Implantation rates of embryos generated from slow cooled human oocytes from young women are comparable to those of fresh and frozen embryos from the same age group

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Abstract Previous reports of slow cooling of human mature oocytes have shown a reduced clinical efficiency relative to fresh oocytes. This study reports that equivalent fertilization and implantation rates to those obtained using fresh oocytes and cryopreserved embryos can be achieved with human mature oocytes dehydrated in 1.5 M propanediol and 0.2 M sucrose at 37°C and cryopreserved using slow cooling rates.

Keywords Human oocytes · Cryopreservation · Slow cooling · Implantation

Introduction

Over the last decade, improvements in slow cooling of human oocytes has seen a transition from research into clinical practice [1] with over 400 births [2] reported from this

Capsule Equivalent implantation rates for slow cooled mature oocytes dehydrated in 1.5 M propanediol and 0.2 M sucrose at 37°C and fresh oocytes from young women.

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technology. Oocyte cryopreservation provides an option for young single women about to undergo gonadotoxic treatments [3, 4], for couples with ethical concerns regarding embryo cryopreservation and for salvage of a cycle in which sperm retrieval has failed or is limited. There is also a major benefit from oocyte cryopreservation in an oocyte donation program [5] and, potentially, a unique opportunity for the growing population of women who may wish to delay their biological clock. However, this technology has not been adopted as routine practice in the majority of clinics due to reports of reduced clinical efficiency when compared to fresh oocytes [6, 7]. In contrast, application of oocyte vitrification has gained increasing momentum due to reports of clinical outcomes similar to those from fresh oocytes [8–10].

Modifications to the original slow cooling methodology described for human oocytes resulted in increased survival rates (review [11]) but the most frequently used modification, which involves dehydration in 1.5 M propanediol with 0.3 M sucrose [12], appears to be associated with reduced implantation rates when compared to fresh oocytes [6, 7]. This may be a consequence of the slower embryo development [13] and organelle damage [14] reported for this method. Although parameters such as reduced time between collection and cryopreservation [15] and assisted hatching of subsequent embryos [16] may result in some improvement, the clinical efficiency has remained low (reviewed in [11]). In contrast, modifying the sucrose concentration to 0.2 M during dehydration also increased survival [17–20] but implantation did not appear to be impaired [19, 21] suggesting more potential [11] with this modification, although a reduced implantation rate relative to fresh oocytes has also been reported recently [20]. This again raised the criticism that slow cooling is inappropriate for human oocytes

and that only vitrification can achieve similar clinical outcomes to those from fresh oocytes [8–10].

It is well established that embryo selection, whether by the use of early developmental/morphological markers or by applying extended culture to the blastocyst stage, influences the clinical efficiency of all ART procedures [22], including oocyte cryopreservation. In contrast to the vitrification studies published where these approaches have been applied [9], the majority of reports on slow cooling of oocytes have been from Italian clinics where embryo selection was precluded by legislative restrictions, with obvious potential impact on clinical efficiency.

The most critical challenge with slow cooling of human oocytes has been to achieve complete dehydration in a population of oocytes known to have large variation in the membrane hydraulic permeability coefficient [23]. The aim of higher concentrations of non permeating cryoprotectants is to increase the rate of dehydration, thereby achieving dehydration in the vast majority of oocytes and circumventing this variation. Also fundamental to this concept is that the membrane permeability coefficient is dependent on temperature [24] which regulates the movement of permeating cryoprotectants [25].

Therefore, the aim of this study was to compare the clinical outcomes from human mature oocytes, dehydrated in a combination of propanediol and 0.2 M sucrose at 37°C prior to slow cooling, to those from fresh oocytes and frozen embryos in the same clinical programme.

