A Prospective Pilot Study Comparing Fertilization and Embryo Development Between Fresh and Vitrified Sibling Oocytes

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ABSTRACT—Objective: To evaluate the outcome of a newly established oocyte vitrification program in women undergoing in vitro fertilization (IVF) within a short timeframe by simultaneously evaluating embryos derived from vitrified and fresh oocytes from the same stimulated cycle.

Design: Cohort prospective controlled trial and case-control study.

Setting: University-based tertiary fertility center.

Patient(s): Fourteen women who fulfilled the inclusion criteria underwent controlled ovarian hyperstimulation and Intracytoplasmic sperm injection (ICSI) treatment.

Intervention(s): Oocyte vitrification.

Main Outcome Measure(s): The primary outcome measures were oocyte survival, fertilization and cleavage rate, and subsequent embryo development, compared between vitrified and fresh oocytes. Secondary outcomes were implantation, clinical pregnancy, miscarriage and live birth rates using embryos derived from the vitrified oocytes for transfer. This was compared with age-matched controls who met similar inclusion criteria as the study patients and who underwent IVF during the same time period. Neonatal data on all newborns was also collected.

Results: From October 2009 until November 2010, a total of 17 patients were enrolled in this study (mean age 31.9 ± 2.9 years). Three subjects withdrew prior to retrieval and one subject did not have a transfer from vitrified oocytes. A total of 164 metaphase II (MII) oocytes were retrieved (mean 11.7 ± 3.7), 83 were vitrified with 86.7% survival. Fertilization rate was similar between vitrified and fresh oocytes (69.4 vs 78.2%, P=0.8). Cleavage on day two, however, was lower in the vitrified oocytes (88% vs 100%, P=0.004). Implantation rate (IR) was 25% (7/28) with a mean number of 2.0 ± 0.5 embryos transferred. Live birth rate/embryo transfer (ET) was 46.1% (6/13) after transferring embryos derived only from vitrified oocytes, (six live births, seven babies, one set of twins). One additional ongoing pregnancy has been established after transfer of a cryopreserved blastocyst derived from a vitrified oocyte (combined pregnancy rate/ET: 50%; 7/14).

Conclusions: This study provides a viable model to quickly assess the efficacy of a newly established egg vitrification program following American Society for Reproductive Medicine (ASRM) guidelines in an investigational protocol. Embryos resulting from oocyte vitrification resulted in optimal live birth and implantation rate. The lower cleavage rate noted in this study may indicate a possible detrimental effect of the vitrification process, which may be overcome with additional experience and refinement of the technique.

Introduction

The need to cryopreserve human oocytes successfully, with the goal of achieving term pregnancies at rates equivalent to those obtained with fresh oocytes has been receiving increasing attention. Cryopreservation of oocytes is desirable because it allows infertility patients to store excess oocytes instead of embryos, eliminating some of the ethical and religious
Vitrification was demonstrated as an alternative to slow freezing of embryos in 1985 by Rall and Fahy. Vitrification is a method to cryopreserve biological specimens that are sensitive to chilling injury such as oocytes and embryos, and it has been employed with increased survival rate and live births. The success of IVF with cryopreserved oocytes has been systematically reviewed in a recent meta-analysis of five randomized controlled trials. The rates of ongoing pregnancy, top-quality embryos, embryo cleavage, and fertilization did not differ between the vitrification and the fresh oocytes groups. The oocyte survival rate was higher in vitrified vs slow-frozen oocytes (odds ratio [OR] 2.46, 95% confidence interval [CI] 1.82–3.32). The fertilization rate, the rate of top-quality embryos, and the embryo cleavage rate were also higher in vitrified vs slow-frozen oocytes. Vitrification also resulted in a higher rate of top-quality embryos and embryo cleavage rate. Overall, vitrification is emerging as the method of choice for oocyte cryopreservation.

Despite promising advances in oocyte cryopreservation, the Practice Committee of the Society for Assisted Reproductive Technology (SART) and the Practice Committee of the American Society for Reproductive Medicine (ASRM) still define oocyte cryopreservation as an experimental procedure. Moreover, the Practice Committee of the ASRM also indicated that procedures classified as experimental or investigational require further research that should be performed only with the specific review of a properly constituted Institutional Review Board (IRB).

The characterization of oocyte cryopreservation as an experimental procedure has been recently challenged by Noyes et al., in view of the technologic advances made in the past decade. Nevertheless, the authors also stated that regardless of IRB approval, clinics should be directed to offer patients reliable data regarding their own oocyte cryopreservation experience and outcomes. Since oocytes may remain in storage for several years before they are utilized, providing reliable outcome data may not always be feasible. In this study we aimed to provide a viable model to quickly assess the efficacy of a newly established egg vitrification program following ASRM guidelines in an investigational protocol.

