Editorial Articles

Advances in oocyte cryopreservation - Part II: rapid cooling using vitrification

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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, for ethical or religious reasons (not having excess embryos frozen) or for women nearing the age of lower fecundity but not married and not ready to use donor sperm. Applying cryopreservation techniques for oocytes used for embryos resulted in very poor pregnancy results. A rapid flash freezing technique has rekindled interest in oocyte freezing known as vitrification. *Methods:* Certain modifications, especially minimizing the volume, have resulted in marked improved pregnancy rates with vitrified thawed oocytes. The lower volume allows decreased exposure to the toxic cryoprotection. Commercial interests have developed an effective device called cryotop but some concerns about microorganism contamination exist because it is an open system. Modifications have been made to make available the cryotip, a closed device which addresses the contamination issue. *Results:* Frozen oocyte survival rates upon thawing fertilization rates and subsequent pregnancy rates after embryo transfer have been reported comparable to data with frozen thawed embryos. *Conclusions:* Because of the uncertainty of the programmable freezer used for the slow cool method and because there has been more commercial interest in the vitrification method, the "flash" freeze protocol seems to have an edge over the slow cool method for oocyte freezing.

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

The Vitrification Method of Cryopreserving Oocytes

Rall and Fahy described vitrification as a potential alternative to slow-cooling. Vitrification involves exposure of the cell to about twice as high cryoprotectant concentrations compared to the slow-cool procedure for brief periods of time usually at or near room temperature followed by rapid cooling in liquid nitrogen [1]. The high osmolarity of the vitrification solution rapidly dehydrates the cell and the submersion into liquid nitrogen quickly solidifies the cell so that the remaining intracellular water does not have time to form damaging ice crystals.

The initial poor success with oocyte freezing using the slow-cool technique that had worked well in many in vitro fertilization (IVF) centers with embryos led researchers to try to modify this old technique of vitrification which was considered prior to the modification as not likely to be effective because of the toxicity of the highly concentrated cryoprotectants and the temperature at which they were used [2, 3].

The first modified technique of vitrification leading to successful deliveries was first described with cows [4]. The first live healthy delivery of a little girl involving fertilization of a vitrified thawed egg and subsequent embryo transfer using a similar technique as the bovine studies was reported in 1999 by Kuleshova *et al.* [5]. It is my belief that it was the relatively poor success of the slow freezing rapid thaw technique (the first live human birth with the slow technique was reported in 1997 by Porcu *et al.*) that led to the commercial push to try to modify the vitrification method [6].

The modification of the vitrification method included decreasing the length of time of exposure to toxic cryoprotectants so that the exposure to the final and highest concentration was 30 seconds or less. This faster cooling increases survival [7-9].

The concept of vitrification proposes that if a cell is dehydrated and then cooled fast enough everything will "freeze" in place and damage will not have time to occur as a vitrified amorphous glass-like solid will instead form of crystals. Similarly thawing must take place at a relatively fast rate to prevent crystal organization upon rewarming. If cells die during vitrification it may or may not be because the cryoprotectant concentration was toxic, or ice did in fact form, or the cooling rate was too slow.

One method to allow an increased rate of cooling and subsequent thawing is to minimize the volume of the vitrification solution which allows bovine concentration of cryoprotectants [10, 11]. The most critical time period is the initial cooling [12]. Minimizing the volume of the vitrification solution containing oocytes also decreases the chance of ice crystal nucleation formation in the small sample [13]. Furthermore, minimum volume vitrification may also help to

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avoid zona pellucida damage and embryo fracture which frequently occurs when oocytes are cryopreserved in standard insemination straws and warmed rapidly afterwards.

Commercialism has led to the cryotop method, a minimum volume vitrification method. A special tool consisting of a narrow film strip attached to a hard plastic holder has been developed to protect the tool from mechanical damage during storage. A 3-cm plastic tube cap can be attached to cover the film part. The vitrification solution contains ethylene glycol, dimethyl sulfoxide and sucrose.