Material and methods

Patients

Oocytes were cryopreserved from patients undergoing controlled ovarian stimulation cycles between January 2001 and August 2011. During this period over 150 patients had oocytes cryopreserved but only 31 have subsequently thawed some or all of their oocytes. Infertility patients requesting thawing had oocytes frozen for the following reasons: a) failure to retrieve sperm ($n=13$), b) objection to embryo cryopreservation ($n=8$), c) ovarian hyperstimulation ($n=2$), and d) egg donation ($n=1$). The majority had been diagnosed with male infertility ($n=19$), the others were diagnosed with endometriosis ($n=2$), unexplained infertility ($n=2$) and age related infertility ($n=1$). Non infertility patients requesting thawing ($n=7$) had oocytes cryopreserved for social reasons ($n=5$) and for fertility preservation ($n=2$). Data for all patients with thawed oocytes is shown in Table 1.

Oocyte cryopreservation

Retrieved oocytes were denuded of cumulus and corona cells using hyaluronidase (20 IU/ml; Hylase, Sanofi Aventis Australia) in Quinn's Advantage HEPES-buffered medium (QHEPES; SAGE BioPharma, USA). Metaphase II oocytes were held in Quinn's Advantage Fertilisation medium (QFERT; SAGE BioPharma, USA) containing 4 mg human

Table 1 Oocyte thaw data

	<38	≥38
Number of patients (pts)	17	14
Mean Age ± SD (range)	30.9±3.8 (24–37.9)	39.9±1.9 (38–45)
Mature oocytes harvested/pt	16.9	9.4
Mature oocytes Cryopreserved/pt	14.3	9.2
Oocytes thawed/pt	11.2	7.1
Number of thaw cycles	25	23
Total no. of embryos transferred (mean/pt)	40 (2.3)	26 (1.9)
No. embryo transfer procedures	30	17
Embryos/transfer procedure	1.3	1.5
Fresh embryos transferred	31 (1.5 per procedure)	24 (1.6 per procedure)
Fresh embryo IR (FH/embryo transferred)	25.8% (8/31)	4.2% (1/24)
Frozen embryos transferred	9 (1.0 per procedure)	2 (1.0 per procedure)
Frozen embryo IR (FH/embryo transferred)	44.4% (4/9)	0%
Babies	10 (8 singleton, 1 twin) + 39 weeks ongoing	1
Gestational weight (g)	3284±539	3100
Gestational age (weeks)	38.7±1.5	37
Gender	6 female, 4 male + 39 weeks ongoing	1 female

IR implantation rate, FH fetal heartbeat

serum albumin (HSA)/ml (SAGE) for a minimum of 1 h between denuding and dehydration. This concentration of HSA was used throughout unless stated. Cryopreservation basal medium was QHEPES supplemented either 20% heat inactivated maternal serum or 20 mg HSA/ml. The maximum time between oocyte collection and cryopreservation was 5 h.

All dehydration solutions were pre-warmed to 37°C and maintained at 37°C on a warmed stage throughout the procedure. Oocytes were dehydrated in 1.5 M 1, 2 - propanediol (PROH) for 5 min (min) followed by 1.5 M PROH + 0.2 M sucrose (SigmaUltra, Sigma) for 5 min before loading into 0.25 ml plastic straws (IMV Technologies, France) and sealed. Straws were loaded into a programmable freezer (Kryo 10; Planer Products, UK) and cooled to −7°C at a rate of 2°C/min. At this point, ice seeding was induced and cooling continued to −30°C at 0.3°C/min and finally at a rate of 50°C/min to ~−150°C. Oocytes were stored under liquid nitrogen.

Thawing

Straws were thawed in air for 30 s followed by 40 s at 30°C. Oocytes were rehydrated through decreasing concentrations of PROH and sucrose (1.0 M PROH + 0.2 M sucrose for 5 min, 0.5 M PROH + 0.2 M sucrose for 5 min, 0.2 M sucrose for 2.5 min and 0.1 M sucrose for 2.5 min). Rehydration was performed at 37°C and the basal medium was QHEPES with 20 mg HSA/ml. Oocytes were transferred to QHEPES for 10 min followed by incubation in QFERT for a minimum of 1 h before ICSI was performed. The ICSI procedure has been previously reported [26]. Oocytes were subsequently cultured in QFERT and assessed for pronuclei at 16–18 h post injection.

