

# Prospective controlled study to evaluate laboratory and clinical outcomes of oocyte vitrification obtained in in vitro fertilization patients aged 30 to 39 years

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**Objective:** To determine whether the process of oocyte vitrification affects oocyte viability in in vitro fertilization (IVF) patients between 30 and 39 years of age.

**Design:** Prospective controlled study.

**Setting:** Private IVF practice.

**Patient(s):** A total of 30 women assigned and 22 qualified.

**Intervention(s):** Denudation of oocytes, cryopreservation of oocytes using vitrification method in a medium with 15% ethylene glycol (EG), 15% dimethylsulfoxide (DMSO), and 0.5 M sucrose.

**Main Outcome Measure(s):** Oocyte survival, fertilization, day-3 embryo quality, blastocyst formation, clinical pregnancy, implantation, and live-birth rates.

**Result(s):** After denudation of oocytes, mature sibling oocytes were randomly allocated to the fresh and vitrified groups. The survival rate was 79.6% after vitrification/warming. Overall, no statistically significant differences were found in fertilization, day-3 embryo quality, or blastocyst formation rates between the fresh and vitrified groups. The positive  $\beta$ -human chorionic gonadotropin, clinical pregnancy rate, and implantation rate were 13 (59.0%) of 22, 10 (45.4%) of 22, and 16 (30.1%) of 53 for the vitrified group. The overall efficiency in achieving a live birth was 11 (5.9%) of 186 per vitrified oocyte.

**Conclusion(s):** The impact of vitrification can be reduced to a minimal level, making it possible to achieve high pregnancy and implantation rates in this age group of IVF patients. (Fertil Steril® 2013;99:1891-7. ©2013 by American Society for Reproductive Medicine.)

**Key Words:** Cryopreservation, fertility preservation, oocyte vitrification

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In women, aging is the most significant factor influencing the ability to conceive. At approximately 37.5 years of age, acceleration of atresia in

ovarian follicles begins (1). As atresia continues, both the number and quality of oocytes fall below a critical level. This accelerated loss is poorly understood

and is often associated with a monotropic rise in the level of follicle-stimulating hormone (FSH) and decreased fertility (2, 3), as well as an increased rate of aneuploidy, a finding that is related at least in part to problems of the meiotic spindle resulting in chromosome nondisjunction (4, 5). Because oocytes that are lost cannot be replaced, women who do not plan to conceive until after this age may wish to consider options to preserve fertility. Oocyte cryopreservation appears to be a practical fertility preservation method

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for reproductive-age women with social and/or medical reasons to delay starting a family until their late thirties (6).

Cryopreservation is known to have significant effects on oocyte physiology, which may compromise the viability of resulting embryos, especially when slow-freezing protocols are applied (7–9). The alternative approach is vitrification, which is a process of ultrarapid cooling that produces a glass-like solidification. Water is prevented from forming intercellular and intracellular ice crystals during cooling as a result of oocyte dehydration and the use of highly concentrated cryoprotectants (10). Studies have suggested that vitrification can be applied successfully to human oocyte cryopreservation. Even though oocytes can be cryopreserved without ice crystal formation through vitrification, it is still not clear whether other manipulations (temperature change, osmotic stress, cryoprotectant toxicity, and/or phase transitions) will cause negative effects on later embryo developmental potential and subsequent clinical outcomes. Therefore, a systematic evaluation is necessary to ensure the efficacy and efficiency of oocyte vitrification.

To investigate the effect of oocyte vitrification, it is important to exclude factors other than the technique of oocyte vitrification itself, which might influence the clinical efficiency of the process. Besides vitrification, several factors may have an effect on embryo development and clinical outcomes, including the intrinsic quality of the gametes (resulting from female age, ovarian stimulation, and sperm source) and the impact of any *ex vivo* manipulations (oocyte handling, fertilization technique, and *in vitro* embryo culture). Although the efficiency of human oocyte cryopreservation has been improved dramatically by vitrification, providing excellent clinical outcomes (11–19), it is still crucial to determine whether the process of vitrification could cause damage or side effects to embryo development and the subsequent clinical results. Most studies of oocyte vitrification were performed using donor oocytes where quality is expected to be near optimal; however, we know little about vitrification outcomes when autologous oocytes are used in an infertile patient group. Therefore, in this study we recruited infertile patients of middle to high maternal age (30 to 39 years-old) who agreed to vitrify half of their oocytes and use the other half (sibling oocytes) as a fresh control. Vitrified oocytes were warmed within half an hour of vitrification and served as a perfect match to the fresh group within the same cycle and time period. The resulting information presented here may be particularly valuable to female patients who decide to preserve their fertility to delay child-bearing until their thirties.

