Prospective randomized comparison of human oocyte cryopreservation with slow-rate freezing or vitrification

Gary D. Smith, Ph.D.,[a,b,c,d] *Paulo C. Serafini, M.D., Ph.D.,*[e,f] *Joyce Fioravanti, B.S.,*[e] *Isaac Yadid, M.D.,*[g] *Marcio Coslovsky, M.D.,*[g] *Pericles Hassun, Ph.D., D.V.M.,*[h] *José Roberto Alegretti, B.S.,*[e,h] *and Eduardo L. Motta, M.D., Ph.D.*[e,i]

[a] Department of Obstetrics and Gynecology, [b] Department of Urology, [c] Department of Physiology, and [d] Department of Reproductive Science Program, University of Michigan, Ann Arbor, Michigan; [e] Huntington Medicina Reprodutiva, Sao Paulo; [f] Department of Obstetrics and Gynecology, Universidade de Sao Paulo, Sao Paulo; [g] Huntington Medicina Reprodutiva, Rio de Janeiro; [h] Genesis Genetics Brazil, São Paulo; and [i] Department of Gynecology, Universidade Federal de Sao Paulo, São Paulo, Brazil

Objective: To compare cryopreservation of mature human oocytes with slow-rate freezing and vitrification and determine which is most efficient at establishing a pregnancy.
Design: Prospective randomized.
Setting: Academically affiliated, private fertility center.
Patient(s): Consenting patients with concerns about embryo cryopreservation and more than nine mature oocytes at retrieval were randomized to slow-rate freezing or vitrification of supernumerary (more than nine) oocytes.
Intervention(s): Oocytes were frozen or vitrified, and upon request oocytes were thawed or warmed, respectively.
Main Outcome Measure(s): Oocyte survival, fertilization, embryo development, and clinical pregnancy.
Result(s): Patient use has resulted in 30 thaws and 48 warmings. Women's age at time of cryopreservation was similar. Oocyte survival was significantly higher following vitrification/warming (81%) compared with freezing/thawing (67%). Fertilization was more successful in oocytes vitrified/warmed compared with frozen/thawed. Fertilized oocytes from vitrification/warming had significantly better cleavage rates (84%) compared with freezing/thawing (71%) and resulted in embryos with significantly better morphology. Although similar numbers of embryos were transferred, embryos resulting from vitrified oocytes had significantly enhanced clinical (38%) pregnancy rates compared with embryos resulting from frozen oocyte (13%). Miscarriage and/or spontaneous abortion rates were similar.
Conclusion(s): Our results suggest that vitrification/warming is currently the most efficient means of oocyte cryopreservation in relation to subsequent success in establishing pregnancy. (Fertil Steril® 2010;94:2088–95. ©2010 by American Society for Reproductive Medicine.)

Key Words: Prospective randomized study, human oocyte cryopreservation, slow-rate freezing, vitrification, pregnancies

Assisted reproductive technologies (ARTs) to treat infertility and obtain healthy offspring have become an important means of procreation. The use of ARTs have increased both nationally and internationally, with an estimated 55,600 children born in 2006 within the United States (1) following in vitro fertilization (IVF)/embryo transfer (ET) and frozen embryo transfer. Although success rates of ARTs have increased over the last 3 decades, the ability to cryopreserve oocytes has been met with limited success. Numerous clinical utilities and practical advantages of oocyte cryopreservation have been recognized and consist of the following: [1] preservation of fertility in women at risk of losing fertility because of chronic disease and/or treatment; [2] allowing flexibility for assisted reproductive programs if initial treatment cycles had to be halted; [3] reducing management burden within an oocyte donation treatment cycle; [4] potential fertility option for patients who choose/require postponing childbirth until advanced age; [5] decreasing the expense of infertility treatment; and finally, [6] providing another option to patients concerned with ethical and legal issues of embryo cryopreservation.

Currently, there are two methods to cryopreserve mammalian oocytes: slow-rate freezing and vitrification (2, 3). Both methods can affect oocyte cellular functions and compromise the ability to develop normally following the cryopreservation process. Documented and/or theoretic specific cellular structures and functions that are/may be compromised by cryopreservation, as well as subsequent effects on oocyte and embryonic developmental competence, have been previously reviewed (4). During cryopreservation, cells are exposed to numerous stresses including mechanical, thermal, and chemical (5, 6), which can lead to compromised cell function and death. In general, it has been demonstrated that oocytes are more sensitive to cryodamage than later embryonic stages (7).