Embryo development

Oocytes were then transferred to Quinn's Advantage Cleavage medium. Fertilised oocytes were assessed at 23 h post injection for entry into syngamy/first cleavage and again at 42 h post injection for embryo cleavage [27]. Embryo selection for transfer on day 2 was based on criteria previously reported [28]. All remaining embryos suitable

for cryopreservation were subsequently cryopreserved on day 2 using the 1.5 M PROH + 0.2 M sucrose embryo method previously reported [29].

Fresh oocytes & frozen embryos

Results obtained from fresh oocytes/embryos and cryopreserved embryos transferred in our clinic post 2004 were compared to those from cryopreserved oocytes. Implantation was defined by detection of an intrauterine fetal heartbeat. Data was analysed by chi square.

Results

The results for cryopreserved oocytes are presented in Table 2 and are stratified according to female age at cryopreservation (<38 and ≥38). All metaphase oocytes were cryopreserved irrespective of morphological appearance. Despite this, the post thaw survival rate was 75.8% (Table 2). The subsequent fertilisation rate for the thawed oocytes which were injected (67.6%) was similar to that observed for fresh oocytes undergoing ICSI in our clinic (70.8%).

A mean of 9.3 oocytes were thawed per patient. In patients where oocytes had been cryopreserved due to lack of available sperm, all or most of the oocytes were thawed and supernumerary embryos were cryopreserved. Where oocyte cryopreservation had been performed to avoid embryo cryopreservation, a maximum of 2 oocytes per cycle were thawed.

Of 40 day 2 embryos, derived from cryopreserved/thawed oocytes, transferred in women under 38, the overall implantation (FH) rate was 36.6% (12/40; Table 2). Four of the 12 implantations resulted from the transfer of frozen/thawed embryos which had been generated from cryopreserved oocytes. We have previously described one of these cases in which implantation and live birth resulted from the transfer of a cryopreserved embryo which had been generated from injection of a cryopreserved sperm into a cryopreserved oocyte [30]. From January 2004 onwards, the implantation rate from over 7000 ICSI generated day 2 embryos in women of the same age in our clinic was 26.0%. A similar rate was observed for embryos transferred fresh which had been generated from cryopreserved oocyte (25.8%; Table 1).

Table 2 Oocyte cryopreservation

Age at oocyte freeze	Patients	Frozen oocytes	Oocytes thawed	Survival (%)	Normally fertilised (%)	Cleaving embryos (%)	Embryos transferred	Fetal hearts	Overall implantation rate	Clinical pregnancies/transfer
<38	17	244	190	144 (75.8)	107 (74.3)	96 (89.7)	40	12	30.0%	11/30 (36.6%)
≥38	14	129	99	75 (75.8)	41 (54.7)	38 (92.7)	26	1	3.8%	1/17 (5.9%)
Total	31	373	289	219 (75.8)	148 (67.6)	134 (90.5)	66	13	18.2%	12/47 (25.5%)

The implantation rate of cryopreserved/thawed day 2 embryos in women under 38 in this period was 19.1%. Although the number was low (9 embryos transferred) a comparable IR (44.4%) was observed for frozen/thawed embryos generated from cryopreserved oocytes.

The implantation rate of day 2 embryos formed from oocytes cryopreserved when the woman was past her 38th birthday was 3.8% (1/26; Table 2). Fresh day 2 ICSI embryos and cryopreserved day 2 embryos in this age group have implantation rates of 10.1% and 7.6% respectively in our clinic.

