Chapter 22

Cryopreservation of Early Cell Stage Human Embryos

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Investigations of the cryopreservation of mammalian embryos began as early as the 1950s. Smith successfully cryopreserved rabbit ova fertilized in vivo and frozen at the unicellular stage.[11] Since that time, more advanced techniques have arisen from this basic research. The first successful reports of mammalian embryo cryopreservation were reported in 1972.[18,19] Application of these techniques is essential to every successful in vitro fertilization program. The most obvious reason for freezing human embryos is the freedom to inseminate all mature oocytes available from an ovum aspiration procedure so that the patient has the optimal number of embryos available for transfer. However, if all of the oocytes fertilize, any embryo remaining in culture after the transfer must then either be donated or discarded. Freezing protocols now enable the patient to have the optimal number of embryos transferred and the remainder frozen, to be transferred during a subsequent cycle should a pregnancy not occur the first time. Before cryopreservation, a limited number of oocytes were inseminated after a retrieval for fear of creating excess embryos, which would then be discarded. This unquestionably reduced the patient's chance of achieving a pregnancy because the embryologist cannot determine which mature oocyte may or may not have fertilizing capacity.

Cryopreservation gives the physician several options. The replacement of three embryos offers the optimum chance of conception without multiple gestation. Some centers believe that the better embryos, as determined by gross appearance, should not be transferred on the stimulated cycle but should be cryopreserved and replaced in a subsequent natural cycle.[6,9] Better synchronization between embryo and endometrium can also be achieved by utilizing cryopreservation.[1] Indeed, many centers report a significantly higher pregnancy rate in menopausal recipients of donated ova who are on an artificial estrogen-progesterone regimen than the pregnancy rate for the stimulated cycles. In our own center the pregnancy rate for stimulation cycles is 14% per induction, whereas it is 23% in those patients with ovarian failure whose embryos are derived from donated ova. Some recent data suggests that, if the endometrial thickness is insufficient, as evidenced by sonography, the embryos should be cryopreserved and the transfer deferred to a natural cycle.[7]

Using the liquid nitrogen storage-tank capacity, it is feasible for patients who become pregnant on their first cycle to continue to have their excess frozen embryos stored for future transfers. From a practical standpoint, this is the ideal situation, both financially and emotionally, reducing the added cost and stress associated with each oocyte retrieval.

PRINCIPLE OF OSMOTIC EQUILIBRATION

The initial step in cryopreserving an embryo is to remove the embryo from the bicarbonate-buffered growth media at the appropriate cell stage and to transfer it to the same phosphate-buffering system as the cryoprotectant solution. The embryo equilibrates with the new buffer for 5 to 10 minutes and is then transferred to a cryoprotectant solution of high osmolarity. The embryo must now adapt to the highly concentrated environment by diffusion through its permeable cell membranes. The intracellular water begins to leave the blastomeres at a faster rate than the extracellular cryoprotectant can enter. As a result, the embryo initially shrinks rapidly as the water escapes and then swells back to its original size as the cryoprotectant and water reenter the blastomeres. Because this process is temperature-dependent, it must be performed at room temperature (between 20 °C and 25 °C). The entire process should be completed within 4 minutes.

THREE COMMONLY USED FREEZING TECHNIQUES

There are various methods of embryo cryopreservation presently in use by in vitro fertilization (IVF) programs worldwide. One method is termed the *ultra-rapid freezing* technique and utilizes dimethyl sulfoxide (DMSO) as the cryoprotectant.[14] The embryos are exposed to 3M DMSO and drawn up into 0.25 mL plastic freezing pailettes, heat sealed, and (after 2 to 4 minutes) plunged

directly into liquid nitrogen.[15] The accompanying thawing procedure involves gentle expulsion of the straw into a phosphate-buffered solution containing 20% fetal calf serum and 0.25 M sucrose for 10 minutes at room temperature. The embryos are then placed in culture medium and incubated for 2 to 4 hours before transfer into the recipient uterus.