Slow-rate freezing attempts to control biophysical properties of freezing, like cooling and warming rates, in conjunction with cryoprotective agents to minimize adverse cellular events. This method, considered an equilibrium approach, allows cells to be cooled to very low temperatures while minimizing intracellular ice crystal formation, and at the same time attempting to minimize detrimental

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Reprint requests: Eduardo L. Motta, M.D., Ph.D., Department of Gynecology, Universidade Federal de Sao Paulo and Huntington Medicina Reprodutiva, Av. República do Líbano, 529 Ibirapuera, São Paulo, SP, Brasil 04501-000 (FAX: 5511 30596100; E-mail: emotta@huntington.com.br).

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influences of increased solute concentrations and osmotic stress (7). Because some ice formation occurs with freezing, the inverse procedure is considered "thawing" for reanimation of cells. Slow-rate freezing has a long history of success for cryopreservation of human zygotes and embryos (8), and more recently with human oocytes (9–13). Conversely, vitrification is a form of rapid cooling, uses high concentrations of cryoprotectant that solidify without forming ice crystals. Vitrification can be considered a nonequilibrium approach to cryopreservation, originally developed for tissues and embryos (14–16). The vitrified solids therefore contain the normal molecular and ionic distributions of the original liquid state, and can be considered an extremely viscous, supercooled liquid (17). Thus, without ice crystal formation vitrified samples are not thawed, but "warmed" to obtain the cryopreserved cells. Human oocytes (18–21), pronuclear zygotes (22), cleavage-stage embryos (23), and blastocysts (24–26) have been successfully vitrified. Excellent reviews of vitrification history, utilization, and potential advantages are available (27, 28).

Although a meta-analysis has been performed comparing freezing and vitrification processes in the outcomes of human oocyte cryopreservation (29), no investigational comparative study for both methods currently exist. Thus, our objective was to compare human mature oocyte freezing and vitrification in a prospective randomized manner with a focus on oocyte survival, embryo development, and pregnancy outcome measures.

MATERIALS AND METHODS

This prospective study was performed at Huntington Medicina Reprodutiva in Sao Paulo and Rio de Janeiro, Brazil, in accordance with requirements of the Declaration of Helsinki. Couples that expressed concern regarding ethical or social issues of embryo cryopreservation were informed of the investigational option of oocyte cryopreservation, the two technical options available and published [slow-rate freezing (30) and vitrification (31)], and the lack of knowledge of which technique was superior. Potential couples were then provided informed consent and invited to participate in this institutional review board approved study.

Before beginning this trial, yearly data from 2004 were analyzed to determine the number of mature oocytes retrieved following controlled ovarian stimulation and its impact on subsequent pregnancy success. It was found that when the number of oocytes exceeded nine the resulting pregnancy success plateaued. Thus, oocyte cryopreservation was offered to those couples that conveyed concerns with embryo freezing and had greater than nine mature oocytes retrieved in their controlled ovarian stimulation cycle. Two hundred thirty patients participated in this trial. This patient population displayed: [1] infertility attributable to tubal factor, severe male factor or unexplained factor; [2] regular, spontaneous menstrual cycles of 25 to 35 days; [3] acceptable follicular phase serum concentrations of follicle stimulating hormone (FSH; $\leq$10 IU/L), luteinizing hormone (LH; $\leq$13.5 IU/L), and estradiol (E_2; $\leq$60 pg/mL); [4] body mass index $\leq$30 kg/m^2; [5] presence of both ovaries and normal uterine cavity; and [6] willingness to participate in the study and comply with procedures. Patients gave their written informed consent and were not participating in any other trial. Patients were excluded from the study when they had [1] previous history of ovarian hyperstimulation syndrome; [2] previous history of intolerance to any of the agents used in the study; [3] clinically significant conditions/disease or active substance abuse; [4] abnormal gynecologic bleeding of unknown origin; and [5] if their fertility treatment entailed preimplantation genetic screening. Potential participants were informed that other hormonal treatments, anti-inflammatory medication, or psychotropic agents with known effect on ovarian stimulation were to be avoided.

All participating patients had an ultrasound scan and serum evaluation of FSH, LH, and E_2 on day 2 of their menses and were stimulated with conventional antagonist protocols (32). Briefly, patients were started on day 3 with r-FSH (Gonal-F, Merck-Serono Labs, Geneva, Switzerland) and monitored with serial transvaginal ultrasounds to monitor and control follicular growth and endometrial thickness (development). From day 5 of stimulation (day 7 of menstrual cycle) until the end of the stimulation protocol, adjustments of r-FSH dose were allowed. When the leading follicle reached 14 mm, 0.25 mg of GnRH-antagonist (Cetrotide; Merck-Serono Labs) was administered daily until final follicular maturation. When at least three mature follicles with mean diameter $\geq$19 mm were detected on ultrasound, 250 μg of recombinant hCG (Ovidrel; Merck-Serono Labs) were administered. This was followed by transvaginal ultrasound-guided oocyte retrieval approximately 35 to 36 hours later. After oocyte aspiration, cumulus cells were removed from all oocytes and nine mature (metaphase II, MII) oocytes were randomly selected for insemination by intracytoplasmic sperm injection (ICSI) (33), embryo growth, and transfer in attempting pregnancy in the controlled ovarian stimulation cycle.