Information regarding the pregnancy outcomes from cryopreserved oocytes are reported in Table 1. All cryopreserved oocyte pregnancies except one continued to term and no birth abnormalities were reported. One patient had a miscarriage at 8 weeks; with a normal karyotype. This patient has also had a fresh oocyte/embryo pregnancy which miscarried at 10 weeks.

Discussion

The survival rate achieved in the present study is similar to that previously observed with the 0.3 M sucrose method [7, 15, 16, 31–37] and the 0.2 M sucrose method [17, 19, 21]. In some of the above studies only oocytes with optimal morphology were cryopreserved [37, 38] which is not the case in the present study.

Theoretically, based on the temperature dependent membrane kinetics [39] and the low permeability [24] to water of human oocytes, exposure to the same concentration of cryoprotectant for an equivalent time will achieve a greater degree of dehydration at 37°C than at room temperature. As a consequence of the variability in the hydraulic permeability coefficient of individual human mature oocytes [23], a higher proportion of oocytes will be fully dehydrated at the higher temperature. This may be expected to impact on cryosurvival and may be an explanation for the difference observed in survival between the present study (75.8%) and a recent multi centre study using 0.2 M sucrose at room temperature (55.8% survival) [20].

In the present study, oocyte cryopreservation had no impact on subsequent fertilisation, cleavage or implantation of the subsequent embryos when compared to outcomes from fresh oocytes. Equivalent outcomes from cryopreserved/thawed compared to fresh oocytes have also been reported after rehydration in 0.2 M sucrose at 22°C with higher sucrose concentrations in the initial post thaw rehydration phase (0.3 M) [19] and in a donor program following dehydration at 37°C [21]. These results are in contrast to those reported for the 0.3 M sucrose dehydration method where an occasional group has achieved good blastocyst development [40] but the majority of data reported indicates a reduction in

implantation relative to fresh controls [32]. Electron microscopic assessment of cryopreserved/thawed oocytes showed an increase in cytoplasmic abnormalities in those dehydrated in 0.3 M sucrose but not in those exposed to 0.2 M sucrose [14]. Slower embryo development on day 2 has also been observed with 0.3 M [31] but not with 0.2 M sucrose [19].

Previously, we have demonstrated a reduced fertilisation rate associated with a delay of >8 h between initial oocyte collection and insemination of cryopreserved oocytes [41]. Although the effect on fertilisation has been circumvented by the universal application of ICSI, similar delays have now been demonstrated to manifest in reduced implantation rates [42, 43]. Although failure to obtain sperm was the main reason for oocyte cryopreservation in the present study, and would often delay the decision to cryopreserve the oocytes, we have adhered to a strict limit of cryopreservation within 5 h of oocyte retrieval. Both limiting this time interval and applying similar criteria when selecting embryos for transfer, have contributed to the similar implantation rates observed for embryos from both cryopreserved and fresh oocytes in this study. Unfortunately, this has not been an option in most of the previous oocyte cryopreservation studies due to legal restrictions in Italy. However, in one Italian study where at least two 4 cell embryos, derived from cryopreserved oocytes, were transferred [44] and in a report from an oocyte donation program [21], similar implantation rates to this study were observed, in both cases using the 0.2 M sucrose dehydration procedure.

It would appear from the present study that the only parameter detracting from application of slow freezing with this method for oocyte cryopreservation is the survival rate of 76%. In comparison, a survival rate of 90% [5, 8, 9, 45] has been achieved with vitrification in 2.7 M ethylene glycol + 2.1 M dimethyl sulphoxide + 0.5 M sucrose in an open system initially reported by Katayama et al., (2003) [46]. This level of survival has repeatedly been obtained [10, 47–49] but not by all groups (77%; [50], 81%; [51] and when used in conjunction with a closed system has resulted in a reduced survival (75%; [52]). Manipulation of the equilibration of the permeable cryoprotectant and water prior to vitrification in a closed system has resulted in a high rate of survival (95%) only previously observed in open systems but blastocyst development appeared to be reduced [40], possibly as a consequence of ultrastructural damage [53]. It has also been shown that a significant reduction in survival results following transport at −196°C of oocytes vitrified in the above open system [54].