### Materials and Methods

**Patients:** This was a prospective randomized trial where a total of 17 patients were enrolled between October 2009 and November 2010. Approval for the study was obtained from The University of Connecticut Institutional Review Board. Inclusion criteria were: 1) age 21 to 37 years, with a normal serum follicle stimulating hormone (FSH) concentration <10 mIU/ml and estradiol (E2) concentration <70 pg/ml obtained on day two or three of the menstrual cycle; 2) BMI <35 kg/m² with no physical or gynecological abnormalities (including major uterine surgery) constituting a medical contraindication to embryo transfer and pregnancy including any known significant genetic disorders; and 3) nonsmoker for at least three months prior to study enrollment and an antral follicle count (AFC) of ≥ 10. Patients were excluded if they had more than one previous miscarriage; more than one previous failed IVF attempt; previous poor response to ovarian stimulation (peak E2 level <1,000 pg/ml or <4 oocytes retrieved); presence of nontreated hydrosalpinx; Stage III or IV endometriosis; intent to have preimplantation genetic diagnosis (PGD) of embryos; or having a male partner requiring surgical sperm retrieval. After initial screening and consents were obtained, one subject voluntarily withdrew from the study, and two had their cycle cancelled due to poor response to stimulation. Patient’s demographics are illustrated in Table 1. A total of 14 patients underwent oocyte retrieval, and 13 underwent embryo transfer (ET) from embryos derived only from vitrified oocytes. One subject did not have embryos available from vitrified oocytes due to lack of embryo development (this patient conceived after transferring embryos derived from fresh oocytes).

Following controlled ovarian hyperstimulation, final oocyte maturation was triggered with human chorionic

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gonadotropin (hCG) 10,000 IU when the two largest follicles reached at least 17 mm in diameter by ultrasound. The cycle was cancelled if less than six follicles >15 mm were seen on ultrasound the day of hCG administration, and the patient was withdrawn from the study. Oocyte collection was performed transvaginally under ultrasound guidance, 35 hours after hCG administration.

One subject returned for an additional frozen-thawed embryo transfer 19 months after delivering a live birth. One cryopreserved blastocyst that originated from a vitrified oocyte was warmed and transferred during the same cycle. One cryopreserved blastocyst that originated from a vitrified oocyte was warmed and transferred during the same cycle. One cryopreserved blastocyst that originated from a vitrified oocyte was warmed and transferred during the same cycle.

Outcomes of IVF after oocyte vitrification were compared with 639 age-matched women (mean age: 33.1 years ± 5.3; p=0.3) who underwent IVF in our program using fresh oocytes during the same time period, and also underwent a day three ET.

Oocyte Selection and vitrification: After incubation for at least one hour following ovum pick-up, oocytes were denuded to identify all the mature (metaphase II) oocytes. Complete removal of the cumulus and corona cells was performed using hyaluronidase (80 IU/ml Sigma H375, Sigma Aldrich, St. Louis, MO). All oocytes were cultured separately in Quinn’s Advantage Cleavage Media (Cooper-Surgical, Pasadena, CA) under Tissue Culture Oil (Sage 2010, Cooper-Surgical, Pasadena, CA) in 5% CO2.

Study participants had half of their mature oocytes vitrified utilizing the Cryolock device (Biodiseno, Colombia) for a minimum of 20 minutes. Vitrification was performed using the method described by Katayama et al.13 The other half of the mature oocytes were kept in the incubator, in preparation for ICSI. All the vitrified oocytes were warmed after at least 20 minutes in liquid nitrogen, and all the surviving oocytes underwent ICSI along with the patient’s fresh mature oocytes. If an odd number of mature oocytes were obtained, the extra oocyte was vitrified. If an immature oocyte at time of denuding matured to a metaphase II (MII), the oocyte was placed with the fresh oocytes for ICSI.