Another method described in the literature utilizes 1,2-propanediol (PROH) as the cryoprotectant.[12] The embryos are placed in 1.5 M PROH in a phosphate buffered saline with 20% human-cord serum for 30 minutes at room temperature. They are then placed in plastic straws with or without 0.1 M sucrose. The embryos are slowly cooled (2 °C/minute) in a rate controlled freezer down to a temperature of −7 °C, at which time seeding is induced. Cooling continues (0.3 °C/minute) until −30 °C is reached, after which the embryos are then rapidly frozen until the end point of −190 °C is reached.[12]

The straws are then transferred to liquid nitrogen for long-term storage. Thawing requires the straw to be held at room temperature for 40 seconds and then placed in a 30 °C waterbath until thawing is complete. The embryos are examined and the cryoprotectant removed by gradually decreasing the concentration of PROH by successive 5-minute baths. Viable embryos are then cultured for 24 hours before transfer into the recipient uterus.

Cryopreservation of prezygotes is another method that is relatively new to human-embryo freezing. This method also utilizes 1.5 M PROH as the cryoprotectant.[1] Prezygotes to be frozen are left in growth media at room temperature for 10 minutes and are then exposed to 1.5 M PROH and 1.5 M PROH in 0.1 M sucrose in a stepwise manner. The prezygotes are placed in glass ampules and heat sealed. The vials are cooled in a preprogrammed rate-controlled biological freezer and stored in liquid nitrogen. Upon thawing, the prezygotes are kept in culture for 12 hours until cleavage has occurred. They are then transferred back to the recipient uterus.

ONE-STEP METHOD FOR EMBRYO FREEZING AND THAWING

The procedure we have chosen to cryopreserve excess embryos was developed by Leibo and associates.[8] This method, the one-step method, was first developed for the cryopreservation of bovine embryos and has since been adapted for human application. The acquired name describes the ease of application for field use when transferring bovine embryos into recipient cattle. We find this to be a highly successful, as well as a time and cost efficient, method of freezing and thawing human embryos. Depending on the number of embryos to be frozen, the entire procedure takes approximately 2 hours. It is not reliant on the costly liquid nitrogen vapor-cooled biological freezer, but rather uses the simple rate-controlled alcohol-bath freezer. The simplicity of the procedure lies in the thawing technique, which takes just a matter of minutes to complete and needs

minimal instrumentation. The survival of each embryo can be determined immediately upon thawing, and appropriate decisions can be made regarding how many additional embryos may need to be thawed, if any. Below is a condensed version of the one-step freezing protocol, which was developed by Leibo and adapted for human needs by Rall and Meyer along with Baker, who prepared the protocol for our IVF laboratory (Figure 22-1).

All the necessary reagants are prepared in advance and stored at 4 °C until the day of embryo freezing. The phosphate-buffered nutrient solution, known as PB1,[17] is phosphate-buffered saline (Gibco #310-4280), which contains glucose, sodium pyruvate, and phenol red. To this solution, we add 3 mg/ml bovine serum albumin (BSA) (Sigma AB022) as our source of protein supplementation, dissolving it without agitation to avoid protein denaturation. Penicillin and streptomycin are also added to prevent bacterial contamination. The solution is then filter sterilized and stored in a sterile bottle. Because the embryos are cultured in bicarbonate buffered Ham's F-10 medium with 5 mg/ml BSA, the PB1 media is used as a holding medium to allow the embryos to come to equilibrium before transfer into the cryoprotectant.

The cryoprotectant solution we use is 1.5 M 1,2-propanediol in a modified PBS. Dulbecco's PBS (Gibco #310-4280) contains D-glucose, sodium pyruvate, and phenol red. To this we add propanediol (Sigma #P1009) and swirl the flask slightly in a 37 °C waterbath to help it dissolve into solution. Next, 3 mg/ml BSA (Sigma A-8022) is added for protein supplementation and penicillin and streptomycin are added to prevent contamination. The pH is adjusted with 1N NaOH to 7.3 to 7.5, and the solution is filter sterilized and placed in a sterile bottle. This is the solution that will enter the individual embryo blastomeres and preserve them during the decrease in temperature that occurs during freezing.

