Editorial Articles

Advances in oocyte cryopreservation - Part I: slow cool rapid thaw technique

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Summary

Purpose: The need for freezing oocytes has been established for females undergoing potential therapy that could damage their ovarian egg reserve, ethical or religious reasons (not having excess embryos frozen) or women nearing the age of lower fecundity but not married and not ready to use donor sperm. Applying the cryopreservation techniques for oocytes as used for embryos resulted in very poor pregnancy results. *Methods:* Changes in methodology including fertilization by intracytoplasmic sperm injection because of zona hardness and using a sodium-deplete choline substitute freezing media are some of the changes made for the slow cool rapid thaw method. *Results:* These modifications have led to significant improved survival rates of frozen oocytes not to mention fertilization rates and subsequent pregnancy rates. *Conclusions:* Since some in vitro fertilization (IVF) centers do poorly with frozen embryo transfer pregnancy rates despite good pregnancy rates following fresh embryo transfer, there is suspicion that the culpability may lie in the programmable freezer used in the slow cool technique. A simplified slow cool freezing technique using a biocool freezer instead of a programmable freezer has been described which has resulted in consistently good results with embryos. It would be interesting to see if this technique would work well with oocytes with the new changes to the freezing method.

Key words: Cryopreservation; Oocytes; Slow cooling; Choline.

Oocyte Freezing

Part 1: Slow cool - fast thaw technique

Though there may be a trend toward decreasing the number of oocytes stimulated for the purpose of in vitro fertilization (IVF), most protocols create multiple follicles and thus a lot of eggs are retrieved. If one fertilizes all of these eggs this leads to extra embryos which are then cryopreserved for future use. If the couple conceives on the fresh transfer then they can transfer frozen-thawed embryos in the future without having to go through controlled ovarian hyperstimulation and oocyte retrieval. Frozen embryos still function quite well when transferred even when they have been frozen more than ten years. If the couple failed to conceive the first time with fresh embryos they can immediately proceed with frozen embryo transfer without going through the expense of IVF-ET.

However, sometimes this present delivery is all the children that the couple wants. The question is what to do with the extra embryos? Options are to keep them frozen in case the couple changes their mind or transfer them and just hope that the transfer does not work. Other options include anonymously donating the embryos to another couple or destroying them. Some couples feel better about donating them to research, e.g., stem cell research. Generally most IVF centers charge some fee for the storage of embryos.

There are some couples also for religious or ethical reasons who cannot choose the option of destroying the extra embryos or donating them to research. Though donating them to another couple would circumvent the problem of destroying life, many couples are not comfortable with the fact that this could result in siblings of their children and they cannot deal with that emotionally.

One strategy used by some couples is to limit the number of retrieved eggs that are to be inseminated by sperm. However, if the embryo transfer does not result in a pregnancy then the couple regrets having to go through another controlled ovarian hyperstimulation cycle and not having frozen embryos to transfer. Sometimes a couple has fulfilled their dreams of a sufficient family only to have some tragedy occur to one of their children and then the couple desperately wants another. Possibly the woman is now at an age when achieving a pregnancy would be difficult with her own eggs. However because the uterus in humans ages more slowly than the ovaries, had she had frozen embryos left over from a younger age, another pregnancy would be feasible. In this case she might wish that she had embryos left.

The ethical and moral dilemmas about what to do with remaining embryos which are considered "life" would be obviated if the extraneous oocytes were not fertilized but frozen as eggs. If a divorcee or widow remarries at the age of 45

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to a man who has no children and they desire children including his and her genetic makeup there is very little chance at this age that she can conceive with her own eggs. Thus she would have to consider donor eggs. If there were embryos from a younger age fertilized by her previous male partner that would not solve the problem. However, if there were frozen eggs available from a younger age that would provide the opportunity to fulfill their dreams if the state of the art for egg freezing would not only provide a good chance of surviving the thaw but a good chance of fertilization and pregnancy after embryo transfer.

If technology improved to the point where egg freezing was successful there are many other scenarios where the preference would be to freeze eggs rather then embryos. These include, but are not limited to, young women about to undergo potential treatment that would damage the ovaries for the future, e.g., radiation therapy, chemotherapy, surgery for cancer, career women who have not married yet but have reached their mid 30's wanting to preserve their ability to conceive in the future, or a sister who is undergoing IVF-ET who wants to leave eggs not embryos for possible use by her older sister who is struggling to conceive.

The problem was that the freezing technique used to freeze embryos was not very successful for freezing eggs. The first pregnancy from fertilization of frozen-thawed oocytes using the slow cool rapid thaw method used for embryo freezing was reported in 1986 by Chen [1]. However it was not viable. More than a decade passed before the first live delivery from the fertilization of an oocyte frozen by the slow cool rapid thaw technique was reported by Porcu *et al.* in 1997 [2].

When one freezes oocytes the zona pellucida becomes hardened [3-5]. This hardening (possibly related to premature release of cortical granules) when freezing oocytes inhibited fertilization of the oocytes. Furthermore the fertilization rate of cryopreserved-thawed oocytes was poor (or more than one sperm fertilized the egg, i.e., polyspermy, related to premature cortical granule release). The advent of intracytoplasmic sperm injection (ICSI) in 1992 provided a significant advancement in the oocyte cryopreservation problem.