For vitrification, oocytes were placed into a 50 µl drop of HEPES buffered media (Sage 1023, Cooper-Surgical, Pasadena, CA), 20% SPS (Sage 3011, Cooper-Surgical, Pasadena, CA) for two minutes before emerging with an adjacent 50 µl drop of equilibration solution containing 7.5% ethylene glycol (Sigma E9129, Sigma Aldrich, St. Louis, MO) and 7.5% DMSO (Sigma D2650, Sigma Aldrich, St. Louis, MO) for approximately five minutes. Once adequate shrinkage and reexpansion occurred, a second 50 µl drop of equilibration solution was merged with the drop containing the oocytes for an additional five minutes. After waiting for shrinking and reexpansion, the oocytes were then placed into a separate drop containing vitrification solution with 15% ethylene glycol, 15% DMSO, and 0.5 M sucrose (Sigma S1888, Sigma Aldrich, St. Louis, MO) for approximately 40 seconds. All the procedures were performed at room temperature, 22-23º C. The oocytes were then loaded onto the open face of the Cryolock in approximately 1µl and then plunged directly into liquid nitrogen. Working under liquid nitrogen the cap filled with liquid nitrogen was then placed over the tip of the carrier. No more than two oocytes were loaded onto the carrier.

Warming of Oocytes and ET: After 20 minutes to one hour in liquid nitrogen, the oocytes were warmed by gently twisting the Cryolock and rapidly transferring it to a 1M sucrose medium. After one minute, oocytes were transferred to a 0.5M sucrose medium for three minutes, then to a 0.25M sucrose medium for an additional three minutes, and then transferred to HEPES buffered media with 20% SPS. All the procedures were performed at 37ºC. The oocytes were then rinsed into Stage Cleavage Medium with 10% SPS and placed in the 5% CO2 incubator for three-four hours until ICSI was performed. The next day, 16-18 hours after ICSI, fertilization was assessed and the zygotes were cultured in 20 µl droplets of Quinn’s Advantage Cleavage Media supplemented with 10% SPS and overlaid with Tissue Culture Oil for an additional 48 hours. On days two and three all embryos were evaluated for cell number and embryo morphology. Each embryo was evaluated for blastomere size and fragmentation. Embryos exhibiting equal blastomere size and no fragmentation were considered G1. G2 embryos have blastomeres of equal size with slight fragmentation while G3 embryos have blastomeres of unequal size but no fragmentation. G4 embryos have blastomeres of equal or unequal size and moderate fragmentation and G5 grade embryos are those with unrecognizable blastomeres and severe fragmentation.

The patients underwent a day-three transfer using only embryos resulting from the vitrified oocytes. One to three embryos were transferred, depending on the embryo quality and availability. Any viable embryos developed from the fresh oocytes as well as any excess embryos resulting from vitrified oocytes were cryopreserved either at the cleavage or at the blastocyst stage, depending on their quality and developmental stage. Embryos with borderline quality that didn’t meet the criteria for cryopreservation on day three were kept in culture until days five or six to allow for blastocyst development. Those embryos were cultured in Quinn’s Blastocyst Media (Sage 1029, Cooper-Surgical, Pasa-
dena, CA) supplemented with 10% SPS until they were either cryopreserved on day five or six were discarded.

**Statistical Analysis:** Chi square or Fisher’s exact test were used for comparison of proportions. *P*≤0.05 was considered statistically significant. Data were expressed as mean ± SD.

**Results**

A total of 164 MII oocytes were retrieved (mean 12.5 ± 3.6), 83 were vitrified, with 72 (86.7%) surviving after warming. Fertilization rate was similar between vitrified (50/72; 69.4%) and fresh oocytes (72/92; 78.2%, *P*=0.8; Fig 1). Cleavage on day two, however, was lower in the vitrified oocytes (44/50; 88% vs 72/72; 100%, *P*=0.004). Implantation rate was 25% (7/28) with a mean number of 2.0 ± 0.5 embryos transferred. Clinical pregnancy rate was 53.8% (7/13) after transferring embryos derived only from vitrified oocytes. Live birth rate was 46.1% (6/13), with six live births (seven babies, one set of twins), and one early miscarriage (Table 2). One additional ongoing pregnancy has been established after transfer of a cryopreserved blastocyst derived from a vitrified oocyte (50% total ongoing pregnancy/ET). One additional subject had two cryopreserved cleavage stage embryos derived from vitrified oocytes, which she elected to discard after a live birth.

