitivity to cooling rates maybe due to its unique structure.

1) A large surface area to volume ration.
2) Small cytoplasmic volume with high protein and low water content.
3) High cellular permeability to water and cryoprotectants.
4) Presence of a heterogeneous mixture of sperm that vary in shape, maturation status and functional potential.

Vitrification medium contains high percentage

of permeable cryoprotectants (30-50% compared to 5-7% with slow-freezing) which have lethal osmotic effects on spermatozoa (Holt, 1997; Katkov et al., 1998; Mazur et al., 2000). Concentrations of cryoprotectants used in conventional freezing protocols resulted in severe toxic effects (Nawroth et al., 2002). Isachenko et al (2003a) demonstrated that vitrification of spermatozoa without cryoprotectant resulted in higher motility after warming in comparison to conventional freezing with cryoprotectant. They also suggested that employing very rapid cooling and warming rates along with a very small specimen size by using different carrier systems (OPS, grids and cryoloops) aids the vitrification of spermatozoa; even at such a rapid cooling rate, the integrity of sperm DNA was intact (Evenson et al., 1991).

Survival of spermatozoa without permeable cryoprotectant could be due to:

1) Presence of large amounts of osmotically inactive water bound to several macromolecular structures such as DNA, histones, hyaluronidase (Gao et al., 1997).

2) Presence of high molecular weight components in spermatozoa which affect the viscosity and glass transition temperature of the intracellular cytosol (Isachenko et al., 2004). Thus, vitrification of human spermatozoa in the absence of cryoprotectants is feasible.

Oocytes: Cryopreservation of oocytes offers:

- Ease of availability of oocyte for research
- Allows sufficient time for detailed donor screening in oocyte donation programme.
- Preservation of the fertility potential of women undergoing chemotherapy.
- Storage for patients with OHSS.

Chen (1986) reported the first pregnancy from cryopreserved oocytes but subsequently there were very few pregnancies in the next 9 years. This slow progression in development of oocyte cryopreservation methods may be the result of a high incidence of chromosomal anomalies seen in freeze thawed oocytes. Reports also show that cooling oocytes to low temperatures significantly disrupts the cytoskeletal elements, cortical granules and plasma membrane (Pickering et al., 1990; Vincent and Johnson, 1992; Aman and Parks, 1994). Studies by Zenzes et al., 2001; Songsasen et al., 2002; Liu et al., 2003, show that chilling mature oocytes from various species viz. human, rhesus macaque, porcine and bovine results in rapid and often irreversible disruption of the metaphase II spindle fibers.

The high ratio of surface area to volume significantly influences the oocyte freezing process. The oocyte is 180 times larger than the spermatozoa and requires longer time to reach the osmotic balance with cryoprotectant solution (Al Hasani and Diedrich, 1995) and hence is more prone to temperature and cryoprotectant induced damage. So far conventional cryopreservation protocols resulted in survival rates of 50-70 % (Borini et al., 2004, 2006), progression rate of 5.6% blastocysts (Cobo et al., 2001) and only 9.7% pregnancy rate per transfer (Borini et al., 2006).

In contrast to slow freezing protocols, vitrification methods involve usage of high concentration of cryoprotectants and ultra low temperatures and these conditions pose a threat to survival of the oocytes. However the toxicity of cryoprotectants can be reduced by pre-equilibrating the oocytes in lower cryoprotectant concentration prior to a very short (<30s) incubation in the final vitrification solution (Chung et al., 2000; Wu et al., 2001). Studies by Kuleshova et al., 1999; Chen et al., 2000a, report 70% survival rates after oocyte vitrification. A post-warming survival rate of 91% (Kuwayama

and Kato 2000) and a pregnancy rate of 21.4 % have been reported after vitrification (Yoon et al., 2003). This indicates that survival and pregnancy rates with vitrification were better than with slow rate freezing. The effect of vitrification on genetic apparatus of oocytes is a concern as meiotic spindles of mouse oocytes disappeared upon exposure to a vitrification solution of 5.5 M ethylene glycol and 1.0 M sucrose (Chen et al., 2000b). However subsequent reports showed that oocytes vitrified in small amounts of vitrification medium were able to retain the integrity of meiotic spindles (Chen et al., 2001). Results with vitrification of oocytes are encouraging compared to slow rate freezing protocols. Moreover the time required to vitrify oocytes was 11.5 minutes and 15 minutes for warming as compared with 108 minutes for freezing and 30 minutes for thawing respectively using the slow freezing method. Hence vitrification may be a useful and better alternate to slow freezing for cryopreservation of oocytes. Modifications in vitrification protocols may improve the results..

