

Clinical evaluation of the efficiency of an oocyte donation program using egg cryo-banking

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Objective: To evaluate the efficiency of oocyte donation cycles using egg “cryo-banking.”

Design: Study conditions for vitrified/warmed oocytes for 20 non-autologous recipients (from 10 donors) were set prospectively, and outcomes of it were later compared retrospectively to nine fresh donations cycles.

Setting: Private assisted reproductive technology program.

Patient(s): Ten donors and 20 infertile recipients.

Intervention(s): Oocytes were vitrified 3 to 4 hours after collection and cryo-stored. Intracytoplasmic sperm injection was performed 3 hours after warming, and embryos were in vitro cultured for 5 days. Two or three blastocysts were transferred per patient.

Main Outcome Measure(s): Oocyte survival, fertilization, development, clinical pregnancy, and implantation rates.

Result(s): A total of 153 oocytes were warmed and 134 survived. A total of 117 fertilized and 68% developed to blastocyst stage. A total of 47 embryos were transferred (2.35 embryos per recipient) and 26 implanted. Fifteen patients achieved ongoing pregnancies initially, and two additional pregnancies were obtained after transfer of supernumerary vitrified/warmed embryos. Nine of the 10 donors from the current study had previous fresh donations cycles from where seven clinical pregnancies were established in nine recipients, providing the base for comparison.

Conclusion(s): Oocyte donation using vitrified/warmed oocytes can provide high pregnancy and implantation rates, and thus can be considered as efficient treatment procedure with additional benefits to recipients. (Fertil Steril® 2009;92:520–6. ©2009 by American Society for Reproductive Medicine.)

Key Words: Oocyte, cryopreservation, vitrification, donation, fertilization, embryo, implantation, pregnancy

Cryopreservation has a pivotal role in infertility treatment. It makes assisted reproduction treatment both more flexible and more efficient. Freezing of sperm and embryos can be performed efficiently, and both have been part of routine IVF procedures for a long time (1, 2). However, cryopreservation of oocytes, the female gamete, has not become a routine procedure until recently, even though the first successful pregnancies were pioneered >2 decades ago in Australia and Europe (3, 4) and more than a decade ago in the United States (5). Since then, a large number of human studies (6–12) have been conducted, scrutinizing various technical approaches, many of them enhancing the experience; however, none have demonstrated consistently high success rates justifying routine use.

There are several areas where an efficient oocyte freezing and cryo-storage program would be fundamental, including:

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women with malignant/premalignant conditions where the disease or treatment may negatively impact their future ability to have children (13, 14), government restrictions on IVF (15), social reasons such as delayed child birth (16, 17), for “regular” IVF patients who prefer oocytes instead or embryos to be cryo-stored for moral/ethical reasons (18), and for other practical reasons, such as unexpected semen collection problem on the pickup day.

Additionally, successful oocyte freezing/thawing technology would potentially aid donation programs (6), which are strongly limited currently in their efficiency by different factors including availability of qualifying donors, expense, delay in synchronization, US Food and Drug Administration regulations, and ethical concerns regarding supernumerary embryo disposition. Oocyte cryo-bank would also aid the more efficient sharing of donor eggs between multiple recipients, improving the cost effectiveness through better use of resources. More recent study reports and our own experience have indicated that efficiency of oocyte cryopreservation is improving (19, 20), especially when employing some of the alternative approaches of vitrification (18, 21–24). Consequently, we aimed to evaluate how improved oocyte cryopreservation technology (24) would help to provide a more efficient approach in egg donor–recipient treatment procedures via “egg cryo-banking.”

MATERIALS AND METHODS

Participants and Ovarian Stimulation

In the frame of this prospective study, oocytes were obtained from 10, all but 1, previously proven egg donors. All donors were healthy women under the age 35 years, and were screened and tested according to regulations (25). This study has been approved by the corresponding institutional review boards, and all participants signed the corresponding consent forms.

