PRESERVATION OF GAMETES

DR POONAM KUMARI
DEPT OF ZOOLOGY
M.SC SEMESTER III CC 02

Cryopreservation—the ability to freeze and thaw (process of erosion that happens in cold areas where ice forms) with retention of viability—provides flexibility in human infertility therapy when gametes or embryos are handled *in vitro* because frozen tissue can be stored indefinitely in liquid nitrogen at -196°C. The freezing of human sperm is an established procedure that has resulted in the birth of thousands of progeny, as many as 30,000 per year. Not only can partner or donor sperm be frozen for therapeutic insemination (TI) at a subsequent date, but sperm banking provides the assisted reproductive technologies (ART) couple with a backup option if a sample cannot be collected on demand or if sample quality is poor on the day eggs are available. In the case of donor insemination, samples are quarantined for 6 months before use, thereby minimizing the risk of infectious disease transmission. In other words, the donor is screened for sexually transmitted diseases at the time of collection, and the frozen sample is released for use 6 months later only after the donor passes rescreening. Pregnancies have been established with sperm stored at low temperatures for more than 10 years; however, it is generally recognized that fecundity using cryopreserved sperm is lower than that obtained with nonfrozen sperm with the possible exception of cryopreserved sperm delivered by intracytoplasmic sperm injection (ICSI).

The most important hurdle in cryopreservation is avoiding the phase transition between water and ice inside the cell. Because water is everywhere in a cell and is important for the function of macromolecules and larger structures such as lipid membranes, the formation of large ice crystals upon cooling destroys cellular components and ruptures intact membranes. Interestingly, pure water will supercool substantially and form ice only at approximately –39°C , which is much lower than the temperature at which ice is thermodynamically stable (<0°C). Impurities, such as dust, act as ice nucleators, initiating large crystal formation at temperatures well above –39°C. However, allowing ice crystals to form at

temperatures more than a few degrees below zero during cooling has been shown to damage embryos and oocytes. To initiate crystal formation in a solution at higher temperatures, the solution must be seeded with ice either by the addition of an ice crystal or by touching it with something colder, such as a forceps dipped in liquid nitrogen, as is done in the laboratory. As ice crystals grow, the volume of the unfrozen solution decreases, thus increasing the concentration of solutes (e.g., salt), which not only helps to reduce ice formation inside the cell but also severely dehydrates cells and can cause cell damage and death.

Cryoprotectants are defined functionally as any compound that increases cell survivability when used in a cryopreservation method. There are many different types of cryoprotectants, including alcohols, sugars, oils, and starches, and each type acts through different mechanisms. However, a good cryoprotectant is one that can preserve cell structures and is not toxic. James Lovelock first described the mechanism of action of cryoprotectants in his experiments showing that erythrocytes (red blood cells) freeze at lower temperatures when combined with glycerol. He found that the increase in salt concentration resulting from ice crystal formation causes hemolysis. His work highlights the delicate balance between the many factors that must be considered when developing a successful cryopreservation method.

Currently, there are three widely used permeating cryoprotectants in fertility preservation: dimethyl sulfoxide (DMSO), ethylene glycol (EG), and propylene glycol (PG). These cryoprotectants have similar properties: solubility in water at low temperatures, cell permeability, and relatively low toxicity. However, each of these cryoprotectants also has different degrees of membrane permeability, as has been shown with mammalian oocytes. Mouse oocytes seem very hearty and are capable of being cryopreserved using several different cryoprotectants; however, evidence suggests that EG is less toxic to mouse oocytes that have resumed meiosis and reached metaphase-II (MII). In contrast, rhesus monkey oocytes are much more sensitive to cryoprotectants and have been shown to be less permeable to glycerol than to PG, EG, and DMSO, and at room temperature, the oocytes are more permeable to PG than to the other cryoprotectants. Interestingly, it was found that PG causes potentially lethal effects when used in human oocyte cryopreservation protocols designed for DMSO. By incubating oocytes in PG at a

higher incubation temperature for a shorter time, it may be possible to prevent oocyte lysis.

Conventional cryopreservation is the process of slow-rate freezing in which a relatively low concentration of cryoprotectant is used, showing little toxicity to cells or tissue. As cryoprotectant is added to cells, it results in initial cellular dehydration followed by a return to isotonic volume with the permeation of cryoprotectant and water. Generally, cells are cooled slowly using a controlledrate freezing machine, which allows samples to be cooled at various rates; ovarian tissue is generally cryopreserved at 2°C/min prior to ice seeding and 0.3°C/min after crystallization to ensure the tissue is dehydrated before intracellular ice formation occurs. Optimal rates to minimize intracellular ice formation vary among cell and tissue types; for example, stem cells survive better at a freezing rate of 1°C/min and red blood cells at a rate of 1,000°C/min.

As mentioned above, extracellular ice nucleation is triggered manually and must be performed above the temperature of intracellular ice formation. The temperature at which optimal nucleation is performed is determined by the cryoprotectant used as well as the characteristics of the cell. It has been shown that the optimal seeding temperature for human oocytes is different from that for primate and mouse oocytes and varies depending on the meiotic stage of the oocyte.

Slow cryopreservation has been used for a number of years; however, in some instances, it can be inconsistent and require expensive equipment. More recently, the process of vitrification has shown to be more successful for some cell and tissue types. Vitrification is a process that uses very high rates of cooling, so fast that water is solidified without crystallization, “like glass.” Some investigations of vitrification have shown that extremely high concentrations of cryoprotectants do not crystallize when cooled, even if it is done slowly. This approach is attractive from a technical standpoint; unfortunately, cyroprotectant solutions are toxic to cells at very high concentrations. Solute toxicity is a major drawback of using vitrification for preservation, even with high cooling rates. To reduce toxicity, concentrations of cryoprotectants can be lowered as long as cooling is fast enough to preclude ice formation. For example, the cooling rate for embryos exposed to 8.5 M ethylene glycol must be at least 100,000°C/min for vitrification to occur. To

achieve an extremely rapid rate of cooling, a small volume of solution must be used. Recently, specialized storage devices designed to achieve rapid rates of cooling have been designed for the vitrification of eggs and embryos. They hold a very small volume of solution, usually less than 1 μl. These devices have various configurations; the ideal device is covered by a thin wall of plastic and can be submerged directly into liquid nitrogen, thus maximizing the cooling rate. Vitrification is more difficult for sizeable tissue samples that require larger amounts of cryosolution, simply due to the inability to be cooled so quickly.

Although many cells and tissues can be successfully cryopreserved using slow-freeze methods or vitrification methods without intracellular ice formation and can be stored in liquid nitrogen indefinitely, there is still a risk of ice formation during the thawing process if it is conducted improperly. If samples are thawed slowly, ice crystals can form and/or grow causing more damage; however, if samples are thawed rapidly enough, there is little time for ice nucleation and growth to occur. After thawing, there is further risk of damage during the course of removing cryoprotectants. If cells are immediately put into a significantly lower concentration of cryoprotectant, water will rapidly move into the cell and the cells can swell and burst. Therefore, it is usually advised that a series of decreasing concentrations of cryoprotectant is used to slowly remove the cryoprotectants and gently rehydrate cells. As an alternative, it can also be very effective to use a non-penetrating cryoprotectant such as sucrose to reduce osmotic shock during the step-down process.