## MATERIALS AND METHODS

The oocytes were obtained from infertile patients aged between 30 and 39 years who had at least one failed *in vitro* fertilization (IVF) treatment. Patients with fewer than eight mature oocytes retrieved were excluded from the study. To eliminate potential negative paternal effects on embryo development, surgically extracted spermatozoa and severe oligoasthenoteratozoospermia (motile sperm count < 1,000,000/mL before preparation) were also excluded

from this study. A total of 30 patients were enrolled in the study. Eight patients did not have eight mature oocytes at the time of oocyte retrieval and thus were excluded from the study. Before enrollment, all participants signed consent forms that had been reviewed and approved by an institutional review board.

Controlled ovarian hyperstimulation was performed using one of two protocols: gonadotropin-releasing hormone (GnRH) agonist long protocol or GnRH-antagonist protocol with recombinant follicle-stimulating hormone (FSH, Gonal-F; EMDSerono). Recombinant human chorionic gonadotrophin (hCG, Ovidrel; EMDSerono) was administered to trigger nuclear maturation of oocytes when two or more follicles had reached 18 mm. Oocyte retrieval was performed 36 hours after hCG administration. The oocytes were denuded by 30 seconds of exposure to intracytoplasmic sperm injection (ICSI) cumulus (Origio) followed by mechanical denudation 1 to 2 hours after oocyte retrieval. The maturity of denuded oocytes was judged by the presence of the first polar body (PB), which is defined as the metaphase II (MII) stage. The MII oocytes were allocated randomly to the fresh (control) or the vitrified (study) group. Randomization was performed at the moment of oocyte denudation. In the vitrified group, the oocytes were vitrified around 2 to 3 hours after oocyte retrieval, and those oocytes were subsequently warmed after storage for 10 to 20 minutes in liquid nitrogen.

## Oocyte Vitrification

The basal medium used for oocyte cryopreservation was HEPES-buffered M199 medium with Earle's salts, 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and NaHCO<sub>3</sub> (M7528; Sigma) supplemented with 20% (v/v) serum protein substitute (SPS) (Cooper Surgical/Sage). The denuded oocytes were vitrified by minimum volume cooling (MVC) method, as described originally by Kuwayama et al. (11) with some modifications. Briefly, the oocytes were equilibrated in equilibration medium—basal medium with 7.5% (v/v) ethylene glycol and 7.5% (v/v) dimethylsulfoxide (DMSO)—at room temperature for 15 minutes. Oocytes were transferred into the vitrification medium—basal medium with 15% (v/v) ethylene glycol, 15% (v/v) DMSO, and 0.5 M sucrose—at room temperature for 45 to 60 seconds. The cryoprotectant-treated oocytes were placed onto a fine polypropylene strip (Cryotop; Kitazato Bio Pharma Co.). As a final step, polypropylene strips carrying oocytes were then submerged into liquid nitrogen.

## Oocyte Warming

Polypropylene strips with vitrified oocytes were immersed directly into 8.0 mL of warming solution—HEPES-buffered embryo culture medium with 20% (v/v) SPS and 1.0 M sucrose—at 37°C for 1 minute. Oocytes were then picked up and transferred into 1.0 mL of the dilution solution—HEPES-buffered embryo culture medium with 20% (v/v) SPS and 0.5 M sucrose—for 3 minutes at room temperature. The oocytes were subsequently washed in 1.0 mL washing solution—HEPES-buffered embryo culture medium with

20% (v/v) SPS—for 10 minutes at room temperature. Oocytes were incubated in Quinn's advantage cleavage medium (SCM, Cooper Surgical/Sage) with 15% SPS for another 2 to 3 hours before ICSI.