Controlled ovarian hyperstimulation was performed as follows. On day 2 or 3 after menses, subcutaneous rFSH, follitropin beta (Follistim; Organon Pharmaceuticals USA Inc., Roseland, NJ) administration was started (after keeping the donors on an oral contraceptive for a minimum of 14 days). Ovarian stimulation was monitored by the measurement of serum E₂ concentration and by ultrasonographic assessment of follicle diameter every 1 to 2 days. Gonadotropin-releasing hormone antagonist (ganirelix acetate) treatment was started on day 6 or day 7 of rFSH administration and continued until hCG was given. The antagonist treatment began when the lead follicle was >12 mm. Recombinant hCG (250 µg subcutaneously; Ovidrel; Serono, Inc., Rockland, MA) was administered within 36 hours after the last dose of gonadotropin, when the patient had at least one follicle >18 mm, and half of the follicle cohort had a 14-mm or larger diameter. Oocyte retrieval was performed 36 hours after hCG administration, using transvaginal ultrasound guidance and under anesthesia.

Laboratory Procedures

The collected oocytes were washed and incubated for 30 to 60 minutes, then were denuded by 30-second exposure of Cumulase (Halozyme therapeutics, San Diego, CA), followed by mechanical denudation. Oocyte maturity was assessed by light microscopy evaluation; those that presented a polar body were categorized as mature (metaphase-II stage; MII) and were eligible for freezing. In the current study, only in vivo matured MII oocytes were used. The steps of the vitrification and warming procedures were as reported earlier (24), but briefly it was as follows. The oocytes were first equilibrated in 7.5% Ethylene Glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) for 15 minutes and transferred into the vitrification solution containing 15% EG and 15% DMSO and 0.5 mol/L sucrose, using a minimal volume approach. The base medium used was HEPES-buffered embryo culture medium (Cooper/Sage, Bedminster, NJ) supplemented with 20% (v/v) serum protein substitute (Cooper/Sage). Oocytes were placed onto a fine polypropylene strip (Cryotop, Kitazato Bio Pharma Co., Japan). Oocyte warming was performed by serial dilutions as follows: 1.0 M, 0.5 M, and 0 M sucrose.

Oocytes that survived were inseminated by intracytoplasmic sperm injection (ICSI) 2 to 3 hours after warming using sperm from the husband of the corresponding recipient. The procedure of ICSI was performed as reported earlier (26), the

only difference being that a narrower diameter of ICSI injection pipette was used to minimize any damage during microinjection (MIC-CUST-30; Humagen Fertility Diagnostics, Charlottesville, VA).

The day after ICSI, oocytes were assessed for the presence of pronuclei, and those displaying two pronuclei and two polar bodies were cultured further. Embryos were cultured until day 5, when morphologic selection by light microscope was performed and two or a maximum of three blastocysts with the highest grades were selected for transfer. Supernumerary embryos with sufficient developmental and morphologic status were vitrified on day 5 or day 6.

Recipients were eligible to participate in the study if they were already on the waiting list for oocyte or embryo donation (before this study, for reasons of previously failed cycle[s] related to advanced maternal age and/or impaired ovarian reserve), and they agreed and signed the corresponding institutional review board consent. For those 20 recipients who participated in this initial study, all IVF-related charges were waived.

Preparation of Recipients

Preparation of recipients was performed using a standard protocol of leuprolide acetate, estrogen, and progesterone. In brief, leuprolide acetate was started in the midluteal phase of the cycle previous to embryo transfer, at a daily dosage of 1 mg. After confirming down-regulation, by measuring the serum estrogen and progesterone levels, estrogen was commenced using Estrace pills (micronized E₂; Bristol-Myers Squibb Co., Princeton, NJ) at 1 mg twice a day for 5 days, then increased to 2 mg twice a day for 4 days, and finally to 2 mg orally three times a day for 4 days. Endometrial thickness was monitored by transvaginal ultrasound and serum E₂ levels were measured every 2 to 4 days. On the 15th day ("cycle day 15") after estrace start (with adequate endometrial thickness), Progesterone in Oil, 25 mg, was administered. The day after, Progesterone in Oil, 50 mg, was administered, and on that day oocyte warming was performed, followed by the ICSI insemination procedure. Progesterone in Oil, 50 mg, was then continued daily until the first pregnancy test. Additionally, 100 mg of doxycycline for 7 days, and 16 mg of methylprednisolone on a daily basis for 7 days was given, starting on the 16th day. Administration of leuprolide acetate was suspended the day before embryo transfer. The first ultrasound was performed 20 to 22 days after embryo transfer. A clinical pregnancy was defined as the presence of fetal cardiac activity on transvaginal ultrasound.