The diluent we use is 1.08 M sucrose in PB1 (see above). Sucrose (Mallinckrodt #8360) is added to Dulbecco's PBS (Gibco #310-4280) and allowed to thoroughly dissolve. Again, 3 mg/ml BSA (see above) and penicillin and streptomycin are added. The pH is adjusted and the solution is filter sterilized and stored in a sterile bottle. This solution will be used to immediately dehydrate the embryos upon thawing. Sucrose is too large a molecule to pass through the cell membranes but the cryoprotectant and water will diffuse out of the blastomeres while in the highly concentrated sucrose diluent.

Each of the three solutions described above is taken from the refrigerator and allowed to warm to room temperature to avoid shocking the viable embryo during incubation. The solutions are then filtered through a 0.2 μm acrodisc filter into 35 mm petri dishes. Each embryo to be frozen has its own dish of PG and PB1. The dishes are labeled and marked with a grid on the bottom for easy location of the embryo during the procedure.

While the dishes are equilibrating to room temperature, the freezing straws can be prepared. We use the 1/4 cc straws to store the embryos and 1/2 cc straws as handles. For identification, we find that colored electrical tape and alcohol-

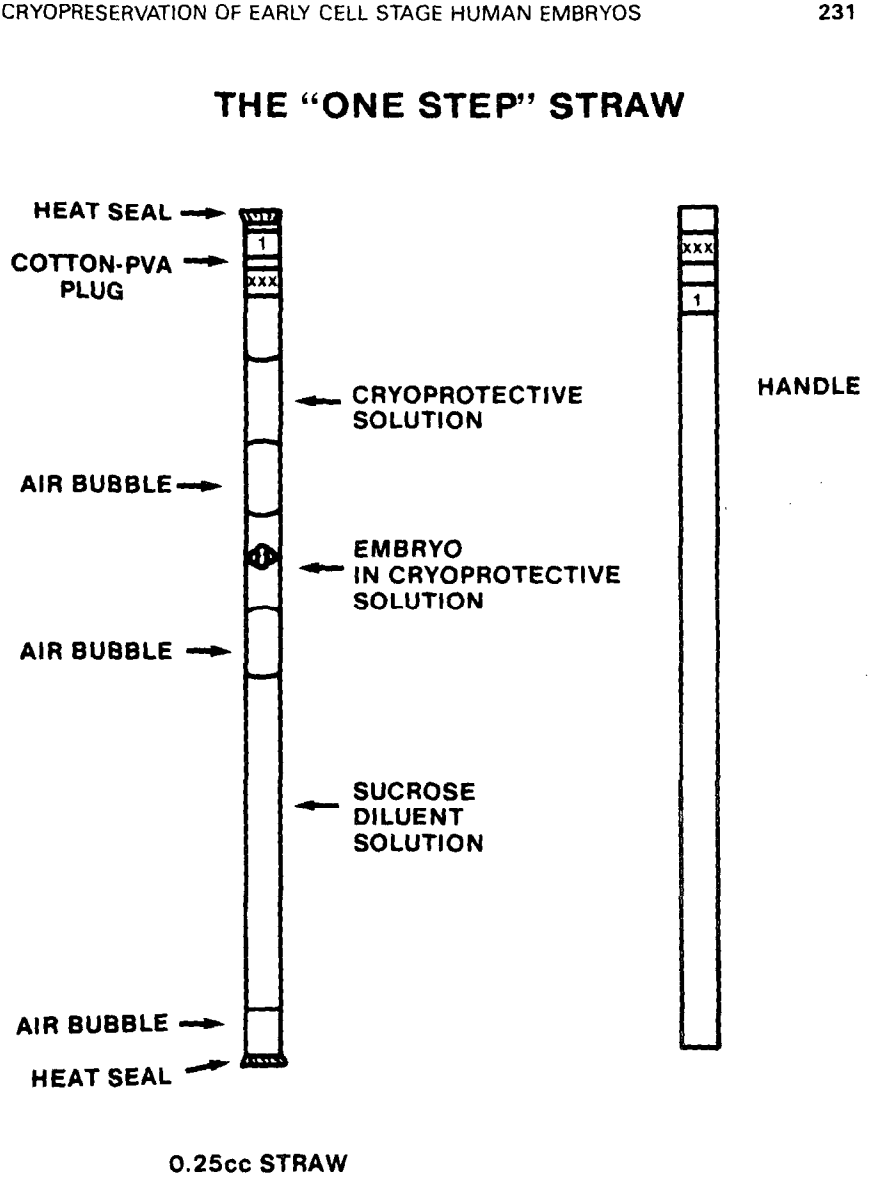

Figure 22-1 Above is a sketch of the one-step method freezing straw and handle. Each air pocket acts as a separator between the solutions and cushions the embryo during expulsion. Clear identification of the straw promotes quick and easy location upon thawing.

insoluble marker are durable in both the alcohol bath and liquid nitrogen. Each patient is identified with a different color of tape and number. This is the same color scheme we use for the storage of their embryos. Once the straws are labeled, they can be filled with a 1 cm column of PG or cryoprotectant and 1 cm of air. This is accomplished by drawing up the PG into the straws with a monoject TB syringe, which fits snugly on the labeled end of the straw. Now the straws are ready to be loaded with one embryo per straw.

Each embryo is now placed in the PB1 dish that was prepared earlier. The embryo will remain in this solution for 10 minutes. Particular care must be given to keep all working environments at room temperature. This procedure is temperature-dependent, and temperature is critical to successful freezing; thus it is important to turn down or off the microscope light source when the embryo is not being observed. When 10 minutes have passed, the embryo can be placed can be placed in the cryoprotectant solution, PG. The dish is now swirled gently to dilute out the PB1 carried over with the embryo in the pipette.