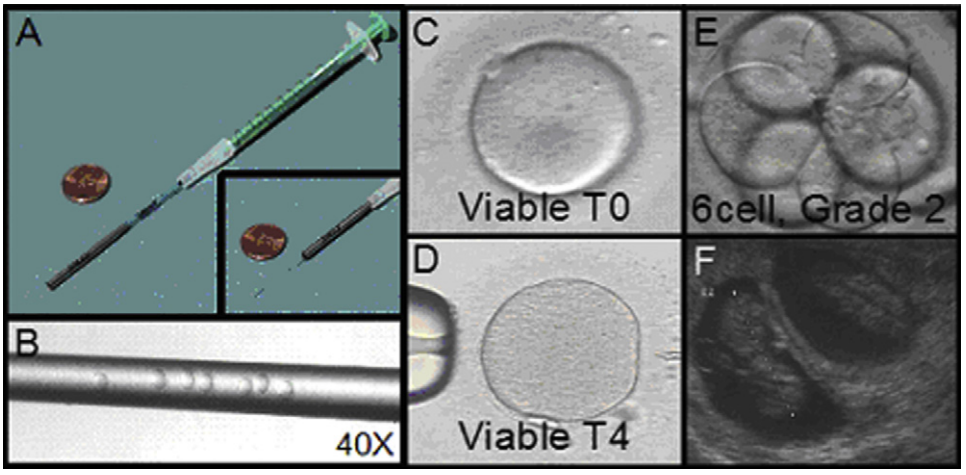
Oocyte Cryopreservation

Patients with supernumerary mature oocytes (more than nine mature oocyte recovered after cumulus cell removal from the controlled ovarian stimulation IVF/ICSI cycle), who consented to participate in oocyte cryopreservation, were randomly allocated by random number generator to oocyte cryopreservation by either slow-rate freezing or vitrification. Oocyte freezing or vitrification were performed by fully trained individuals using previously published techniques (21, 30). Briefly, for freezing denuded mature oocytes were first placed into Dulbecco's phosphate-buffered solution (PBS) with 12% synthetic serum substitute (wt/vol; SSS; Irvine Scientific, Santa Ana, CA) and 1.5 M propanediol at 22°C for 10 min. Oocytes were then transferred to PBS, 12% SSS, 1.5 M propanediol, and 0.3 M sucrose at 22°C for 5 min. Within this solution one to four oocytes were loaded into a 0.25-cc cryopreservation straw (Cryo Bio Systems, SPECTRUN Ltd, Sao Paulo, Brazil) and placed into a programmable freezer (CL5500, CryoLogic, Victory, Australia) at 20°C. The program decreased temperature to −7°C at 2°C/min, held at −7°C to allow manual seeding, and subsequently dropped to −30°C at 0.3°C/min. Samples were plunging in liquid nitrogen and stored until thawing. At thawing straws containing oocytes were removed from liquid nitrogen, held at 22°C for 30 seconds, and then immersed into water at 30°C for 40 seconds. Oocytes were then expelled from straws into PBS, 12% SSS, 1.0 M propanediol, 0.3 M sucrose for 5 minutes exposure at 22°C. Oocytes were subsequently moved through PBS, 12% SSS, 0.5 M propanediol, 0.3 M sucrose for 5 minutes, then PBS, 12% SSS, 0.3 M sucrose for 10 minutes, and finally PBS, 12% SSS for 10 minutes before transfer into pre-equilibrated 37°C G1 media (Vitrolife, Goteborg, Sweden) supplemented with SSS to a total protein content of 12% (wt/vol) and placed into a humidified, 37°C environment with 7.0% CO_2 and air until ICSI was performed.