In our experience, the implantation and clinical pregnancy rate was significantly higher in the vitrification as compared with slow freezing group.

Embryos: The extensive use of gonadotrophins in ART programmes frequently results in the production of multiple oocytes and subsequently embryos. Transfer of more than two embryos result in multiple pregnancies causing pre- and post-natal complications.

Successful cryopreservation of mouse embryos was reported in 1972 by Whittingham *et al.* In 1983, Trounson and Mohr reported a pregnancy following freezing of a 8-cell human embryo using slow -freezing protocols.. Rall and Fahy in 1985 developed a vitrification protocol using mouse embryos. High survival rates have not been consistently achieved either by slow cooling or vitrification. This could be due to sensitivity of embryos to chilling, lower permeability of cell membrane and toxicity of cryoprotectant.

Recently, ultra rapid vitrification, in which the cooling and warming rate has markedly increased by minimizing the volume of the cryoprotectant solution and the container. This method employs loading of embryos in carriers like the electron microscopic grids, open pulled straws, hemi straws, cryoloops, cryotops and micro drops. Applying this method, critical temperatures at which cells are damaged by chilling injury are quickly bypassed and intracellular ice formation is avoided by forming a glassy state due to extreme cooling rates. Cryoprotectant toxicity is minimized bcause of the small volume used.

In 1998, Mukaida et al reported high survival rate of 8-cell human embryos using 40% ethylene glycol. El-Danasouri and Selman (2001) reported successful pregnancies and deliveries after vitrification of day 3 human embryos using OPS. Studies from the author's facility (Rama Raju et al., 2005) showed significantly high post-thaw survival rates of human embryos in the vitrification group (95.3%, $P < 0.05$) than those frozen using the slow rate protocols (60.0%). The implantation rate of 14.9% ($P< 0.05$) in the vitrification group was significantly higher than the rate of 4.2% in the slow freezing, and the number of clinical pregnancies was higher in the vitrification (35.0%) compared to the slow-freezing group (17.4%). Our protocol has minor

modifications compared to protocol described by El-Danasouri and Selman (2001). The exposure time to 10% ethylene glycol was decreased from 10 min to 5 min the rehydration steps increased to 4, instead of 2,, i.e. 1 M, 0.5 M, 0.25 M, 0.125 M sucrose solution, with an exposure time of 2.5 min in each step. The shorter exposure time during vitrification (Vander Elst et al., 1995 and Walker et al., 2004) and increasing the number of warming steps may have had a role in the better survival of embryos.

Pronuclear stage oocytes (zygotes): Cryopreservation of zygotes by slow freezing is a routine practice in countries where freezing of cleavage stage human embryos is not permitted by law or due to ethical reasons. Zygotes are more sensitive to osmotic shock than cleavage stage embryos because of lower permeability to cryoprotectants because of which they undergo volume change during equilibration with cryoprotectants. The rate of volume change and the temperature at which it occurs plays a critical role in the survival of zygotes.

Vitrification of zygotes results in 80 % survival, cleavage rate of 77-85% on day 2 and blastocyst formation of 31% (Park et al., 2000; Jelinkova et al., 2002; Selman and El-Danasouri, 2002). The ability of zygotes to cope up the vitrification and warming conditions maybe zona hardening due to cortical reaction which gives the ooplasm membrane the stability to withstand low temperature and osmotic shock (Liebermann et al., 2003).

Blastocysts: Culture of embryos up to the blastocyst stage and their transfer has become a promising option to improve the pregnancy rates of ART (Gardner et al., 1998). This in turn has led to the need for improvement of blastocyst cryopreservation protocols. The advantages of blastocyst freezing includes; 1) Reduction of the number of embryos to be frozen 2) Culture to blastocyst before freezing increases final cell number 3) Selection of embryos is possible.

Successful cryopreservation using slow freezing protocols has been reported (Menezo et al., 2000; Ludwig, 1999) although there has been no consistency in the results. Vitrification of blastocysts with straws has been attempted by Vanderzwalmen et al (1999) and Yokota et al (2001). However success rate in terms of survival was quite low. The probable reasons could be that human blastocysts are less permeable to cryoprotectant and they shrink relatively slowly in the cryoprotectant suggesting injury from intracellular ice formation. Ultra rapid vitrification of embryos using OPS (Vajta et al., 1998a, cryoloops (Lane et al., 1999) and gel loading tips (Tominaga et al., 2001) has been reported. This approach is beneficial for less permeable cells as very rapid cooling reduces the intracellular ice formation. Using this approach, Mukaida et al (2001) vitrified 60 blastocysts from 21 patients and achieved a pregnancy rate of 32%. These results indicate that cryoloop vitrification is an effective and practical method for cryopreservation of human blastocysts.