### ICSI Fertilization, Embryo Culture, and Embryo Transfer

The partner's sperm of the female patient was prepared by density gradient centrifugation, and ICSI fertilization was performed as previously described elsewhere (20). The oocytes from both fresh and vitrified groups were all inseminated by ICSI at about the same time around 41 to 42 hours after hCG administration, and fertilization was judged by the formation of two pronuclei 18 hours after ICSI. Embryos were cultured in Quinn's advantage cleavage medium (Cooper Surgical/Sage) with 15% SPS (Cooper Surgical/Sage) and incubated in 6% CO<sub>2</sub>, 5% O<sub>2</sub>, and 89% N<sub>2</sub>. Embryos were evaluated on days 3, 5, and 6, and were assessed for development and quality. Embryo transfer (ET) was always performed with the embryos derived only from vitrified/warmed oocytes. Good-quality day-3 embryos were defined as six to eight cells and ≤20% fragmentation. If there were at least three high-quality embryos present in the vitrified group, the embryo culture would be extended to day-5 for ET.

At 72 hours after insemination, embryos were transferred to Quinn's advantage blastocyst medium (Cooper Surgical Sage) with 15% SPS. At 116 hours after insemination, blastocyst formation was assessed and graded for ET. Embryo transfer was only performed with the embryos derived from the vitrified group.

All ETs were performed either on day 3 or day 5 with a Cook catheter (Cook Medical) under ultrasound guidance. The luteal phase was supported by use of Estrace (estradiol; Warner Chilcot) and intramuscular progesterone. Pregnancy was confirmed by a serial rise in serum hCG concentrations on two consecutive occasions 10 or 12 days after embryo transfer. Clinical pregnancy was determined by ultrasound demonstration of fetal cardiac-activity 30 days after ET. Implantation rate was defined as the number of fetuses with cardiac activity divided by the total number of embryos transferred.

Statistical analyses were performed using chi-square tests when appropriate.  $P < .05$  was considered statistically significant.

## RESULTS

### Outcome Comparison: Fresh Versus Vitrified Oocytes

A total of 30 patients were enrolled in the study. Eight patients did not have eight mature oocytes at the time of egg retrieval and were excluded from the study. A total of 390 oocytes were obtained from 22 IVF patients, who were an average age of 35.4 years. A total of 204 (52.3%) of 390 oocytes were allocated to the fresh group and 186 (47.7%) of 390 were allocated to the vitrified group. The survival rate was 148 (79.6%) of 186 after vitrification/warming. A summary of the fertilization and embryo development from both groups

is shown in Table 1. No statistically significant differences were found between the fertilization rates of fresh and vitrified oocytes (75.0% vs. 66.6%, not statistically significant [NS]) (see Table 1). There was no difference in the good-quality embryo rates between the fresh and vitrified oocytes (49.5% vs. 48.4%, NS) (see Table 1). There also was no difference in the blastocyst formation (day-5/6) rates between the fresh and vitrified groups (53.2% vs. 55.1%, NS) (see Table 1).

All embryos of adequate quality derived from the fresh group were cryopreserved by vitrification. Embryo transfer was only performed with the embryos derived from vitrified group. A total of 53 embryos were transferred in 22 patients (9 patients with day-3 ET and the other 13 patients with day-5 ET), with a mean number of  $2.41 \pm 0.85$  embryos per transfer. After ET, positive serum  $\beta$ -hCG was detected in 13 of 22 embryo recipients. A total of 10 patients had detectable fetal heartbeats confirmed by ultrasound 30 days after embryo transfer.

Of the 53 transferred embryos, 16 implanted, corresponding to an implantation rate of 30.1%. The clinical pregnancy rate was 45.4% per patient (10 of 22). Two of the ten patients with clinical pregnancies had a miscarriage during the first trimester. The remaining eight patients delivered 11 healthy newborns.

There were five singleton and three multiple gestation live births (three twins), resulting in 11 healthy newborns (five males and six females). Five of the eight pregnancies resulted in term deliveries. Three (37.5%) of the eight gestations delivered between 34 to 37 weeks of gestation. The mean gestation age at delivery was 37.4 weeks for singleton and 36.6 weeks for twin gestations. The mean birth weight for singletons and twins were  $3,191 \pm 674$  g and  $2,555 \pm 1,089$  g, respectively. The overall efficiency to achieve live birth was 5.9% (11 deliveries/186 vitrified oocytes). In other words, it took about 16.9 vitrified oocytes to achieve a live birth in this age range (30–39 years old).