For vitrification, denuded MII oocytes were initially placed into a 20-μL drop of M-199 HEPES-buffered medium (M-199-H), 12% SSS (wt/vol) for 1 minute before merging with an adjacent 20-μL drop of equilibration solution (7.5% [vol/vol] ethylene glycol, 7.5% [vol/vol] dimethylsulfoxide [DMSO], 12% [wt/vol] SSS in M-199-H). After 2 minutes a second 20-μL drop of equilibration solution was merged with drops containing oocytes. Two minutes later oocytes were removed and pipetted into a fresh 20-μL drop of equilibration solution for 3 minutes. Oocytes were subsequently pipetted into separate 20-μL drops of vitrification solution (15% [vol/vol] ethylene glycol, 15% [vol/vol] DMSO, 0.5 M sucrose, 12% [wt/vol] SSS in M-199-H) for 5 seconds, 5 seconds, 10 seconds, and 90 seconds. All solution exposures were performed at 22°C. During the final 90 seconds in vitrification solution, oocytes to be cryopreserved were loaded into pulled straws (CryoTip, Irvine Scientific) with this solution, heat sealed at the thin end, had protective metal jackets positioned over the thin portion of the straw, were heat sealed at the large end (Fig. 1A and B), and submerged in liquid nitrogen. Closed-pulled straws were maintained in liquid nitrogen before warming. For warming, straws containing oocytes were rapidly transferred from liquid nitrogen into a 37°C water bath for 3 seconds. After straws were wiped with sterile gauze, both ends were opened and the contents expelled as approximately a 1-μL drop (straw content drop). Straws were rinsed with $\sim$1 μL of initial warming solution (1.0 M sucrose, 12% [wt/vol] SSS in M-199-H), expelled as a drop and merged with the straw content drop for 1 minute. Oocytes were transferred into a 20-μL drop of initial warming solution for an additional minute followed by movement through 2 $\times$ 20 μL

FIGURE 1

Representative photographs, micrographs, and sonograph of various aspects of oocyte vitrification, fertilization, embryo development and clinical pregnancies. (**A**) Photograph of closed-pulled straw system and (**B**) micrograph of oocytes within the narrow portion of a close-pulled straw which is ~200 μm in diameter. (**C**) Representative micrograph of a viable oocyte at initial warming (T0). Oocyte diameter ~135 μm. (**D**) Representative micrograph of a vitrified oocyte that is still viable 4 hours after warming (T4), just before ICSI. (**E**) Day 3 human embryo following oocyte vitrification/warming, insemination by ICSI, and embryo culture. This embryo was six-cell grade 2 (11%–20% fragmentation) and representative of average embryo morphology on day of transfer following oocyte warming. (**F**) Sonogram representing a 10-week twin clinical pregnancy following transfer of three embryos resulting from oocyte vitrification/warming.



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drops of dilution solution (0.5M sucrose, 12% [wt/vol] SSS in M-199-H) for 2 minutes each, and finally through 3 × 20 μL drops of wash solution (12% [wt/vol] SSS in M-199-H) for 2 minutes per drop. All solution exposures were again performed at 22°C. Oocytes were then moved into preequilibrated 37°C G1 media supplemented with SSS to a total protein content of 12% (wt/vol) and placed into a humidified, 37°C environment with 7.0% CO_2 and air until ICSI was performed.

All patients who failed to achieve a pregnancy in the fresh cycle and had supernumerary oocytes cryopreserved were provided the option to transfer embryos derived from frozen/thawed or vitrified/warmed oocytes. Uterine preparation for embryo transfer following oocyte thawing or warming was similar to established protocols for frozen embryo transfer (34). Briefly, all patients underwent a transvaginal ultrasound evaluation for adnexal cysts and serum evaluation of progesterone (P) and E_2 on menstrual cycle day 1. If cysts were ≤2 cm, serum P ≤1.0 ng/mL, and E_2 ≤60 pg/mL patients began 6 mg of oral 17-β-estradiol (Estrofem; Medley Labs, Brazil) for a period between 12 and 24 days until a trilaminar endometrium exceeding 8 mm in thickness was identified. At this time, two micronized P capsules (Utrogestan 200 mg; Farmoquimica Labs, Brazil) were administered vaginally three times daily for a period of 4 days to prepare for day 3 embryo transfer; and this continued up to 12 weeks' of pregnancy along with the E_2.

In Vitro Fertilization, Embryo Growth, Outcome Measures, and Statistics

Oocytes were assessed for viability at 0 and 4 hours after completion of the thawing or warming process. Oocytes were considered viable at initial thaw or warming (T0; Fig. 1C) at the light microscope level if they contained an intact oolemma, were not shrunken or darkly granulated, and fully expanded with minimal perivitelline space between the oolemma and zona pellucida. Surviving mature oocytes were inseminated by ICSI 4 hours (T4) after completion of the thawing/warming procedure (Fig. 1D). At 16 to 20 hours postinsemination oocytes/presumptive zygotes were assessed for evidence of fertilization. Zygotes with two pronuclei and two polar bodies were considered normally fertilized and transferred into a 50-μL microdrop of embryo growth media (G1 media; VitroLife), overlaid with mineral oil, and cultured as a group under humidified conditions in 5% CO_2 and air at 37°C. Embryo development was assessed every 24 hours. On day 3 after ICSI a final embryo grade was assigned based on morphologic classifications comprised of blastomere number and percentage of fragmentation; Fig. 1E). Grades of fragmentation were 1: ≤10% fragmentation, 2: 11 to 20% fragmentation, 3: 21 to 50% fragmentation, and 4: >50% fragmentation (35). Selected embryos were subjected to laser-assisted hatching and transferred into modified human tubal fluid (HTF-HEPES + 50% serum substitute supplement; Irvine Scientific), loaded into a Sydney IVF embryo transfer catheter of 23 cm (Cook IVF, Brisbane, Australia), and transferred into the uterine lumen under trans-abdominal ultrasound guidance. Serum β-hCG levels were measured 12 days after embryo transfer to determine biochemical pregnancy. Transvaginal ultrasound was performed at 6 weeks of gestation to confirm a clinical pregnancy consisting of a gestational sac and heartbeat.