Statistical Analyses

One-way analysis of variance, chi-square test, and Fisher exact test were applied whenever appropriate for comparing fresh and vitrified/warmed oocyte donation outcomes. Results were considered to be significant with a value of $P < .05$.

RESULTS

A total of 153 vitrified MII oocytes were warmed for the 20 recipients: a mean of 7.65 per recipient; a minimum of 4, and a maximum of 12. From those warmed oocytes, 134 survived (89%), an average of 6.7 per recipient (minimum three and maximum nine). All surviving oocytes were inseminated and 117 fertilized normally (87%; lowest fertilization was 60% and the highest 100%; half of the recipients had maximum fertilization). A total of 115 zygotes were developing on day 3 (98%; all patients but two had 100% cleavage stage development). A total of 78 embryos developed to the blastocyst stage by day 5 (68%). There were 47 embryos selected for embryo transfer (an average of 2.4 embryos per recipient). Seven patients had three embryos transferred and 13 had two embryos transferred. There were a total of 31 supernumerary embryos with sufficient quality, which were frozen using a vitrification protocol. Initial hCG levels were positive and rising for 16 recipients; 4 recipients had negative hCG tests. One of the patients with initially rising serum hCG had a decline after the third measurement, and later no fetal cardiac activity was detected. In 15 patients a total of 26 fetal cardiac activities were detected, corresponding to a 55.3% implantation rate. Six of the pregnancies were singleton, seven were twin, and two were triplet implantations. All pregnancies were uneventful and all 26 infants have been born. One club foot was noted in a triplet delivery; no other major or minor alterations were detected. The details on each individual recipient are presented in Table 1.

Results from these frozen–thawed egg donor cycles were compared with those from the donors' prior fresh donation cycles. As mentioned, all donors but one had prior donation cycle(s).

In the fresh donation cycles a total of 322 oocyte–cumulus complexes were obtained (average of 35.8 per cycle), and 182 of them were inseminated with ICSI (an average of 22.7 oocytes compared with 6.7 oocytes in the frozen oocyte cycle; Table 2). A total of 137 oocytes fertilized after ICSI normally in the fresh donation (a mean of 67%), and 117 out of 134 fertilized in the frozen oocyte group (a mean of 87%). Nineteen embryos were transferred and nine of them implanted in the fresh cycle compared with 26 out of 47 in the frozen oocyte cycles. Initially six patients become pregnant in the fresh cycles; however, one of the pregnancies was an ectopic; thus, only five were ongoing in the nine recipients. A mean of 17.3 (a total of 156, including conventionally inseminated eggs) embryos were cryopreserved in the fresh cycles compared with the mean of 1.6 in the frozen oocyte cycles (a total of 31). In both groups, there were two additional recipients who achieved pregnancies after transferring frozen/thawed (warmed) embryos; thus, the total pregnancy rate was 78% following fresh oocyte donation (a total of seven patients had clinical pregnancy out of the nine donation cycles), whereas a total of 17 patients achieved clinical pregnancy after frozen/warmed oocyte donation cycles (total pregnancy rate of 85%; Table 2).

DISCUSSION

The current study suggests that oocyte donation cycles may be more efficacious if performed with frozen eggs (alternatively called “oocyte cryo-banking”).

Clearly, the most crucial prerequisite for a successful egg cryo-banking program is to have an efficient oocyte freezing/thawing (warming) technology. In regard to oocyte freezing, there have been various concerns. One of the questions raised was on the effect of cooling/freezing on spindle integrity and chromosome composition of derived embryos (27). Different studies have demonstrated that although the spindle may disassemble during the freeze–thaw process, it can correctly reassemble again in most oocytes (28–30). Accordingly, there was no apparent increased incidence of chromosomal abnormalities in embryos derived from frozen eggs, a fear that is even further mitigated when vitrification is used (30, 31–33). Other studies have correlated alterations to the membrane, the cortical granules, the zona pellucida, and mitochondria to cryopreservation, especially when a slow-rate cooling method was applied (34–37). However, some of these alterations, such as zona pellucida hardening, and related concerns were proven to be alleviated using ICSI as the insemination technique (7, 38).