### Outcome Comparison: Young Age Versus Advanced Age Vitrified Oocytes

A total of 97 mature oocytes were vitrified from 11 patients in the young age group (range: 30 to 36 years; mean age:  $32.91 \pm 1.97$ ), and 89 mature oocytes were vitrified from 11 patients in the more advanced age group (range: 37 to 39 years; mean age:  $37.90 \pm 0.83$ ). A total 80 (82.5%) of 97 oocytes from the young age group and 68 (76.4%) of 89 from the advanced age group survived after warming (Table 2). No statistically significant differences were found between the fertilization rates of young age and advanced age groups (70.1% vs. 62.9%) (see Table 2).

At 72 hours after insemination, embryo development and quality were assessed. The good-quality embryo rates were statistically significantly different between young and advanced age groups (55.6% vs. 40.4%) (see Table 2). Embryo replacements were only performed with the embryos derived from the vitrified group. A total of 24 embryos were replaced in 11 embryo transfer cycles (mean:  $2.18 \pm 0.6$ ) in the young age group ( $n = 11$ ), and a total of 29 embryos were replaced in

**TABLE 1****The outcome of comparison between fresh versus vitrified oocytes.**

|   | Fresh          | Vitrified            | P value |
|---|----------------|----------------------|---------|
| No. of patient  |                | 22                   | –       |
| Mean age (mean $\pm$ SD)  |                | 35.4 $\pm$ 2.9       | –       |
| Mean basal FSH (mean mIU/mL $\pm$ SD)   |                | 6.20 $\pm$ 1.54      | –       |
| GnRH-agonist protocol (%)   |                | 12/22                | –       |
| Antagonist protocol (%)   |                | 10/22                | –       |
| Days of stimulation (mean $\pm$ SD)   |                | 12.50 $\pm$ 1.00     | –       |
| Total gonadotropin amount, IU (mean $\pm$ SD)                                 |                | 1,739 $\pm$ 865      | –       |
| No. of MII oocytes (mean $\pm$ SD)  |                | 390 (17.8 $\pm$ 9.0) | –       |
| No. of MII oocytes allocated  | 204            | 186                  | –       |
| Survival (%)  | –              | 148 (79.6)           | –       |
| No. of fertilized oocytes (%) [using oocyte starting number as a denominator] | 153/204 (75.0) | 124/186 (66.6)       | NS      |
| No. of good-quality embryos on day 3 (%)                                      | 101/204 (49.5) | 90/186 (48.4)        | NS      |
|   |                |                      | .8399   |
| No. of patients with day 3 ET   |                | 9                    | –       |
| No. of patients with day 5 ET   |                | 13                   | –       |
| No. of blastocysts formed on day 5/6 (%), n = 13                              | 65/122 (53.2)  | 59/107 (55.1)        | NS      |
|   |                |                      | .7919   |
| No. of embryos transferred (mean $\pm$ SD) <sup>a</sup>                       | –              | 53 (2.41 $\pm$ 0.85) | –       |
| No. of positive $\beta$ -hCG (%)  | –              | 13 (59.0)            | –       |
| No. of clinical pregnancies (%)   | –              | 10 (45.4)            | –       |
| No. of implantations (%)  | –              | 16 (30.1)            | –       |
| No. of deliveries (%)   | –              | 8 (36.3)             | –       |
| No. of live births  | –              | 11                   | –       |
| Percentage of oocytes to achieve a live birth (%)                             | –              | 11/186 (5.9)         | –       |

Note: ET = embryo transfer; FSH = follicle-stimulating hormone; GnRH = gonadotropin-releasing hormone;  $\beta$ -hCG =  $\beta$ -human chorionic gonadotropin; MII = metaphase II; NS = not statistically significant; SD = standard deviation.

<sup>a</sup> Only the resulting embryos from vitrified group were transferred.

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11 embryo transfer cycles (mean: 2.64  $\pm$  1.0) in the advanced age group (n = 11). A total of seven patients had detectable fetal heartbeats confirmed by ultrasound 30 days after embryo replacement in the young age group (7 of 11), and

a total of three patients had detectable fetal heartbeats after embryo replacement in the advanced age group (3 of 11).