Statistical analyses were performed for both parametric and nonparametric outcome measures. Parametric outcomes were analyzed by unpaired Student's *t*-test with differences considered significant at $P<.05$. Nonparametric outcomes were subjected to chi-square analysis and differences considered significant at $P<.05$.

RESULTS

From initiation of the study in January 2005 until April of 2009, 230 patients consented to have their oocytes cryopreserved. From those patients, 78 did not get pregnant within their fresh IVF cycles and returned to the clinic requesting an oocyte thaw or warming procedure to achieve a pregnancy. This equated to 30 cases of oocyte thawing and 48 cases of warming. Average ages of female patients, at the time of oocyte collection and cryopreservation, that had oocytes thawed or warmed, were 31 ± 1 and 32 ± 1 years, respectively, and not significantly different. Table 1 shows patient ovarian stimulation and noncryopreserved IVF cycle characteristics for patients who subsequently had oocytes thawed or warmed. There were no

TABLE 1

Female patient demographics and fresh IVF cycle ovarian stimulation and laboratory outcomes from which supernumerary oocytes were cryopreserved by either slow-rate freezing or vitrification.

Demographics or outcome measures	Slow-rate freezing/thawing	Vitrification/warming	*P* value
Patients (N)	30	48	ns
Age (y)	31 ± 1	32 ± 1	ns
Previous IVF attempts	0.4 ± 0.1	0.2 ± 0.1	ns
Starting does of FSH/day (IU)	198 ± 14	196 ± 11	ns
Total FSH use/cycle (IU)	1,659 ± 137	1,731 ± 100	ns
Total gonadotropin (FSH + LH)/cycle (IU)	1,832 ± 101	1,897 ± 93	ns
Days of gonadotropin stimulation	10 ± 0.2	9.8 ± 0.2	ns
Oocytes collected	21 ± 1.8	19.2 ± 1.3	ns
Mature oocytes (%)	88	83	ns
Fertilized (%)	79	83	ns
Cleavage (%)	88	88	ns
Day 3 embryo cell number	7.0 ± 0.1	6.8 ± 0.1	ns
Day 3 embryo grade	2.0 ± 0.1	2.1 ± 0.1	ns

Note: Values are means ± SE unless otherwise indicated. FSH = follicle stimulating hormone; LH = luteinizing hormone; ns = not statistically significant ($P>.05$).

Smith. Freezing and vitrification of oocytes. Fertil Steril 2010.

significant differences in number of previous IVF attempts, exogenous gonadotropin stimulation doses used, gonadotropin stimulation duration, resulting oocyte fertilization, or embryo development.

Once an oocyte thaw or warming cycle was initiated, a semen sample was collected from male partners and analyzed. No significant differences existed in seminal parameters of volume, sperm concentration, percent motility, or total motile sperm per ejaculate between semen samples prepared for insemination of thawed or warmed oocytes (Table 2).

Oocyte survival, fertilization, embryo development, and resulting pregnancy rates were analyzed following oocyte freezing/thawing and vitrifying/warming (Table 3). The average number of oocytes thawed or warmed were 7.9 ± 0.5 and 7.3 ± 0.3, respectively, and not significantly different. Initial survival of cryopreserved oocytes was significantly greater in the vitrification/warmed group compared with the frozen/thawed group ($P<.001$). Based on information from oocyte cryopreservation and metaphase spindle depolymerization and repolymeration in the mouse model system (35, 36); we incubated thawed and warmed oocytes in culture media at 37°C within CO_2 incubators for 4 hours before insemination by ICSI. Survival of oocytes at 4 hours after thawing or warming was still significantly higher in the vitrification group ($P<.01$). Of note, three couples from the oocyte frozen/thawed arm of the study had no surviving oocytes to inseminate, whereas all couples from the vitrified/warmed arm of the study had oocytes that survived so as to be injected. Percentage of inseminated cryopreserved and surviving oocytes that fertilized normally were significantly lower following thawing compared with warming ($P<.03$). These fertilization rates were similar to rates obtained in fresh oocytes (Table 1), and lysis rate following ICSI was <3%, and well within ART standards. Two couples had no fertilization of thawed oocytes, whereas one couple had no fertilization of warmed oocytes. None of these patients had failed fertilization in their noncryopreserved cycle.