Ovarian tissue: Cryopreservation of ovarian tissue is an alternative to cryopreservation of oocytes or embryos for patients with a risk of premature ovarian failure due to chemotherapy, radiotherapy, genetic causes or other diseases. Cryopreservation of ovarian tissue has several advantages over preservation of oocytes and embryos mainly because the primordial germ cell population is abundant at birth in contrast to a woman at age of 40; Ovarian tissue can be collected by laparoscopy at any time irrespective of stage of menstrual cycle. It is

less cryosensitive than mature oocytes. Ideal for model investigations as ovarian tissue has no species specific morphological properties.

However cryopreservation of ovarian tissue is problematic compared to isolated cells as it contains many different cell types such as stromal cells, follicles containing oocytes, granulosa cells and theca cells. In addition the intactness of vascular structures and nerves are important for function after transplantation. While selecting the cryoprotectant, its ability to penetrate through the stroma and granulosa cells to oocytes, its toxicity, and its specificity for each cell and tissue type have to be considered.

Newton et al (1996) reported the cryopreservation of ovarian tissue with slow freezing techniques. Frozen–thawed follicles from these tissues survived for up to 2 weeks in organ culture as cortical slices and grew as well as the non-frozen follicles (Hovatta et al., 1997). These follicles showed functionality after sub-cutaneous transplantation to immunodeficient mice or under the kidney capsule (Van den Broecke et al., 2001). The first human live birth after transplantation of

Variables	Vitrification	Slow freezing
Time consumed	Less (10 mins)	More (3 hrs)
Instruments	Inexpensive	Expensive
Mechanical damage	Less or none	More
Intracellular ice formation	Less	More
Chemical damage	More	Less
Concentration of cryoprotectant	High	Low
Accessibility and regulation		
Can be observed	Yes	No
Can be analysed	Yes	No
Interaction with the oocyte or embryo	Yes	No
Control of solute penetration	Yes	No
Control of dehydration rate	Yes	No
Maintenance of physiological temperature during equilibration procedure	Yes	No
Duration out of incubator	~10 mins	~3 hrs
Prolonged temperature shock	No	Yes
Interference with oocyte or embryo	Low	High
Fracture of zona pellucida	No	Possible
Capture by growing ice crystals	No	Possible

Advantages and disadvantages of vitrification vs slow freezing

frozen–thawed tissue has been reported by Donnez et al (2004) using standard slow freezing protocols.

Successful vitrification of ovarian tissue in mice has been reported (Salehnia, 2002; Tokieda et al., 2002; Migishima et al., 2003) but data on human ovarian tissue is limited. Isachenko et al., (2003b) and Rahimi et al., (2004) suggest parameters to be considered while attempting the vitrification of ovarian tissue. However more studies are needed to standardize the vitrification of human ovarian tissue.

Limitations of vitrification

Vitrification is still an experimental procedure. There are two major concerns about vitrification viz., toxicity of high concentration of cryoprotectants used and microbial contamination of liquid nitrogen.

Recently Menezo (2004) voiced concern about use of high concentrations of ethylene glycol used during vitrification. However till date no developmental abnormalities in animals or human have been reported following its use. Nevertheless, it is a fact that some viruses may contaminate and survive in liquid nitrogen (Tedder et al., 1995). There are reports of possible transmission of pathogens to bovine embryos vitrified and stored in liquid nitrogen (Bielanski et al., 2003).

Preventing contamination during storage

Proper sealing of straws and cryovials containing embryos is as an effective measure against contamination during storage. An alternative preventive step described by Vajta et al. (1998b) is filtration of liquid nitrogen and the application of accessory protective storage containers. A straw in straw method for successful storage of vitrified blastocysts reported by Lieberman et al. (2002) and Vanderzwalmen et al. (2003) reduces risk of viral contamination from liquid nitrogen. Cross-contamination may be prevented by storing embryos from patients with known infections in separate liquid nitrogen tanks. Preventive measure against contamination in liquid nitrogen should be taken although there have been no reports of contamination.

Conclusions

Vitrification is a simple, inexpensive and rapid procedure compared to other cryopreservation methods. Success with vitrification of various stages of cells in reproductive medicine is encouraging the acceptance of this cryopreservation method in ART programmes. Increasing number of publications as well as the commercial vitrification products that are being introduced reflects the acceptance of this method. Development of universal protocols for oocytes, zygotes, embryos and blastocysts may not be feasible as response of each cell stage depends on their structure, metabolism and sensitivity to cryoinjuries. Standardization of definite protocols for each cell stage is preferred.

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