Cryotop is an open method where direct contact between liquid nitrogen and the solution containing oocytes is required. Though an open system would theoretically allow contamination by microbes, those advocating open vitrification systems argue that at such a low temperature proliferation of any serious pathogens is unlikely. Nevertheless some studies show that certain bovine viruses can infect the embryos in an open system but not in a closed system [14, 15]. Some countries will not approve an open vitrification system because of the theoretical contamination of embryos by pathogens. A closed system known as Cryotip has thus been produced. Cryotip is a narrow capillary that can be sealed after loading with a minimum volume solution [16]. There is no direct contact between the biological solution and the liquid nitrogen.

Kuleshova *et al.* reported the first birth from vitrified human oocytes in 1999 after vitrification of 17 oocytes by using ethylene glycol (40%) and 0.6M/l sucrose in open pulled straws [5]. The first large series of human oocyte vitrification was published by Yoon *et al.* in 2003 [17]. They cryopreserved 474 cumulus-oocyte complexes (mature and immature oocytes) by using vitrification with 5.5 M ethylene glycol and 1.0 M sucrose as cryprotectants. To maximize cooling rates, the oocytes were loaded on an electron-microscope grid. These investigators reported a survival rate of 68.7%, a fertilization rate of 71.7%, an implantation rate of 6.4%, and a clinical-pregnancy and live-birth rate per transfer of 6/21 (21.4%). Chian *et al.* [18] used a combination of ethylene glycol, PROH, and sucrose to vitrify 180 oocytes in an open container called a Cryoleaf [18]. They reported a survival rate of 20.4% and a clinical pregnancy rate per patient of 7/15 (46.7%) [18].

Both Kuwayama *et al*. and Antinori *et al*. showed the best success rates following thawing of cryopreserved ocoytes and subsequent embryo development and pregnancies [11-19]. Kuwayama *et al*. reported with the Cryotop method that 148 of 153 vitrified oocytes had normal morphology upon thawing, 91 of 153 oocytes cleaved into embryos and 35 (22.9%) developed into blastocysts (comparable data for fresh oocytes were cleavage of 118 of 153 eggs (77.6%) and 68 (44.7%) became blastocysts. Pregnancies were achieved in 12 of 29 (41.3%) of embryo transfers involving fertilization of vitrified thawed oocytes [11].

Lucena *et al.* also used the Cryotop method for oocyte vitrification and reported an overall pregnancy rate of 13/23 (56.5%) per patient [20]. These high pregnancy rates may be attributed partly to the fact that the majority of transfers used donor oocytes and involved the transfer of a high number of embryos (mean of 4.5) [20].

Antinori *et al.* tried to independently assess the protocol of vitrification described by Kuwayama *et al.*. using Cryotop [19]. Antinori *et al.* [19] found that 328 of 330 vitrified oocytes (99.4%) survived upon warming. Following ICSI 305 of 328 (93.0%) fertilized and 295 of these 305 2PN embryos cleaved. These 295 cleaved embryos resulted in 120 embryo transfers. There were 39 (32.5%) clinical pregnancies and 28 ongoing pregnancies (23.3%). The implantation rate per transferred embryo was 13.2% and the implantation rate per thawed oocyte was 11.8% [19].

Ri-Cheng Chian and colleagues at the 14th World Congress on in vitro fertilization in Montreal, 2007, presented their experience involving vitrification of oocytes with fertilization upon thawing with ICSI. There were 38 women (mean age 31.5) in the trial and there were 463 oocytes vitrified. The survival rate post-thaw was 82.7% (383/462) following insemination by ICSI, and 75% (287/383) were fertilized normally. The pregnancy rate per transfer (mean of 3.7 ± 1.1 transferred) was 44.7% (17/38). To date one has had an ectopic pregnancy, 13 have delivered, and there are two ongoing pregnancies. All babies were normal. The implantation rate was 18.1% (24/133).

Conclusions and Caveats

It is clear that modern technology has allowed the development of cryopreservation techniques for oocytes that can approach the success of embryo freezing. The myriad of potential uses for oocyte freezing have been discussed in part I of this editorial [2, 22]. A women who is considering cryopreserving the only fertilizable eggs she will have for the rest of her life prior to ablative therapy, e.g., for cancer, has to carefully review all options. Even a single young woman without a current partner could still consider fertilizing those oocytes retrieved with donor sperm and freeze them at the 2 pronuclear stage.