Significantly more zygotes resulting from vitrified oocytes cleaved after fertilization, compared with those from oocytes that had been frozen ($P<.01$). For five patients, all of their oocytes that had been frozen, thawed, and fertilized, failed to cleave; whereas all of those patients whose oocytes had been vitrified and fertilize had zygotes that cleaved. Additionally, one patient with oocytes thawed had embryo development arrest after initial cleavage, which did not occur in the oocyte warming group. Patients that had cleavage failure had no historic indication of cleavage failure in noncryopreservation oocyte cycles. On day 3 (day 0 = day of oocyte thaw or warming) average embryo development was significantly compromised following oocyte thawing compared with warming. Average cell number and grade of embryos derived from oocyte thawing were 5.1 ± 0.2 (mean ± SE) and 2.5 ± 0.1, respectively, compared with embryos derived from oocyte warming, which were 5.9 ± 0.2 and 2.1 ± 0.1 (example: Fig. 1E), respectively. Similar high-quality embryos resulting from oocyte cryopreservation

TABLE 2

Semen parameters of samples used for intracytoplasmic sperm injection of oocytes cyropreserved with slow-rate freezing or vitrification.

Oocyte cryopreservation	Volume (mL)	Concentration ($\times 10^6$/mL)	Motility (%)	Total motile sperm ($\times 10^6$)
Slow-rate freezing (n = 30)	1.9 ± 0.2	33.6 ± 7.3	62 ± 5	46.6 ± 10.8
Vitrification (n = 48)	2.2 ± 0.2	44 ± 6.9	55 ± 4	64.4 ± 11.4

Note: Values are means ± SE unless otherwise indicated. All values were not statistically significant ($P>.05$).

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TABLE 3

Oocyte survival and functionality following cryopreservation by slow-rate freezing/thawing or vitrification/warming.

	Slow-rate freezing/thawing	Vitrification/ warming	*P* value
Thaws/liquefactions cycles	30	48	NA
Oocytes thawed/warmed	238	349	NA
Oocyte thawed/warmed per treatment (mean ± SE)	7.9 ± 0.5	7.3 ± 0.3	NS ($P=.3$)
Survival T0 (%)	159/238 (67%)	281/349 (81%)	$P<.001$
Survival T4 (%)	155/238 (65%)	260/349 (75%)	$P<.01$
Patients with no oocytes for ICSI (#)	3	0	NA
Fertilization (%)	104/155 (67%)	200/260 (77%)	$P<.03$
Patients with no fertilization (n)	2	1	NA
Cleavage D1 to D2 (%)	74/104 (71%)	168/200 (84%)	$P<.01$
Patients with no cleavage (n)	5	0	NA
Patients with embryos arrest D2 (n)	1	0	NA
Patients with no ET (n)	11	1	NA
Biochemical pregnancies/thaw or warming cycle (%)	5/30 (17%)	22/48 (46%)	$P<.01$
Clinical pregnancies/thaw or warming cycle (%)	4/30 (13%)	18/48 (38%)	$P<.02$
Clinical pregnancies/oocytes thawed or warmed (%)	4/238 (1.7%)	18/349 (5.2%)	$P<.03$

Note: NA = not applicable; NS = nonsignificant; Survival T0 = number of oocytes surviving cryopreservation immediately after thaw/warming solution exposure; Survival T4 = number of oocytes surviving cryopreservation after 4 hours of incubation at 37°C following thaw/warming; ICSI = intracytoplasmic sperm injection; D1 = day 1 (day 0 = day of insemination); D2 = day 2; ET = embryo transfer.

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were transferred on day 3, and numbers of embryos transferred were not significantly different between oocyte thawing (3.2 ± 0.3) and warming (3.1 ± 0.1) groups.

Because the objective of this trial was to compare oocyte cryopreservation technologies, both biochemical and clinical pregnancy rates were compared in relation to number of oocyte thawing or warming cycles performed and not with respect to number of embryo transfers performed. Biochemical pregnancy rate/thawing cycles was 17% (5/30) and significantly different ($P<.01$) than biochemical pregnancy rate/warming cycles at 46% (22/48). A similar significant difference ($P<.02$) was observed in clinical pregnancy rates (gestational sac and heartbeat; example: Fig. 1F) at 13% (4/30) per thawed cycles compared with 38% (18/48) per warmed cycles. Spontaneous abortion rates were similar following transfer of embryos from oocyte thawing (n = 1; 20%) and warming (n = 4; 18%). Of the 18 clinical pregnancies resulting after oocyte vitrification/warming 16 were singletons and two sets of twins. Of the four clinical pregnancies resulting after oocyte slow rate freezing/thawing two were singletons, one set of twins, and one set of triplets.