Probably the most critical challenge in cryopreservation is to maintain plasma and membrane integrity by avoiding or preventing ice-crystallization damage (39, 40). Various permeating and nonpermeating cryoprotectants are used at different concentrations to help prevent cryo-injuries. However, some of those cryoprotectants are known to be toxic relative to their concentration and to the temperature (41). As a consequence, an intricate balance is required between the type and concentration of multiple cryoprotectants, the exposure time to those chemicals, and the temperature. During slow freezing, the exposure to a lower concentration of cryoprotectants for a more extended time before the actual “freezing” occurs, and during vitrification there is exposure to a high concentration of cryoprotectants for a relatively short period before submerging the sample to liquid nitrogen.

A recent review (11) showed that pregnancy and implantation rates improved when employing the slow-freezing method, and especially in more recent studies where elevated extracellular sucrose in cryoprotective solutions was used or sodium-depleted choline-based media (19, 20, 42–44). However, at the same time, vitrification, the alternative approach to slow freezing, seemed to emerge as an even more efficient technique in general (45), not only for embryo but also for oocyte cryopreservation (30, 46–49). Employing vitrification, different investigators have reported much improved survival, implantation, and pregnancy rates, using different cryoprotectants (or combinations) and different cryo-storage devices (18, 21–23, 50, 51). Following an initial intensive “in-house” exploration on the different alternative approaches (Chang, unpublished) it was decided to employ the vitrification technique with the minimal volume method in the current study.

TABLE 1

Laboratory and clinical outcomes in the 20 frozen/warmed oocyte recipients cycles (oocytes were obtained from 10 donors).

Recipient	Oocytes warmed	Oocytes survived	% Survived	Ferti-lized	% Fertilized	D3 Cleaved	% D3 Cleaved	D5 Blastocyst	% D5 Blastocyst	ET	HCG	FCA	CRYO
1	8	6	75	5	83	5	100	3	60	3	POS	2	0
2	4	3	75	3	100	3	100	2	67	2	POS	1	0
3	10	8	80	8	100	8	100	6	75	3	POS	3	3
4	8	8	100	8	100	8	100	3	38	3	POS	3	0
5	10	9	90	9	100	9	100	6	67	2	POS	2	4
6	10	9	90	6	67	6	100	2	33	2	POS	0	0
7	6	5	83	5	100	5	100	3	60	3	POS	1	2
8	6	6	100	6	100	6	100	3	50	2	POS	2	1
9	9	7	78	6	86	6	100	5	83	3	POS	2	3
10	8	8	100	7	88	7	100	5	71	3	NEG	0	2
11	9	7	78	5	71	5	100	3	60	2	POS	1	1
12	6	6	100	6	100	6	100	5	83	2	NEG	0	3
13	6	6	100	5	83	5	100	3	60	2	POS	2	1
14	10	9	90	9	100	8	89	6	67	2	NEG	0	3
15	6	6	100	4	67	4	100	3	75	3	POS	2	0
16	8	5	63	3	60	3	100	3	100	2	NEG	0	0
17	6	6	100	4	67	4	100	3	75	2	POS	1	0
18	5	5	100	5	100	4	80	3	60	2	POS	2	1
19	12	9	75	7	78	7	100	7	100	2	POS	1	5
20	6	6	100	6	100	6	100	4	67	2	POS	1	2

Nagy. Oocyte cryo-bank. Fertil Steril 2009.

TABLE 2

Laboratory and clinical outcomes in the vitrified/warmed oocyte group with 10 donors and corresponding data of the same donors in their last fresh oocyte donation cycle.

	Cryo oocyte	Fresh oocyte	P
Number of donors	10	9	NA
Number of recipients	20	9	NA
Mean age (\pm SD) of recipients	39.7 (\pm 4.8)	39.6 (\pm 5.8)	NS
Mean number of oocytes per recipients	7.7	35.8	< .05
Mean number of oocytes for ICSI	6.7	22.7	< .05
Average 2PN ICSI fertilization rate	87%	67%	< .05
Embryos implanted/transferred	26/47	9/19	NS
Mean number of embryos cryopreserved	1.6	17.3	< .05
Clinical pregnancy rate	75%	56%	NS
Fresh + frozen PR	85%	78%	NS
Total number of pregnancies	17	7	NA

One of the donor in the “Cryo oocyte” group was a first time donor; thus, she had no fresh donation outcomes to include.