Young women or somewhat reproductively older women must realize that the present optimistic conclusion that oocyte freezing and embryo freezing are “about” comparable based on limited studies by highly experienced IVF centers with the best subjects. Just because an IVF center is claiming to use the vitrification method using cryotop does not mean that the center should show equal success to that reported by Kuwayama *et al*., especially if that given IVF center has limited experience with the procedure [11]. There is apparently a significant learning curve with vitrification that may take five months or more to master even in a center performing the procedure frequently. Some IVF centers willing to perform oocyte freezing have never even tested whether the oocytes that have been cryopreserved will result in live babies or what the chance of success is.

Thus one option until more extensive experience is achieved (unless the young woman can actually have the procedure performed at one of the experienced centers) would be to fertilize the eggs with donor sperm but advise the woman that a future husband could also continue his genes in his progeny by the fertilization of donated eggs. She should also be advised of the future use of a younger sister's eggs or even the use of her brother's sperm with anonymously donated eggs.

There are not enough data, especially with the slow freeze rapid thaw method, to make a determination as to whether one technique is superior to the other for oocyte cryopreservation [21]. There seem to be more publications lately concerning vitrification especially with the cryotop or cryotip but this may be commercially stimulated. What is needed is more very good IVF centers skilled in both techniques to freeze half of the oocytes retrieved with the slow-freeze rapid-thaw procedure and the other half with vitrification, and compare the outcomes. These centers should include not just ideal patients but others, e.g., slightly older women.

Oocyte freezing should still be considered experimental and should be under the supervision of an institutional review board (IRB). Such an IRB, in my opinion, should require that a given IVF center able to cryopreserve oocytes should first demonstrate in an experimental group (given perhaps some financial considerations), that the fertilization of frozen thawed oocytes by that given IVF center results in an adequate live delivery rate after embryo transfer before they should be allowed to freeze oocytes for young women about to undergo therapy that could jeopardize their future egg supply.

Since oocytes present much more of a challenge to successful cryopreservation than embryos, it seems logical that if technology advances with vitrification so that a successful oocyte freezing program can be established, embryos should follow suit and successful embryo vitrification should also be found.

The majority of IVF centers today use the slow cool rapid thaw method for freezing of embryos. If cryopreservation of oocytes proves superior by vitrification than slow cool, even if the two techniques prove equal for embryo freezing, the IVF programs planning on freezing oocytes would likely switch to vitrification of embryos, especially once skilled in the latter technique in order to unify procedures.

If the two cryopreservation procedures prove to have equal efficacy for oocyte and embryo freezing, new IVF centers may prefer the vitrification procedure because they would not have to invest in an expensive programmable freezing machine. Furthermore, since the slow cool rapid thaw technique is very time consuming, whereas vitrification can be completed by one embryologist within minutes, this factor could also influence the majority of neophyte IVF centers to choose vitrification even if success rates prove similar.

As mentioned, one of the things needed to determine if slow cool or vitrification of oocytes results in higher success rates is to have an expert cryobiologist experienced in both techniques to perform comparative studies. Such data is available with human embryos. One of the most respected cryobiologists in the world is Kuwayama. Using slow-cool vs the cryotop method of vitrification for freezing 2 pronuclear (2PN) embryos, 89% (1730/1944) 2PN embryos survived with slow-cool vs 100% (5881/5881) with vitrification [10]. The cleaved survival rate was 90% (1557/1730) with slow-cool vs 93% (5409/5881) with vitrification. The blastocyst cleaved rate was 51% (796/1557) with slow-cool vs 56% (3058/5469) for vitrification and the blastocyst/cryopreserved rate was 41% (796/1944) vs 52% (3058/5881). Thus vitrification of 2PN embryos resulted in higher survival developmental rates than slow-cooling [10]. The author did not provide comparative pregnancy rates per transfer for this stage of embryo freezing.

For 4-cell two human embryos the survival of rate cryopreservation was 91% (857/942) with slow cool vs 879/897 (98%) with vitrification. The pregnancy transfer rate was 32% (172/536) vs 27% (136/504) for vitrification [10].