DISCUSSION

Decades have past since the first report of a pregnancy after an oocyte cryopreservation (9) originally from slow-rate freezing/thawing, and was followed by many years of limited success. More recently, because of enhanced knowledge of cell biology and biophysical properties of ice crystal formation in relation to cryoprotective agents, resurgent interests and efforts have focused on oocyte cryopreservation by both slow-rate freezing(10–13, 37) and vitrification (19–21, 38). Oocyte cryopreservation holds clinical and practical promise for many patient populations: for example, women at risk of losing fertility because of chronic disease and/or treatments using radiation or chemotherapy (39), and those with genetic predisposition to infertility (40). With increasing survival rates of cancer patients it is recognized that many of them will desire children following their treatment and remission. Oocyte cryopreservation allows flexibility for assisted reproductive programs if initial treatment cycles must be halted for unforeseen reasons. Such reasons could include adverse reactions to hormonal hyperstimulation (41), inappropriate endometrial receptivity, and inability of the partner to produce a viable sperm sample. Oocyte cryopreservation significantly reduces the medical management and financial burden within an oocyte donation treatment cycle. Additionally, the ability to cryopreserve and quarantine oocytes for a period of time compatible with infectious disease testing of donors would enhance protection of recipients. This would allow equality of male and female gamete donation with respect to donor eligibility determination (42). One can envision that in the future patients who choose/require postponing childbirth until advanced age, when oocytes normally decrease in ability to support developmental competence, could cryopreserve oocytes at a young age thus increasing the chances of establishing a pregnancy with their own genetic material at a more advanced age. Oocyte cryopreservation can potentially decrease the expense of infertility treatment. When cryopreservation and in vitro maturation of human oocytes become an efficient means of generating live births (43), this will significantly reduce the cost of infertility treatment by removing the need for exogenous gonadotropin stimulation and multiple follicular development assessments. This will also provide a viable option for family development to individuals currently unable to have their infertility treated because of cost constraints. Finally, oocyte cryopreservation provides another option to patients concerned with ethical and legal issues of embryo cryopreservation. This could result in a reduction in embryo cryopreservation that could benefit society as a whole by elimination of legal issues regarding cryopreserved embryos, custody, and disputes involving death and separation.

Our survival rate of oocytes was significantly elevated following oocyte vitrification/warming compared with freezing/thawing. The initial survival following warming in our study was 81%, and although lower than a few reports using similar techniques in the 95+% (20, 21, 44), it is equivalent as calculated averages in other reports (18–20, 31, 45–47). In addition, a recent critical review documented a computed survival rate for vitrification/warming of

81% (48). Similarly, in our study, initial survival in the frozen/thawed oocyte group was 67%, again comparable to an average of 68% calculated from literature reports (9–12, 45–48). Insemination was only attempted by ICSI because of past reports of zona pellucida modifications upon oocyte cryopreservation that can compromise subsequent normal fertilization (49, 50). Recently, it has been reported that modifications to cryosolutions can prevent premature release of cortical granules upon oocyte cryopreservation (51), which may lead to important considerations in the future.

Our fertilization rates were significantly higher following oocyte warming (77%) compared with thawing (67%), and were quite similar to reported fertilization rates following oocyte warming (74%) and thawing (65%) calculated in a meta-analysis focused on comparison of oocyte cryopreservation techniques (29). Significantly higher cleavage rates and embryo morphologic scores in the first 3 days of development following warming of oocytes in comparison to thawing were also demonstrated (84% vs. 71%). This better development suggests the impact of mechanical, chemical, and thermal (5, 6) stressors on oocytes; and how these influences could be minimized by the vitrification/warming process, resulting in enhanced embryo development. Finally, this study delineates the important issues of step-wise efficiencies, loss of attrition, cumulative impact of oocyte perturbations, and subsequent embryo development and influence on final measures of success. Even though similar average numbers of oocytes were thawed or warmed per cycle, the reduced efficiency in survival, fertilization, and cleavage following thawing equated to 11 patients who did not have embryos for transfer, whereas only 1 patient in the oocyte warming arm did not have an embryo transfer.