The clinical pregnancy rate was 63.6% per patient in the young age group and 27.3% in the advanced age group (see

**TABLE 2****The outcome comparison between young age versus advanced age patients' oocytes after vitrification.**

|   | Young age group<br>30–36 y (n = 11) | Advanced age group<br>37–39 y (n = 11) | P value |
|---|-------------------------------------|--|---------|
| Patient age (mean y $\pm$ SD) <sup>a</sup>              | 32.91 $\pm$ 1.97                    | 37.90 $\pm$ 0.83                       | < .0001 |
| Mean basal FSH (mean mIU/mL $\pm$ SD)                   | 6.20 $\pm$ 2.26                     | 6.20 $\pm$ 0.92                        | NS      |
| Survival rate (%)                                       | 80/97 (82.5)                        | 68/89 (76.4)                           | > .9999 |
| Fertilization rate (%)                                  | 68/97 (70.1)                        | 56/89 (62.9)                           | NS      |
| No. of good-quality embryos on day 3 (%) <sup>a</sup>   | 54/97 (55.6)                        | 36/89 (40.4)                           | .3639   |
| No. of embryos transferred (mean $\pm$ SD) <sup>b</sup> | 24 (2.18 $\pm$ 0.6)                 | 29 (2.64 $\pm$ 1.0)                    | NS      |
|   |                                     |  | .2056   |
| No. of clinical pregnancies (%)                         | 7/11 (63.6)                         | 3/11 (27.3)                            | NS      |
| No. of implantations (%)                                | 10/24 (41.7)                        | 6/29 (20.7)                            | NS      |
| No. of take home babies (%)                             | 6/11 (54.5)                         | 2/11 (18.2)                            | NS      |
| No. of live births                                      | 8                                   | 3                                      | NS      |
| Percentage of oocyte to achieve a live birth (%)        | 8/97 (8.2)                          | 3/89 (3.3)                             | NS      |
|   |                                     |  | .1827   |
|   |                                     |  | .2173   |

Note: NS = not statistically significant.

<sup>a</sup> Statistically significantly different.

<sup>b</sup> Only the resulting embryos from vitrified group were transferred.

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Table 2). In the young age group, of the 24 transferred embryos, 10 implanted (41.7%). In the advanced age group, of the 29 transferred embryos, 6 implanted (20.7%).

Six of the 11 young age group patients delivered eight healthy babies, and two of the 11 advanced age group patients delivered three healthy babies. The overall efficiency to achieve live birth per vitrified oocyte was 8 (8.2%) of 97 versus 3 (3.3%) of 89 in the young versus advanced groups, respectively. In other words, it took about 12.1 vitrified oocytes to achieve a live birth in the younger group versus 29.6 vitrified oocytes to achieve a live birth in the advanced age group.

### Outcome Comparison: Agonist Versus Antagonist Stimulation

A total of 90 mature oocytes were vitrified from 12 patients in the agonist group (mean age:  $36.42 \pm 2.19$  years), and 96 mature oocytes were vitrified from 10 patients in the antagonist group (mean age:  $34.20 \pm 3.39$  years). A total 73 (81.1%) of 90 from the agonist group and 74 (77.0%) of 96 from the antagonist group oocytes survived after warming. No statistically significant difference was found between the fertilization rates of the agonist and antagonist groups: 61 (67.7%) of 90 versus 62 (64.5%) of 96, respectively. The good-quality embryo rates were not statistically significantly different between agonist and antagonist groups in day-3 development: 48 (53.3%) of 90 versus 42 (43.7%) of 96, respectively.

Embryo transfers were only performed with the embryos derived from vitrified oocytes. A total of 31 embryos were transferred in 12 ET cycles (mean number:  $2.58 \pm 1.00$ ) in the agonist group ( $n = 12$ ), and a total of 22 embryos were transferred in 10 ET cycles (mean number:  $2.20 \pm 0.63$ ) in the antagonist group ( $n = 10$ ). A total of 5 of 12 patients had detectable fetal heartbeats confirmed by ultrasound 30 days after embryo replacement in the agonist group; a total of 5 of 10 patients had detectable fetal heartbeats after embryo replacement in the antagonist group. The clinical pregnancy rate was 41.6% per patient in the agonist group and 50.0% in the antagonist group. In the agonist group, 9 (29.0%) of the 31 transferred embryos implanted. In antagonist group, 7 (31.8%) of the 22 transferred embryos implanted.

Four of the 12 patients in the agonist group delivered five healthy babies, and four of the 10 antagonist group patients delivered six healthy babies. The overall efficiency to achieve live birth per vitrified oocyte was 5 (5.55%) of 90 versus 6 (6.25%) of 96 in the agonist and antagonist groups, respectively. There were no statistically significant differences in any outcome as a function of the stimulation protocol.