For blastocysts the survival of rate vitrification was 84% (131/156) with slow cool vs 90% (5695/6328) with vitrification [9]. The pregnancy rate per transfer was 51% (50/98) vs 53% (2516/4745) and the live birth rate per transfer was 41% (40/98) vs 45% (2138/745) [10].

If the slight advantage seen with vitrification vs the slow-cool method for various stages of embryo freezing remains similar in other studies by other authors, the differences may not be sufficient for IVF centers doing well with slow-cool techniques and already having invested in the expensive programmable freezer to invest the time and possibly risk lower pregnancy rates initially until the learning curve has been satisfied. These IVF centers could be interested in switching if they become interested in oocyte freezing and if the vitrification methods show an even more impressive outcome with oocytes than slow cool techniques. However it is well known that some IVF centers do not seem to have very good results with their present slow cool procedure for embryo freezing. Clinical success may depend on many factors including patient age and stimulation protocol, quality of embryos selected for freezing, developmental stage at freezing, media formulation including type of cryoprotectants used, and parameters of cooling and warming. Another very important factor, however, is the type and quality control of programmable freezing unit employed. This problem with slow cooling would be completely eliminated with vitrification since this procedure does not require sophisticated equipment of questionable reliability of certain makes and types. Thus, there seems to be enough data available at present to entice those IVF centers without great success with their slow cool embryo freezing procedures to switch to vitrification.

Our division of infertility and reproductive endocrinology is a university medical center which has a very large multidisciplinary oncology center. Our IVF center is the largest in the southern part of New Jersey and is one of the largest in the state. Nevertheless only a few times at most per year does a case of potential egg ablation following cancer ther-

apy come up where there is a request to preserve future fertility with a couple's own gametes. In every instance to date the single young women were content to fertilize the eggs with a boyfriend's sperm or donor sperm. Though we advised them of other centers freezing the oocytes they chose to merely freeze the embryos. I suspect that some would have preferred egg freezing if we had it available. Possibly time constraints may have precluded them from contacting these other centers.

There is some variation in success rates among various IVF centers with pregnancy rates following fresh embryo transfer but there are much greater differences in the success rates following frozen embryo transfer. In fact some IVF centers with the best success with fresh embryo transfer do not fare nearly so well with their pregnancy rates following frozen embryo transfer.

My suspicion to explain this apparent paradox is that the poor success with frozen but not fresh embryo transfer is not likely related to a substandard embryology laboratory or poor transfer technique. Instead my hunch has been that the weak point may be the quality of the programmable freezer. Thus we modified a technique that had been used in cattle where a simplified freezing protocol that required a Biocool freezer was used instead of the Planer programmable freezer and a one-step removal of the cryoprotectant 1,2 propanediol [22]. With this simplified freezing protocol we have attained similar pregnancy rates following frozen ET as with our fresh ET pregnancy rates in women who underwent hyperstimulation [23].

This simplified protocol on egg freezing could be attempted using some of the media changes recommended by Porcu *et al.* [6]. However, the lack of requests have prevented my group from performing such a study since, as mentioned, I believe that the ethical thing to do would be to first try the technique on women wanting immediate transfers which would require the normal financial rewards of participating in a research study, i.e., purposely not transferring the proportion of embryos formed from fertilization of fresh eggs but instead the portion formed from fertilization of frozen thawed oocytes.

Since most of the commercial efforts have centered on vitrification methods, if a company funded a study, e.g., as proposed above, or even funded a study comparing our modified slow-cool fast-thaw method to vitrification, and we found an advantage to the latter, then I would consider switching.

For a new IVF center just starting because of much less expense and space occupying equipment to start with and because of avoiding the Achilles heel for some freezing programs, i.e., the programmable freezer, I would advise the neophyte center to consider vitrification from the outset. I would also advise an IVF center not doing well with their present slow cool technique to switch to vitrification.

I do not believe that there is enough need for oocyte freezing to justify every IVF center offering the service. Instead I think the companies selling equipment (e.g., the Kitazato Co., Fujinomiya, Japan which makes the cryotop and cryotip) should designate certain IVF centers to cover certain geographical areas to learn the vitrification method and fund a study to evaluate the proficiency of that center in egg freezing for women who desire immediate replacement of embryos. It would be important to check pregnancy rates of sibling frozen oocytes.

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