One of the main challenges in freezing any live material is the formation of intracellular ice crystals [6-8]. Cryoprotectants are needed to prevent ice crystal formation. Permeating cryoprotectants are small molecules that readily permeate the membranes of cells. They form hydrogen binds with water molecules and thus prevent ice crystallization. At high enough concentrations, these cryoprotectants inhibit the formation of the characteristic ice crystal and lead to the development of a solid glasslike, so-called vitrified state in which water is solidified but not expanded.

The most commonly used permeating cryoprotectant for embryos and eggs is 1,2 propanediol. When used in a concentration high enough to prevent ice crystal formation they are very toxic. To reduce this toxicity either the cell must be exposed to the cryoprotectant for very short time periods (as with the vitrification technique which will be described later) or at a time when the metabolic rate of the cell is low, e.g., very low temperatures.

The use of more than one cryoprotectant can maximize inhibition of ice crystal formation with lower toxicity by not requiring a high concentration of any one cryoprotectant. This is best accomplished by a step-wise addition of cryoprotectants. Generally 1,2 propanediol is added first then a non-permeable cryoprotectant sucrose is added later.

At first the general concept was that the reason why embryos were resistant to ice crystal damage was the difference in the state of the chromosomes between embryos and eggs. The DNA of mature unfertilized oocytes is compacted into chromosomes that are aligned on a metaphase plate. In contrast the majority of DNA in embryos exists as decondensed chromatin at interphase. The thought process was that the meiotic spindle of the oocyte was more susceptible to cryodamage and that the spindle becomes deorganized leading to altered DNA. There is evidence however that with the use of cryoprotectants the meiotic spindle can reorganize in the oocyte and that this is not the main reason for oocytes being less cryo-hearty [9].

As mentioned two of the improvements in oocyte cryopreservation technology were the use of ICSI for fertilization of frozen thawed oocytes with exposure to toxic cryoprotectants at lower temperatures and the use of multiple cryoprotectants to minimize the toxicity of any one specific cryoprotectant. Certain changes in the cryoprotectant when introduced along with using ICSI for fertilization and making certain changes in the freezing media, e.g., substituting choline in the cryopreservation media for sodium also resulted in marked improvement in pregnancy rates with the slow cool rapid thaw technique [10]. Choline may have beneficial effects on the membrane [11, 12].

Thus the slow-cool freezing method relies on low initial cryoprotectant concentrations which are associated with lower toxicity. This is important because the oocyte is still at a temperature at which it is metabolizing. Thus an important strategy is not to increase the concentration of cryoprotectants and other solutes until the oocyte has been cooled down to a temperature at which metabolism is slow. The initiation of adding cryoprotectnats begins at room temperature which is gradually lowered about 2°/minute to the seeding temperature of –6°C. Seeding is the initiation of ice formation in a controlled manner. It involves the minimal introduction of a stable ice crystal to the freeze solution which acts as a template for additional ice formation.

Following seeding at –6°C the solutions are held for 10-30 minutes to allow equilibration. The growing ice crystals result in squeezing out of the oocytes the cryoprotectants and other solution which results in a gradually rising concentration of the cryoprotectants in the remaining solution.

The temperature is slowly decreased to –32°C which allows gradual diffusion into the oocyte of additional permeating cryoprotectants. Ice crystal formation has increased in size in the intracellular medium thus increasing the concen-

tration of the cryoprotectant in the intracellular space. Further dehydration of the intracellular space is accomplished through the use of nonpermeating cryoprotectants, e.g., sucrose.

At the final temperature of –32°C the freezing temperature for 1,2 propanediol has not been reached but its concentration has markedly increased. The metabolic rate of the oocyte is now quite slow further limiting the toxicity of the increasing concentration of the cryoprotectants. The freezing vessel is now plunged into liquid nitrogen.

Substitution of sodium by choline in the cryopreservation media enhances cryopreservation outcome. In addition to solution effects sodium in the culture medium is thought to accumulate intracellularly as a result of impairment of the plasma membrane Na+k+ pump during cryopreservation [13, 14]. Unlike the sodium ion, choline is not thought to cross the cell membrane and thus not contribute to intracellular osmolarity or toxicity.

The largest experience with human oocyte cryopreservation using the slow cool technique has come from an Italian group [10, 15]. The success rate after transfer of embryos derived from the thawed oocytes has approcached the pregnancy rates for transfer of frozen thawed embryos [15].

The basic methodology that was modified for oocyte freezing was the embryo cryopreservation technique described by Lassalle *et al.*, in 1985 [10, 15, 16]. Though some IVF centers show good pregnancy rates with frozen embryo transfer other IVF centers do not seem to fare so well despite good success with fresh embryo transfer. It has been my belief that the weak and variable part of this cryopreservation procedure is the programable freezer. My colleagues and I have had good pregnancy rates following frozen embryo transfer with a simplified freezing protocol with a one-step removal of the cryoprotectant 1,2 propanediol upon thawing that avoids the programmable freezer [17-19]. Possibly pregnancy rates following fertilization of cryopreserved-thawed eggs can be increased using the slow cool methodology that avoids the programmable freezer.

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