Special Article

Cryopreservation of Human Embryos

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Principles of Cryopreservation of Cells

Principles of cryopreservation are similar for living cells. The procedure of cryopreservation includes initial exposure to and equilibration with cryoprotectants, cooling to sub-zero temperatures, storage, thawing, and finally, dilution and removal of the cryoprotectants, with return to a physiologic environment that will allow further development[1,2]. Cells must maintain structural integrity throughout the cryopreservation procedure. Major factors known to affect survival of cryopreserved cells include the species, the developmental stage, the cryoprotectants, and the method of cryopreservation[3].

For the slow cooling method, probably the single most important principle of cryopreservation is that it is necessary to reduce damage caused by intracellular ice formation. The most common method is to remove most of the water from cells before they are cooled. If dehydration is inadequate, large, intracellular crystals of ice may form, which damage cells severely[2,3].

The rapid cooling method normally refers to the techniques of vitrification and ultrarapid freezing. The rapid cooling techniques are still being developed and refined, and may ultimately replace the conventional high-cost and time-consuming slow cooling methods.

Methods for Cryopreservation of Human Embryos

Slow Cooling

The original human embryo freezing techniques involved slow cooling in cryoprotectant solutions to low sub-zero temperatures to avoid the formation of intracellular ice[4]. The cryoprotectants used were dimethyl sulphoxide (DMSO) and glycerol, and the methods were based on those used to cryopreserve embryos of laboratory and domestic animals. Pregnancies and births were reported when early cleavage stage embryos (4-cell to 16-cell) were cooled slowly in 1.5 M DMSO to -40°C[5] or to -80° C[6], and when blastocyst stage embryos were frozen in 8% glycerol to-36°C[7]. The cryoprotectant 1,2 propanediol

(PROH) was also reported to be very successful for freezing pronucleate and early cleavage stage embryos (2-cell to 4-cell) when they were slow cooled in 1.5 M PROH and 0.1 M sucrose to -30° C[8]. While it is difficult to determine which method produces the best result, the majority of IVF clinics presently freeze pronucleate and early cleavage stage embryos by slow cooling in PROH or DMSO[9].

The method for cryopreservation of pronucleate and early cleavage stage embryos in PROH involves exposure of the embryos, at room temperature, to medium containing 1.5 M PROH for 10-15 minutes, and then loading the embryos into freezing straws in medium containing 1.5 M PROH and 0.1 M sucrose[10]. The straws are sealed and cooled at -2°C/minute to -7°C. The straws are seeded at -7°C, slow cooled at -0.3° C/minute to -30°C and then cooled rapidly at -50.0°C/minute to -190°C, before plunging into liquid nitrogen. Embryos are thawed rapidly (30.0°C/minute) by removing the straws from liquid nitrogen and keeping them at room temperature for 40 seconds. The straws are then placed in a water bath at 30° C for 1 minute. The cryoprotectants are removed by stepwise exposure for 5 minutes at room temperature to medium containing 0.2 M sucrose and 1 M PROH, 0.2 sucrose and 0.5 M PROH, 0.2 M sucrose, and finally medium supplemented with 20% human serum. Embryos are then cultured in medium supplemented with 15% human serum. Early cleavage stage embryos are cultured for 2-4 hours prior to replacement, and pronucleate stage embryos are cultured overnight and replaced approximately 24 hours post-thaw.

DMSO may be used to freeze preimplantation embryos of all developmental stages[11]. The embryos are then cooled to -7°C at -2°C/minute, seeded and cooled slowly (-0.3°C/minute) to either temperatures around -30 to -40°C[5] before rapid cooling and storage in liquid nitrogen, or temperatures of -60 to -80° C[6] before storage in liquid nitrogen. Embryos slow cooled to higher sub-zero temperatures are thawed rapidly in a warm water bath (30-37°C), and those slow cooled to lower temperatures are thawed slowly from -80°C to around 0°C at 5-15°C/minute. DMSO is then removed by gradual dilution.

Glycerol is used for later stage embryos, preferably after reaching the blastocyst stage. Embryos can be frozen using the method described by Cohen et al[7]. This involves a stepwise exposure, at room temperature, of the blastocysts to gradually increasing concentrations of glycerol. The blastocysts are then loaded into freezing straws in medium containing 8-10% glycerol. The straws are cooled at -1°C/minute to -7°C, seeded and then cooled at a rate of -0.3°C /minute to -36°C, before being plunged directly into liquid nitrogen. The straws containing blastocysts are thawed by removing them from liquid nitrogen and placing them in a water

bath at 30°C for 1 minute. The cryoprotectant is removed by stepwise exposure of the blastocysts to medium containing gradually decreasing concentrations of glycerol.

The results of human embryo cryopreservation by slow freezing techniques, from 25 member institutes of the Society of Assisted Reproductive Technology in USA, showed the mean number of pronuclear oocytes, early cleavage-stage and blastocyst-stage embryos transferred per pregnancy was 11.5, 16.0 and 46.0, respectively (pregnancy rate per transfer : 17.4, 12.5 and 4.3%, respectively)[12]. These results indicate that pronuclear-stage oocytes have a higher survival rate after freezing than cleavage-stage embryos, a conclusion which has general support among IVF embryologists[13,14]. Concerning the cryoprotectants, it is very difficult to conclude that there is any difference in the success rate of cryopreservation by slow cooling in PROH or DMSO. Comparable results (12.3-14.5% and 16-17% pregnancy rate per transfer)[15,16] were obtained with PROH and DMSO for the cryopreservation of early cleavage-stage embryos, while that of pronuclear oocytes, PROH may produce better results[8]. In addition, combination of sucrose to PROH significantly increased survival of embryos after thawing to 61% compared with 46% without sucrose[17].