A timer is set for 20 minutes, the maximum time to complete the next few steps. The shrinking and swelling motion of the embryo as it osmotically adjusts to the highly concentrated cryoprotectant is observed. This process should be completed within 4 minutes. Now the embryo should be evaluated under the microscope to verify that it has returned to its original size and shape, and then the embryo can be drawn up into the freezing straw with a TB syringe, along with 1 cm of PG. An additional 1 cm of air is aspirated below the embryo column, and the sucrose level is raised to up to 0.5 cm from the plugged end of the straw, which is now placed under the microscope once again to ascertain the position of the embryo within the column of PG. It is best to place the embryos toward the middle of the PG column, away from the miniscus on either end to prevent the embryo from possibly dehydrating from the adjoining air space. Air is now drawn into the straw allowing the polyvinyl alcohol (PVA) powder to become wet with the initial column of PG. At this point, the syringe is no longer be able to create a suction. The straw is then heat sealed on each end and attached to the corresponding handle. Each straw is placed in the alcohol bath, which was preprogrammed to start at $-6°C$. A Dewar flask containing liquid nitrogen is kept close at hand to seed the embryos. A metal spatula is now dipped into the liquid nitrogen and the straw raised out of the ethanol; the sucrose column is touched directly below the column containing the embryo. The straw is sufficiently submerged into the ethanol so that the embryo column is now covered, and the PG column is touched directly above the embryo column with the spatula. Ice crystals should immediately form where the two columns have been touched. The straw in the alcohol bath is positioned so that only the identification tape on the handle is not submerged. The embryos remain this way for 15 minutes, at which time seeding should be complete.

Programmed freezing can now be initiated according to the method recom-

mended by Leibo et al. The starting temperature is −6 °C, and that temperature is held for 15 minutes for seeding to occur. The ramp rate for reducing the temperature is −0.4 °C/minutes until the end point of −35 °C is reached. This temperature is held for an additional 15 minutes before the straws are removed. The straws are quickly transferred individually to a Dewar flask full of liquid nitrogen and carefully loaded into prelabeled, cooled goblets, which are attached to metal canes. The entire cane is then rapidly transferred to the liquid nitrogen storage tank where the embryo will remain until thawing is required.