The experimental design allowed oocyte outcomes comparison between sibling fresh and vitrified oocytes, but not implantation rate (IR) or live birth rates as only embryos derived from vitrified oocytes were transferred. For that reason, we compared the IVF outcomes after oocyte vitrification with matched age women who underwent IVF in our program using fresh oocytes during the same time period who also underwent day three ET. Clinical pregnancy rates were similar (7/13; 53.8% vs

| Table 2. Vitrified vs Fresh Sibling Oocytes vs Age-Matched Controls for the Same Time Period |
|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| **Vitrified oocytes** (n=13) | **Fresh sibling oocytes** (n=13) | **Controls** (n=639) | **P value** |
| Oocyte survival rate (%) | 86.7 | N/A | |
| Fertilization rate (%) | 69.4 (50/72) | 78.2 (72/92) | 0.8 |
| Mean cell number on day 3 | 5.1 ± 1.3 | 6.5 ± 1.8 | 1.7 |
| Embryo grade on day 3 | 3.2 ± 0.8 | 3.1 ± 0.6 | 0.4 |
| Mean number of ET | 2.0 ± 0.5 | N/A | 1.75 ± 1.1 | 0.4 |
| Implantation rate (%) | 25 (7/28) | N/A | 41.8 (489/1,170) | 0.08 |
| Clinical pregnancy rate/ET (%) | 53.8 (7/13) | N/A | 58.0 (371/639) | 0.7 |
| Miscarriage rate (%) | 14.2 (1/7) | N/A | 17.2 (64/371) | 0.8 |
| Live birth rate/ET (%) | 46.1 (6/13) | N/A | 48.0 (307/639) | 0.8 |
| Multiple pregnancy rate (%) | 16.6 (1/6) | N/A | 31.9 (98/307) | 0.4 |
| Mean gestational age at delivery (weeks) | 38.6 ± 2.5 | N/A | 38.1 ± 4.05 | 0.7 |
| Mean birth weight (gr) | 2,954 ± 533 | N/A | 2,898 ± 472 | 0.7 |
The implantation rate/ET was lower after transferring embryos derived from vitrified oocytes (7/28; 25% vs 489/1170; 41.8%, respectively; \( P = 0.08 \)). This difference was not statistically significant, possibly due to the small sample size (Table 2).

We collected neonatal data on all newborns. Mean gestational age at delivery was 38.6 ± 2.5 weeks, and mean birth weight was 2,954 ± 533 gr. These results did not differ significantly from the control group (38.1 ± 4.0 weeks, and 2,898 ± 472 gr; \( P = 0.7 \)).

All singletons were delivered at term, with only one preterm delivery at 34 weeks (twin gestation) delivered by cesarean section. All other patients delivered vaginally without any obstetrical complications. Interestingly, six newborns were male and one female. One subject reported a male infant born with pyloric stenosis requiring corrective surgery. The same patient recently conceived again after transferring a cryopreserved blastocyst created during the same cycle.

**Discussion**

The main aim of this study was to evaluate the outcomes of a newly established oocyte vitrification program in women undergoing IVF within a short timeframe by simultaneously evaluating fertilization rate and development of embryos derived from vitrified and fresh oocytes from the same stimulated cycle. By transferring only embryos derived from vitrified oocytes, we were able to validate our vitrification technique under an IRB approved research protocol, following current ASRM guidelines. The results of this pilot study suggest this could represent a viable model to quickly assess the efficacy of an oocyte vitrification program.

The Cryotop (Kitazato Biopharma, Japan) method of vitrification, which we aimed to investigate in our study, has been reported as the most efficient method for human oocyte cryopreservation.\(^6\)\(^{,14-16}\) Different approaches in the vitrification procedure have been described, which differ by type and concentration of cryoprotectants and/or the device used. We chose to use Kuwayama’s protocol, which provides an efficient approach to minimize the vitrification volume ensuring consistently high cooling-warming rates and reliable results. Since the Cryotop device is not commercially available in the USA, we chose to use the Cryolock, which is an analogous device. The Cryolock device consists of a fine, transparent polypropylene film attached to a plastic handle and equipped with a cover straw, in which oocytes can be loaded in a very small volume.\(^6\) Less than 1 µl of the final vitrification solution containing the sample is loaded on the filmstrip, and then most of the solution is removed, leaving only a thin layer of solution to cover the sample, and eventually the filmstrip is quickly submerged in the liquid nitrogen. Similarly, at warming the device is removed from the liquid nitrogen and immediately submerged in the rehydration medium.\(^17\) The main disadvantage is that the Cryolock is an open system that may raise biosafety concerns regarding the theoretical risk of cross-contamination during storage.\(^18\)