The purpose of this table is to give a basic comparison of the fresh and cryopreserved donor oocyte cycles.

ICSI = intracytoplasmic sperm injection; PR = pregnancy rate.

Nagy. Oocyte cryo-bank. Fertil Steril 2009.

The current study results support the concept that oocyte cryopreservation can be performed successfully in a routine IVF program, providing the foundation to apply it for different indications. As suggested, efficient oocyte cryopreservation as a fertility preservation tool can serve women with severe diseases (such as cancer), where the illness or the therapy would reduce (or destroy) ovarian function (13, 14, 43). Additionally, infertile patients may choose oocyte cryopreservation instead of embryo freezing as a way to deal with ethical/moral dilemmas or to reduce complications of “social situations” (such as ownership of embryos) (18). In some countries, like Italy, oocyte freezing is a “must,” as, by law, no more than three oocytes can be fertilized; thus, any supernumerary IVF oocytes have to be destroyed or to be cryopreserved (15, 22). It is also to be expected that fertility preservation may be used by healthy women, who, for personal or social reasons, are delaying childbearing and want to have an “insurance” against biologic aging of oocytes (16, 17). Furthermore, it is likely that in different, other, yet to be defined, situations, an efficient oocyte freezing technique will be highly instrumental (51, 52).

In the current study, we also wished to explore how oocyte cryopreservation would change the “dynamic” of a donor–recipient program. Most IVF clinics around the world are facing similar challenges with oocyte donation programs, as it has been related earlier (6): lack (or low number) of qualifying donors, expense, difficulty of synchronization, lack of quarantine period, and frequently a large number of supernumerary embryos.

To relate outcomes of the “cryo-egg bank,” we have also tabulated the data of the fresh donation cycles of the same donors. Recipients were different in the fresh and in the

“cryo-oocyte” group. There were only nine fresh donor cycles because one of the donors in the “cryo-oocyte” study had not donated oocytes before.

When comparing results of fresh and frozen oocyte donation cycles, most striking is the number of oocytes allocated per recipient: about five times higher in fresh cycles, on average. It was possible to keep the number of warmed oocytes relatively low in the study group as a consequence of high survival rates, confirming previous observations when vitrification was used (18, 21, 22, 53). Fertilization rates were similarly high in both groups, although a trend may be noted for higher fertilization in the frozen/warmed oocyte group, which may raise the question of parthenogenetic activation (related to the cryopreservation process and/or to the cryoprotectant exposure (54, 55). Embryo development was similarly good in both groups (fresh and vitrified/warmed oocytes), and in fact, a very high percentage of blastocyst development was observed in the study group. To our knowledge, this may be one of the first studies where extended culture of embryos to day 5 was used as a standard approach for all cases after oocyte cryopreservation. It is an important observation, showing that vitrification not only provides high survival/fertilization rates but also good blastocyst development in vitro. Implantation and pregnancy rates were comparably high both in fresh and in vitrified/warmed oocyte donation cases, clearly indicating that good quality oocytes retain a high viability after vitrification. Slightly more embryos (per recipient) were transferred in the vitrification group, resulting in a number of multiple gestations. Initially, three embryos were transferred in the study group because of the reported relatively low implantation rate following egg freezing in earlier studies. However, in a later phase of the study, as experience was gained, most patients

agreed on transferring two embryos, as is the routine in fresh oocyte donation cycles.