As stated earlier, the thawing procedure is the simplest step to perform. The minimal equipment needed includes a 37 °C waterbath, a timer, and a laminar flow hood. Approximately 18 to 20 hours before transfer, each straw is thawed one at a time in a Dewar flask filled with liquid nitrogen until the appropriate number of viable embryos are reached. The straw is removed from the flask by the handle and held horizontally at room temperature (it is important to thaw the straw away from drafts or fans, which can alter the air temperature). The straw is now wiped and inspected for condensation. Thawing continues at room temperature until condensation does not reform on the straw. This usually takes 2 minutes. The handle is removed and the sucrose column is shaken down into the embryo column to dilute the PG while thawing continues. The straw is then placed in a 37 °C waterbath for 3 minutes and then held in room temperature water for 1 minute and wiped dry; the ends are clipped off with scissors. The content of the straw is gently expelled into a small petri dish, and the embryo is recovered and immediately placed in PB1 to rehydrate. Once again, gentle swirling of the dish will dilute out the sucrose and PG and expedite expansion of the blastomeres. After equilibrating in PB1 for 10 minutes, the embryo is scrutinized under the microscope for any signs of freezing damage. The viable embryo is then placed in culture media and allowed to continue developing until the time of transfer.

CHOOSING A CRYOPRESERVATION PROCEDURE

Up-to-date statistics from 25 human-embryo–cryopreservation centers in the United States were gathered by the American Fertility Society.[4] According to this report, most centers recommend cryopreservation when the patient has >3 to 4 zygotes or >2 to 3 cleaved embryos. Three cryoprotectants are reportedly being used currently:

1 1,2-Propanediol (PROH)
2 Dimethyl sulfoxide (DMSO)
3 Glycerol

A variety of developmental cell stages of human embryos are presently being frozen from zygotes or blastocysts. The survival rate of the different cell-

stage embryos appears to be similar. Data indicates that frozen zygotes result in fewer embryos used per pregnancy and higher pregnancy rates per transfer than 2-cell to morula-stage embryos. Published results also indicate that a rapid freezing of human-pronuclear embryos with 1,2-PROH results in 28% (out of 56 pronuclear embryos) continual cleavage in culture,[16] whereas 71% of pronuclear embryos frozen by a slow-freezing method show continual cleavage 24 hours later.[13] Although 7.3% of frozen-thawed zygotes degenerate immediately upon thawing, one center has shown that those zygotes that do survive not only have the same potential to cleave but also develop at the same rate as fresh zygotes at a similar stage in development.[4] As far as embryos are concerned, we have found the greatest success in freezing four-cell stage embryos with little or no cytoplasmic fragmentation that display a nucleus in the blastomeres at the time freezing is initiated. We are, however, in the process of evaluating the efficacy of freezing zygotes. Our standard for "good" survival for thawing-frozen embryos is based on $\geq$ 50% of the blastomeres remaining intact after thawing is complete.

When evaluating the embryo characteristics that best enhance cryopreservation survival, one should scrutinize the actual appearance of the zona and blastomeres to determine the grade of fragmentation, granularity, thickness of zona, and appearance of nuclei (to indicate the resting cell stage). The amount of time an embryo spends in culture may automatically dictate those embryos best suited for freezing. Data from Bourne Hall indicate that approximately 40% of fertilized eggs develop to the blastocyst stage in vitro and that 25% are arrested from the eight-cell stage onward.[3] Thus a longer culture period will exclude those embryos that are arrested, fragmented, or developing abnormally.[14] Almost all centers reported using rate-controlled freezing equipment to lower the temperatures of the embryos to subzero. Plastic straws and glass ampules appear to be the standard for storage of frozen embryos in low-level alarmed liquid-nitrogen tanks. With the increasing advancement of cryotechnology and cryobiology, the new decade holds much promise of fertility for infertile couples.

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