### Outcome Comparison: Day-3 ET Versus Day-5 ET

In the day-3 ET group, a total of 78 mature oocytes were vitrified from 9 patients (mean age:  $36.78 \pm 2.22$  years), and 108 mature oocytes were vitrified from 13 patients in the day-5 ET group (mean age:  $34.46 \pm 3.09$  years;  $P < .05$  between day-3 and day-5 ET age groups). A total 53

(67.9%) of 78 from the day-3 ET group and 94 (87.0%) of 108 from the day-5 ET group oocytes survived after warming. A total 43 (55.1%) of 78 from the day-3 ET group and 80 (74.7%) of 108 from the day-5 ET group oocytes fertilized after ICSI. The good-quality embryo rates on day 3 were 31 (39.7%) of 78 versus 59 (54.6%) of 108 on day 5.

Embryo replacements were only performed with the embryos derived from vitrified oocytes. A total of 23 embryos were replaced in nine embryo transfer cycles (mean number:  $2.56 \pm 1.01$ ) in the day-3 ET group, and a total of 30 embryos were replaced in 13 embryo transfer cycles (mean number:  $2.31 \pm 0.75$ ) in the day-5 ET group. A total of 3 of the 9 patients had detectable fetal heartbeats confirmed by ultrasound 30 days after day-3 ET, and a total of 7 of the 13 patients had detectable fetal heartbeats after day-5 ET. The clinical pregnancy rate was 33.3% per patient in the day-3 ET group and 53.8% in the day-5 ET group (NS). In the day-3 ET group, 5 (21.73%) of 23 transferred embryos implanted. In the day-5 ET group, 11 (36.66%; NS) of 30 transferred embryos implanted.

One of the 9 day-3 ET group patients delivered one healthy newborn, and 7 of the 13 day-5 ET group patients delivered 10 healthy newborns. The overall efficiency to achieve live birth was 1 (1.28%) of 78 versus 10 (9.26%) of 108 in the day-3 ET and day-5 ET groups, respectively ( $P < .05$ ). In the day-3 ET group, there was one singleton gestation, resulting in a term delivery (38.0 weeks' gestation) with a healthy newborn weighing 2,353 g. In the day-5 ET group, there were four singleton and three twin gestation live births, resulting in 10 healthy newborns. Among the pregnancies from the day-5 ET group, 4 of the 7 pregnancies resulted in term deliveries. The mean gestation age at delivery was 37.1 weeks for singleton and 36.7 weeks for twin gestations. The mean birth weight for singletons and twins were  $3,401 \pm 560$  g and  $2,555 \pm 1,089$  g, respectively.

## DISCUSSION

The capacity of a mature oocyte to support the initial stage of embryo development and implantation is termed oocyte developmental competence and is a measure of intrinsic oocyte quality. Cryopreservation is known to have important effects on oocyte physiology (7–9), which may compromise the developmental competence of the resulting embryos. Since the first successful human oocyte cryopreservation was performed (21), techniques have been improving consistently and have greatly impacted clinical infertility treatment (11, 22–24). Even though oocyte cryopreservation was promptly adopted and applied to the human IVF field (25), there are few data regarding oocyte quality after oocyte cryopreservation, especially in infertile women, a group of more advanced reproductive age.

This investigation of the impact of oocyte vitrification on laboratory and clinical outcomes evaluated early embryo development by comparing fresh and vitrified sibling oocytes as well pregnancy and implantation rates (using embryos derived only from vitrified/warmed eggs). The results show that fertilization and early embryo development (defined by

day-3 good-quality embryo rates and blastocyst formation rates) were not statistically significantly different in the vitrified oocytes compared with the fresh sibling oocytes. The clinical outcomes in this study also provide valuable information about the expected efficiency of oocyte vitrification when used for patients who chose fertility preservation for social reasons.

### Impact of Oocyte Quality after Vitrification

To measure the specific effect of oocyte vitrification, it is necessary to exclude all the potential factors that could affect final outcomes. Besides oocyte vitrification, there are several factors that may have an effect on the final clinical outcomes, such as the intrinsic quality of the gametes (particularly as related to female age, ovarian stimulation, and sperm source) and the impact of any *ex vivo* manipulations (oocyte handling, fertilization technique, and *in vitro* embryo culture) (26).