It is our experience that many or most recipients end up having many more embryos in a fresh donation cycle than they would possibly ever use; most of them remaining frozen, adding to the ever-increasing problem of cryo-stored embryos. Compared with that, there were very few supernumerary embryos frozen in the cryo-egg group; nevertheless, two additional pregnancies were achieved using those frozen embryos (24). Although ethical concerns on large number of frozen embryos may be mitigated using cryo-egg banks, a new ethical dilemma may be raised on the potential “ownership” of the frozen donated oocytes, which needs to be addressed in the future. Overall implantation rate calculated on the number of oocytes warmed in this study thus reached 19%; in other words, approximately five warmed oocytes resulted in one implantation. This favorable outcome compares positively to previously reported implantation rates per warmed oocyte, confirming the potential of oocyte vitrification. Possibly the most exciting data obtained from this study is demonstrating that a total of 17 patients achieved ongoing clinical pregnancies from 10 oocyte donation cycles using cryo-oocyte banking compared with seven patients from nine fresh oocyte donation cycles (using the same donors). This outcome suggests that the efficiency of oocyte donation can be significantly improved using cryo-banking.

Additionally, banks of cryopreserved donated oocytes would allow for the quarantine of oocytes until appropriate infectious disease screening of the donor could be completed, as is already the case for frozen semen (25).

The present study demonstrates that oocyte cryopreservation can be considered as a tool to provide highly successful outcomes in an egg donor program. Our results validate the use of oocyte cryo-banking for egg donation purposes. In addition to the high success rates, oocyte cryo-banking can help to eliminate some of the obstacles associated with the current “fresh embryo” transfer policy. Oocyte cryo-banking will also dramatically reduce frozen embryo storage and mitigate related moral/ethical concerns, and may help with donor–recipient synchronization and possibly reduce cost.

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REFERENCES

1. Sherman JK. Improved methods of preservation of human spermatozoa by freezing and freeze-drying. *Fertil Steril* 1963;14:49–64.
2. Trounson A, Mohr L. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature* 1983;305:707–9.
3. Chen C. Pregnancy after human oocyte cryopreservation. *Lancet* 1986;i:884–6.
4. Al-Hasani S, Diedrich K, van der Ven H, Reinecke A, Hartje M, Krebs D. Cryopreservation of human oocytes. *Hum Reprod* 1987;8:695–700.
5. Tucker M, Wright G, Morton P, Shanguo L, Massey J, Kort H. Preliminary experience with human oocyte cryopreservation using 1,2-propanediol and sucrose. *Hum Reprod* 1996;11:1513–5.
6. Tucker MJ, Morton PC, Wright G, Sweitzer CL, Massey JB. Clinical application of human egg cryopreservation. *Hum Reprod* 1998;13:3156–9.
7. Gook DA, Schiewe MC, Osborn SM, Asch RH, Jansen RP, Johnston WI. Intracytoplasmic sperm injection and embryo development of human oocytes cryopreserved using 1,2-propanediol. *Hum Reprod* 1995;10:2637–41.