There are numerous means of assessing success of any ART, including biochemical pregnancy rates, implantation rates, clinical pregnancy rates, and live birth rate. All of these rate measures can be calculated with various denominators (per oocyte retrieved, per oocyte retrieval cycle, per embryo transferred, or per embryo transfer cycle). Which denominator used should be dictated by the experimental question posed. In this trial, the experimental question was "which oocyte cryopreservation procedure was most efficient at establishing a clinical pregnancy?" Thus, calculations in relation to number of embryos transferred or embryo transfer cycles do not address this question. These calculations would miss important data in relation of poor oocyte cryosurvival, fertilization failures, and cleavage failures. Pregnancy rates based on number of thaw or warming cycles capture these data and represent a practical and appreciable evaluation of efficiency of each oocyte cryopreservation technique. Calculations based on total number of oocytes thawed or warmed per clinical pregnancy do not fully address the issue because not all oocytes thawed or warmed resulting in transferable embryos were transferred. Although one goal of this study was to reduce embryo cryopreservation, there were instances when embryos resulting from oocyte thawing or warming required cryopreservation to maintain a medically sound number of embryos transferred. Such double cryopreserved embryos (cryopreserved as oocytes and as embryos) can establish pregnancies (52, 53). However, if one calculates pregnancy efficiency based on number of oocytes thawed or warmed, minus resulting embryo cryopreserved that have the potential of establishing a pregnancy, and divide by number of clinical pregnancies, the resulting values from this study are 16.5 vitrified/warmed oocytes per pregnancy compared with 33.1 frozen/thawed oocytes per pregnancy. This calculates to a clinical pregnancy rate per oocyte thawed or warmed of 1.7% versus 5.2%, respectively. These values are similar to clinical pregnancy rates per oocyte thawed (2.3%) or warmed (4.5%) reported in a meta-analysis (29) and live birth rates per oocyte thawed (2.3%) versus warmed (5.2%) reported in an extensive literature review (54). This can be a useful number in counseling patients.

Although this study has demonstrated that human oocyte vitrification/warming is more efficient at establishing clinical pregnancies in comparison to freezing/thawing, there are important biologic and technical issues that warrant consideration. First, efforts continue in refinement of slow-rate freezing and vitrification. An example of such a modification involves changing sucrose molarity in freeze and thaw solutions (30, 48, 55, 56). With sucrose molarities in both freeze and thaw solutions equal, similar to our protocol, numerous groups have reported clinical pregnancy rates per oocyte thawed of 1.0% to 2.0% (13, 57, 58). Inclusion of a higher concentration of sucrose or similar nonpermeating cryoprotectant in the initial thaw solution compared with the final concentration of nonpermeating cryoprotectant in the freezing solution is believed to be important to enhance cryo-survival [for review see (59)]. Recently, oocyte slow-rate freezing/thawing with dissimilar sucrose concentrations improved oocyte cryopreservation results with clinical pregnancy rates per oocyte thawed of 3.7% (55, 56). Although temping to change our initial slow-rate freezing protocol during study, we felt it was essential to continue using the most evidenced/publish procedure at the time of study initiation in 2005 (30) until study completion. With recent improvements of oocyte slow-rate freezing success future studies should prospectively compare these improved, more effective, slow-rate freeze protocols to vitrification.

In the human ART field overall experience in freezing/thawing, especially in the area of embryo cryopreservation, is greater than experience with vitrification/warming (60). The actual technique of oocyte vitrification/warming is not difficult, but does require practice, technical expertise, and can carry a "technical signature" that can impact success. Additionally, because during oocyte vitrification/warming extremely small volumes are used with rapid cell movement, deviations from protocol can significantly compromise results. Many vitrification/warming protocols use high concentrations of permeating cryoprotectants, such as dimethyl sulfoxide, thus exposure times and temperatures are important. Finally, because vitrification/warming is performed predominantly in very small volumes (<1.0 μL) on/in either electron microscopy grids (61), nylon loops (24), open-pulled straws (62), microdrops on plastic strip (20), or closed-pulled straws (21, 63), extreme caution must be demonstrated in movement of samples within and between liquid nitrogen or liquid nitrogen vapor tanks. These samples can begin warming very rapidly and any inadvertent increase in sample temperature above -130°C can cause irreversible damage to cells within or on these vitrification devices (64).

In conclusion, in this prospective randomized comparison of human oocyte cryopreservation techniques; vitrification/warming resulted in significantly higher cryosurvival, fertilization, embryo cleavage and development, and clinical pregnancy rates in comparison to slow-rate freezing/thawing. Continued evaluation of oocyte cryopreservation and subsequent embryo genetic normalcy, as well as long-term offspring health is needed. Finally, considering accumulating data on influence of ART and fetal/placental epigenetics (65) it will be prudent to consider whether oocyte manipulations during cryopreservation might impact embryo/fetal/placental imprinted gene expression.

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