A few investigators have utilized the sibling-oocyte study design to assess the efficiency of oocyte cryopreservation. Chamayou et al\(^19\) found significantly lower cleavage rate and embryo quality after slow-freezing of oocytes. Patients with infertility indications underwent 11 IVF and 29 ICSI cycles with fresh oocytes. Forty ICSI cycles were performed using frozen-thawed oocytes in 34 couples who did not conceive with fresh oocytes. Clinical pregnancy and live birth rates were extremely low after transferring embryos derived from frozen-thawed as well as from sibling fresh oocytes. Reduced oocyte competence to development has been
reported after oocyte cryopreservation utilizing the slow-freezing technique when compared with sibling fresh cycles, confirming the negative impact of slow-freezing on oocyte potential. It is conceivable that oocyte cryopreservation in general is less effective than the use of fresh oocytes, particularly when the slow-freeze technique is used. In contrast, the reported experience using vitrification of oocytes has resulted in much more favorable outcomes. Most published studies have used a similar open vitrification protocol (Cryotop device, 15% EG + 15% DMSO + 0.5M sucrose). Cobo et al evaluated the outcome of oocyte vitrification using the Cryotop method in an egg donation program by simultaneously evaluating embryos derived from vitrified and fresh oocytes coming from the same stimulated cycle. Embryos derived from vitrified oocytes were preferentially transferred, but some cases of mixed transfers were included. There were no differences in fertilization rates, day two and three cleavage, and blastocyst formation between vitrified and fresh oocytes. Rienzi et al conducted a prospective randomized sibling-oocyte study in a population of infertile patients. They found no differences in fertilization rate, day-two embryo development, and the percentage of excellent quality embryos between vitrified and fresh sibling oocytes. Only the patients who did not conceive in their fresh ET returned to use their vitrified oocytes. Embryo development was assessed on day two, and no information was provided on live birth rates or the incidence of birth defects. Our study therefore differs from previous publications using sibling oocytes, and to our knowledge is the first study in which infertile patients underwent ET with embryos derived from vitrified oocytes without having failed a previous cycle with fresh oocytes. In contrast to Rienzi et al, we found a lower cleavage rate in the embryos derived from vitrified oocytes when compared with their fresh sibling oocytes. In addition, the implantation rate of the embryos resulting from vitrified oocytes was lower when compared with age-matched controls treated during the same time period. These findings could be attributed to the oocyte vitrification learning curve vs an actual difference in outcomes between fresh and frozen oocytes.

The potential lower efficiency of oocyte vitrification has also been recently described by other investigators. Forman et al reported a paired randomized controlled trial in which each patient’s cohort of mature oocytes was divided into two even groups with half undergoing Cryotop vitrification and rapid warming and half serving as controls. The vitrification method was identical to the one used in our study. Interestingly, there was a lower fertilization rate with ICSI (77.9% vs 90.5%), a lower cleavage rate (90.9% vs 99.2%) and a lower rate of usable blastocyst formation obtained from vitrified oocytes when compared with fresh oocytes. In contrast to our study, embryos transferred were mixed and derived from both groups, and implantation rates were assessed by DNA fingerprinting. Similarly to our study, the authors concluded that the IVF process is less efficient after oocyte vitrification, but the equivalent implantation and aneuploidy rates between both groups was reassuring.

Noyes et al performed a literature search on the reported live births resulting from oocyte cryopreservation over the past decade. Of the total 936 live born babies, 1.3% were noted to have birth anomalies. The mean birth weight and the incidence of congenital anomalies are comparable to that of spontaneous conceptions in fertile women or infertile women undergoing IVF treatment. In our small series, we report one case of pyloric stenosis in a male infant. Pyloric stenosis has a multifactorial inheritance pattern, affecting males more commonly than females, with firstborn males affected about four times as often, with an incidence of 2.4 per 1,000 live births in whites. This birth defect was considered an unrelated adverse event, although a possible association with the vitrification procedure cannot be completely ruled out. Likewise, the excess of male infants in our series is intriguing, although this may be due to the small sample size. In the literature review published by Noyes et al, gender information was available for 395 live born babies. The proportion of male infants (47%) was similar to females (53%).

One weakness of our study is its small sample size. The study was designed as a pilot study to quickly assess the effectiveness of oocyte vitrification, including information on pregnancies and live births. The conclusions are strengthened by the prospective, controlled-study design, utilizing a population of infertile patients. Therefore, this information might be more meaningful to counseling patients than reported outcomes obtained from oocyte donation programs, where optimal quality oocytes are being cryopreserved.

In conclusion, the results of this pilot study showed that vitrifying sibling oocytes provides a viable model to quickly assess the efficacy of an oocyte vitrification program, within ASRM ethical guidelines. Since only half of the oocytes are being exposed to the experimental group, participants still have embryos derived from fresh oocytes as a backup. We have shown that embryos derived from oocyte vitrification resulted in optimal live birth rates, although with a lower implantation rate than age matched controls. The lower cleavage and implantation rates noted in this study may indicate a possible detrimental effect of the vitrification process, which may improve with more experience and further refinement of the technique.
REFERENCES