8. Tucker MJ, Wright G, Morton PC, Massey JB. Birth after cryopreservation of immature oocytes with subsequent in vitro maturation. *Fertil Steril* 1998;70:578–9.
9. Porcu E, Fabbri R, Marsella T, Balicchia B, De Cesare D, Giunchi S, et al. Clinical experience and applications of oocyte cryopreservation. *Mol Cell Endocrinol* 2000;169:33–7.
10. Borini A, Lagalla C, Bonu MA, Bianchi V, Flamigni C, Coticchio G. Cumulative pregnancy rates resulting from the use of fresh and frozen oocytes: 7 years’ experience. *Reprod Biomed Online* 2006;12:481–6.
11. Oktay K, Cil AP, Bang H. Efficiency of oocyte cryopreservation: a meta-analysis. *Fertil Steril* 2006;86:70–80.
12. Edgar DH, Gook DA. How should the clinical efficiency of oocyte cryopreservation be measured? *Reprod Biomed Online* 2007;14:430–5.
13. Rao GD, Chian RC, Son WS, Gilbert L, Tan SL. Fertility preservation in women undergoing cancer treatment. *Lancet* 2004;363:1829–30.
14. Del Mastro L, Venturini M. Fertility preservation strategies for breast cancer patients. *J Clin Oncol* 2006;24:4220–1.
15. Boggio A. Italy enacts new law on medically assisted reproduction. *Hum Reprod* 2005;20:1153–7.
16. Jain JK, Francis MM, Marsh EM, McConnell G, Quinn P, Chung K, et al. Oocyte cryopreservation: a promising strategy for circumventing the age-related decline in human fertility. *Fertil Steril* 2006;85:S7.
17. Luna M, Barritt J, Howard M, Jones C, Sandler B, Copperman AB. Oocyte cryopreservation for fertility preservation: the first 149 patient consults and their subsequent cycle outcomes. *Fertil Steril* 2007;88:S339–40.
18. Lucena E, Bernal DP, Lucena C, Rojas A, Moran A, Lucena A. Successful ongoing pregnancies after vitrification of oocytes. *Fertil Steril* 2006;85:108–11.
19. Boldt J, Tidswell N, Sayers A, Kilani R, Cline D. Human oocyte cryopreservation: 5-year experience with a sodium-depleted slow freezing method. *Reprod Biomed Online* 2006;13:96–100.
20. Barritt J, Luna M, Duke M, Grunfeld L, Mukherjee T, Sandler B, et al. Report of four donor–recipient oocyte cryopreservation cycles resulting in high pregnancy and implantation rates. *Fertil Steril* 2007;87. 189.e13-7.
21. Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod Biomed Online* 2005;11:300–8.
22. Antinori M, Licata E, Dani G, Cerusico F, Versaci C, Antinori S. Cryotop vitrification of human oocytes results in high survival rate and healthy deliveries. *Reprod Biomed Online* 2007;14:72–9.
23. Cobo A, Kuwayama M, Pérez S, Ruiz A, Pellicer A, Remohí J. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. *Fertil Steril* 2008;89:1657–64.
24. Chang CC, Shapiro DB, Bernal DP, Wright G, Kort HI, Nagy ZP. Two successful pregnancies obtained following oocyte vitrification and embryo re-vitrification. *Reprod Biomed Online* 2008;16:346–9.
25. Food and Drug Administration. Inspection and enforcement; final rule. Federal Registry. Current good tissue practice for human cell, tissue, and cellular and tissue based product establishments November 2004.
26. Nagy ZP, Liu J, Joris H, Bocken G, Desmet B, Van Ranst H, et al. The influence of the site of sperm deposition and mode of oolemma breakage at intracytoplasmic sperm injection on fertilization and embryo development rates. *Hum Reprod* 1995;10:3171–7.
27. Pickering SJ, Braude PR, Johnson MH, Cant A, Currie J. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil Steril* 1990;54:102–8.