Vitrification

A relatively recent approach to achieve rapid freezing without the use of freezing machines is called vitrification. Vitrification is defined as the physical process by which a highly concentrated solution of cryoprotectants solidifies during cooling without the formation of ice crystals. The solid retains the normal molecular and ionic distribution of the liquid state and is called a glass and can be considered to be an extremely viscous supercooled liquid[18]. Vitrification has certain advantages over freezing because it avoids the damage caused by intracellular ice formation and the osmotic effects caused by extracellular ice formation[19]. The theory behind vitrification as a method for cryopreservation has been reviewed thoroughly by Fahy et al[20]. Basically, the vitrification solution needs to consist of one or more cryoprotectants in excess of 40% (v/v)[9]. The original vitrification solution which consisted of 20.5% DMSO, 15.5% acetamide, 10% PROH and 6% ethylene glycol allowed a successful cryopreservation of mouse 8-cell embryos[18]. These solutions are toxic to cells at ambient temperature, so embryos are usually placed in the final concentrated solutions at low temperatures (0-4°C). Revision of the initial composition of vitrification solutions, i.e. combinations of glycerol (6.5 M) and polyethylene glycol (6%) or glycerol (25%) and PROH (25%), have reduced their toxicity and have made the method a little easier to use. Very recently, mouse embryos have been successfully vitrified in a solution composed of 40% ethylene

glycol, 30% ficoll and 0.5 M sucrose[21].

Most of the studies have been done in animal embryos, especially in mice, with encouraging results but there has not been any reports to date on the success of human embryo vitrification. The only report of vitrification of human embryos has been by Quinn and Kerin[22]. They vitrified 22 embryos and of 11 embryos warmed,only one survived and was transferred, but no pregnancy occurred.

Ultrarapid Freezing

In contrast to vitrification, this procedure involves crystallization of extracellular and, maybe, intracellular water. The freezing method is simple and quick, involves no expensive freezing machines. A prerequisite for successful ultrarapid freezing is the presence of a permeating cryoprotectant such as DMSO and a nonpermeating compound usually sucrose[23]. A simple ultrarapid freezing technique developed by Trounson et al[24-26] requires a 3 minute equilibration of embryos in high concentrations of DMSO (3.0 to 4.5 M) and sucrose (0.25 M) before plunging into liquid nitrogen. Embryos are thawed rapidly in a warm water bath (37° C) and the continued development of frozen-thawed embryos in vitro and in vivo is not significantly different to non-frozen embryos[27]. The outcome of ultrarapid freezing is mostly influenced by the cryoprotectant(s) and the time and the temperature of exposure. One-cell mouse embryos, for example, seemed to survive and develop well after being frozen-thawed either in 4.5 M DMSO for 3 minutes at 22°C or in 4.5 M PROH for 5 minutes at 4°C[28]. Concerning chromosomal abnormalities in mice associated with ultrarapid freezing using DMSO, Shaw et al[29] found that 4.5 M DMSO was safe and efficient, while lower concentrations had this detrimental effect.

In human embryos, the results of ultrarapid freezing from the Monash IVF programme, using 3.0 M DMSO and 0.25 M sucrose, have been disappointing; no live birth was obtained in spite of high survival and developmental rates after thawing (7-12% pregnancy rates)[9,30]. However, these success rates have been reproducible and reported from other groups[31,32]. Very recently, ultrarapid freezing using 3.5 M DMSO and 0.25 M sucrose has also been reported to produce comparable results to the conventional controlled rate technique (slow cooling in 1.5 M PROH)[33]. Concerning the cell stage of the human embryos being rapidly frozen, Diotallevi et al[34] reported higher survival rates of 2- 4-cell stage than 6-8 cells stage (82.3% vs 41.8%). Interestingly enough, the first live birth, using an ultrarapid two-step embryo freezing method, has just been reported[35]. The protocol utilized a permeation 1st step of low concentration (1.5 M DMSO) cryoprotectant for 5 minutes followed by a dehydration 2nd step of high concentration (3.5 M DMSO) cryoprotectant for 2.5 minutes, and then

plunged directly into liquid nitrogen. Following storage in liquid nitrogen, embryos were thawed rapidly (6 seconds at 37° C) and removed from cryoprotectant in a gradual stepwise fashion at room temperature and allowed to culture for 24 hours prior to transfer.

Conclusion

Cryopreservation has been widely incorporated into clinical IVF and presently based on slow cooling methods using DMSO and PROH. optimum strategy at the present time is to freeze pronuclear oocytes in PROH, as this achieves pregnancy rate of around 20% of patients transferred embryos. Slow cooling of early cleavage stage embryos in DMSO results in pregnancy rates of 10-15% of patients transferred embryos. For the human blastocyst, slow cooling in glycerol and rapid thawing is the only method reported with success. The rates of survival from freezing and thawing blastocysts are not sufficiently high, however, to justify the losses associated with prolonged in vitro incubation.

New methods of rapid freezing have been developed using mouse embryos. These methods include vitrification and ultrarapid freezing. It is too early to assess their value in human IVF but they are replacing slow cooling methods for cryopreserving animal embryos due to their simplicity and good results.

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