First, to ensure that gametes with similar quality were distributed across the treatment groups, we retrieved sibling oocytes from the same individual on the same day. To eliminate the differences in *ex vivo* manipulations, the sibling oocytes, fresh or vitrified/warmed, were handled, inseminated, and cultured in the same conditions at the same time. Because a portion of the oocytes did not survive the cryopreservation process, we controlled for possible discrepancies in the efficiency calculations between the fresh and vitrified group by using the oocyte starting number as the denominator to make the comparisons between the fresh and vitrified group (Table 1) (26). Fertilization rates were similarly high in both groups, although a trend might be noted for higher fertilization in the fresh group when the oocyte starting number was used as a denominator. Embryo development was also comparable between both groups (see Table 1).

Two important observations can be derived from this study. First, the fertilization rate was not reduced significantly by oocyte vitrification. Second, embryo developmental competence was not significantly affected by the oocyte vitrification. Implantation and pregnancy rates also remained at high levels (comparable to historical results in similar patients in our practice, as well as in comparison with published studies). It seems clearly that oocytes still retain high viability after vitrification as well (as shown by the implantation rates).

### Age Effect

The age of the woman at the time of oocyte retrieval has a major impact on clinical outcomes and is probably the most prominent factor related to IVF success (27). The impact of increasing female age is not only associated with a statistically significant decrease in human ovarian follicle number (28, 29), but also is related to increasing chromosome abnormalities such as nondisjunction (30) and in relation to the spindle assembly checkpoint (31) in human oocytes. In our study, the patients' baseline ovarian reserve characteristics did not statistically significantly differ between the young and advanced-age groups (Table 2). A trend might be noted that both survival and fertilization rates

were lower in the advanced age group (see Table 2); although due to the sample size we were unable to detect a statistically significant difference.

Our study also shows that embryo developmental potential (the good-quality embryo rate on day 3) was also significantly impacted by the patients' age (see Table 2). This is consistent with existing data that show embryo development rates are a highly significant indicator of implantation potential in both fresh and cryopreserved embryos (32–34). Thus, it should not come as a surprise to observe a trend of higher pregnancy and implantation rates in the younger patients using their own vitrified oocytes when compared with the advanced age group doing the same (see Table 2). Again, our limited sample size prevented the numerical difference from reaching statistical significance.

The overall efficiency of vitrified oocytes could be appraised by the “percentage of oocytes to achieve implantation/live birth” or “number of oocytes to achieve implantation/live birth” (13, 16). In our study, the overall efficiency to achieve a live birth was very similar when comparing agonist versus antagonist stimulation: 5 (5.55%) of 90 versus 6 (6.25%) of 96. There were no statistically significant differences found in the outcomes between agonist and antagonist stimulation cycles. We observed that 8.2% of vitrified oocytes from the younger group resulted in a live birth (12.1 vitrified oocytes per live birth) versus 3.3% of vitrified oocytes from the advanced age patients (29.6 vitrified oocytes per live birth) (see Table 2). These observations and findings suggest that instead of the vitrification process or stimulation protocol, maternal age is the determining factor in our results, with obvious advantages for the younger group of patients (30 to 36 years old) when compared with the advanced age patients (37 to 39 years old).

### Embryo Stage at Transfer

The day-5 embryo transfer pregnancy and implantation rates were not statistically significantly higher (though a trend is clearly observable) compared with the day-3 transfers (probably due to sample size). However, there was a statistically significantly higher live-birth rate per oocyte warmed in the day-5 transfer group compared with the day-3 transfer group. This finding is not unexpected because patients in the day-3 transfer group were older and had fewer high quality embryos than the patients in the day-5 transfer group. Importantly, embryo characteristics obtained from the fresh “sibling” oocytes in the day-3 transfer group were very similar to those embryos obtained after vitrification/warming, suggesting that the lower observed embryo development/quality in this group was unrelated to the cryopreservation procedure. This specific finding from our study is similar to the observation published by Rienzi et al. (35).

### CONCLUSIONS

Our study has demonstrated that the clinical efficiency of oocyte vitrification is high, and both the fertilization and

development rates are comparable to fresh sibling oocytes. These results indicate that the impact of vitrification can be reduced to minimal levels, making it possible to achieve high pregnancy and implantation rates in this group of IVF patients. These results open a wide range of possibilities for a similar age group of general (assumed fertile) population, such as those seeking fertility preservation for social or medical indications. Female age remains the most critical factor that could influence clinical outcomes after oocyte vitrification.

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