28. Gook DA, Osborn SM, Bourne H, Johnston WI. Fertilization of human oocytes following cryopreservation; normal karyotypes and absence of stray chromosomes. *Hum Reprod* 1994;9:684–91.
29. Rienzi L, Martinez F, Ubaldi F, Minasi MG, Iacobelli M, Tesarik J, et al. Polscope analysis of meiotic spindle changes in living metaphase II human oocytes during the freezing and thawing procedures. *Hum Reprod* 2004;19:655–9.
30. Chang CC, Tian XC, Yang X, Shapiro DB, Slayden SM, Nagy ZP. Oocyte spindle preservation during vitrification of mouse oocytes. *Fertil Steril* 2007;88:S89.
31. Cobo A, Rubio C, Gerli S, Ruiz A, Pellicer A, Remohí J. Use of fluorescence in situ hybridization to assess the chromosomal status of embryos obtained from cryopreserved oocytes. *Fertil Steril* 2001;75:354–60.
32. Cobo A, Perez S, Zulategui J, De los Santos MJ, Domingo J, Remohí J. Effect of different oocyte cryopreservation protocols on the morphological appearance of MII meiotic spindle in humans. *Reprod Biomed Online* 2008. in press.
33. Huang JY, Chen HY, Park JY, Tan SL, Chian RC. Comparison of spindle and chromosome configuration in in vitro- and in vivo-matured mouse oocytes after vitrification. *Fertil Steril* 2008. in press.
34. Schalkoff ME, Oskowitz SP, Powers RD. Ultrastructural observations of human and mouse oocytes treated with cryopreservatives. *Biol Reprod* 1989;40:379–93.
35. Fuku EJ, Liu J, Downey BR. In vitro viability and ultrastructural changes in bovine oocytes treated with a vitrification solution. *Mol Reprod Dev* 1995;40:177–85.
36. Johnson MH, Pickering SJ, George MA. The influence of cooling on the properties of the zona pellucida of the mouse oocyte. *Hum Reprod* 1988;3:383–7.
37. Jones A, Van Blerkom J, Davis P, Toledo AA. Cryopreservation of metaphase II human oocytes effects mitochondrial membrane potential: implications for developmental competence. *Hum Reprod* 2004;19:1861–6.
38. Porcu E, Fabbri R, Seracchioli R, Ciotti PM, Magrini O, Flamigni C. Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. *Fertil Steril* 1997;68:724–6.
39. Mazur P. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *J Gen Physiol* 1963;47:347–69.
40. Mazur P, Rall WF, Leibo SP. Kinetics of water loss and the likelihood of intracellular freezing in mouse ova. Influence of the method of calculating the temperature dependence of water permeability. *Cell Biophys* 1984;6:197–213.
41. Fahy GM, Lilley TH, Linsdell H, Douglas MS, Meryman HT. Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms. *Cryobiology* 1990;27:247–68.
42. Stachecki JJ, Cohen J, Garrisi J, Munne S, Burgess C, Willadsen SM. Cryopreservation of unfertilized human oocytes. *Reprod Biomed Online* 2006;13:222–7.
43. Yang D, Brown SE, Nguyen K, Reddy V, Brubaker C, Winslow KL. Live birth after the transfer of human embryos developed from cryopreserved oocytes harvested before cancer treatment. *Fertil Steril* 2007;87:1469.e1–4.
44. Diaz DG, Rodriguez-Karl MC, Moody JE, La A. Survival, fertilization, and cleavage rate of frozen–thawed oocytes using a new modified slow-freeze protocol—preliminary results. *Fertil Steril* 2007;87:S18.
45. Vajta G, Nagy ZP. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reprod Biomed Online* 2006;12:779–96.
46. Hong SW, Chung HM, Lim JM, Ko JJ, Yoon TK, Yee B, et al. Improved human oocyte development after vitrification: a comparison of thawing methods. *Fertil Steril* 1999;72:142–6.
47. Chang CC, Sung CX, Tian XC, Yang X, Kort HI, Nagy ZP. Parallel comparison of parthenogenetic development following oocyte cryopreservation vitrification vs. slow freezing. *Fertil Steril* 2007;88:S350.
48. Gardner DK, Sheehan CB, Rienzi L, Katz-Jaffe M, Larman MG. Analysis of oocyte physiology to improve cryopreservation procedures. *Theriogenology* 2007;67:64–72.
49. Huang JY, Chen HY, Tan SL, Chian RC. Effect of choline-supplemented sodium-depleted slow freezing versus vitrification on mouse oocyte meiotic spindles and chromosome abnormalities. *Fertil Steril* 2007;88:1093–100.
50. Yoon TK, Lee DR, Cha SK, Chung HM, Lee WS, Cha KY. Survival rate of human oocytes and pregnancy outcome after vitrification using slush nitrogen in assisted reproductive technologies. *Fertil Steril* 2007;88:952–6.
51. Elizur SE, Chian RC, Holzer HE, Gidoni Y, Tulandi T, Tan SL. Cryopreservation of oocytes in a young woman with severe and symptomatic endometriosis: a new indication for fertility preservation. *Fertil Steril* 2007 [Epub ahead of print].
52. Homburg R, van der Veen F, Silber SJ. Oocyte vitrification—women’s emancipation set in stone. *Fertil Steril* 2008 [Epub ahead of print].
53. Yoon TK, Kim TJ, Park SE, Hong SW, Ko JJ, Chung HM, et al. Live births after vitrification of oocytes in a stimulated in vitro fertilization–embryo transfer program. *Fertil Steril* 2003;79:1323–6.
54. Shaw JM, Trounson AO. Parthenogenetic activation of unfertilized mouse oocytes by exposure to 1,2-propanediol is influenced by temperature, oocyte age, and cumulus removal. *Gamete Res* 1989;24:269–79.
55. Van der Elst J, Van den Abbeel E, Nerinckx S, Van Steirteghem A. Parthenogenetic activation pattern and microtubular organization of the mouse oocyte after exposure to 1,2-propanediol. *Cryobiology* 1992;29:549–62.