It is clear that critical optimal parameters, whether for vitrification or slow cooling, need to be determined for the human oocyte, in order to consistently achieve high survival and implantation rates. While the debate around whether high success can be achieved with a closed vitrification system continues [55], the slow cooling method reported herein

provides an easy, reproducible, safe closed storage system for human oocytes achieving similar clinical efficiency to fresh oocytes and cryopreserved embryos.

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References

- Noyes N, Boldt J, Nagy ZP. Oocyte cryopreservation: is it time to remove its experimental label? *J Assist Reprod Genet.* 2010;27(2–3):69–74.
- Noyes N, Porcu E, Borini A. Over 900 oocyte cryopreservation babies born with no apparent increase in congenital anomalies. *Reprod Biomed Online.* 2009;18(6):769–76.
- Yang D, Brown SE, Nguyen K, et al. Live birth after the transfer of human embryos developed from cryopreserved oocytes harvested before cancer treatment. *Fertil Steril.* 2007;87(6):1469.e1–1469.e4.
- Porcu E, Venturoli S, Damiano G, et al. Healthy twins delivered after oocyte cryopreservation and bilateral ovariectomy for ovarian cancer. *Reprod Biomed Online.* 2008;17(2):265–7.
- Cobo A, Meseguer M, Remohi J, et al. Use of cryo-banked oocytes in an ovum donation programme: a prospective, randomized, controlled, clinical trial. *Hum Reprod.* 2010;25(9):2239–46.
- Borini A, Bianchi V, Bonu MA, et al. Evidence-based clinical outcome of oocyte slow cooling. *Reprod Biomed Online.* 2007;15(2):175–81.
- Levi Setti PE, Albani E, Novara P, et al. Cryopreservation of supernumerary oocytes in IVF/ICSI cycles. *Hum Reprod.* 2006;21(2):370–5.
- Antinori M, Licata E, Dani G, et al. Cryotop vitrification of human oocytes results in high survival rate and healthy deliveries. *Reprod Biomed Online.* 2007;14(1):72–9.
- Cobo A, Kuwayama M, Perez S, et al. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. *Fertil Steril.* 2008;89(6):1657–64.
- Ubaldi F, Anniballo R, Romano S, et al. Cumulative ongoing pregnancy rate achieved with oocyte vitrification and cleavage stage transfer without embryo selection in a standard infertility program. *Hum Reprod.* 2010;25(5):1199–205.
- Gook DA, Edgar DH. Human oocyte cryopreservation. *Hum Reprod Update.* 2007;13(6):591–605.
- Fabbri R, Porcu E, Marsella T, et al. Human oocyte cryopreservation: new perspectives regarding oocyte survival. *Hum Reprod.* 2001;16(3):411–6.
- Bianchi V, Coticchio G, Distratis V, et al. Early cleavage delay in cryopreserved human oocytes. *Hum Reprod.* 2005;20 Suppl 1:i54.
- Nottola SA, Macchiarelli G, Coticchio G, et al. Ultrastructure of human mature oocytes after slow cooling cryopreservation using different sucrose concentrations. *Hum Reprod.* 2007;22(4):1123–33.
- Parmegiani L, Cognigni GE, Bernardi S, et al. Freezing within 2 h from oocyte retrieval increases the efficiency of human oocyte cryopreservation when using a slow freezing/rapid thawing protocol with high sucrose concentration. *Hum Reprod.* 2008;23(8):1771–7.
- Konc J, Kanyo K, Varga E, et al. Births resulting from oocyte cryopreservation using a slow freezing protocol with propanediol and sucrose. *Syst Biol Reprod Med.* 2008;54(4–5):205–10.
- Winslow K, Yang D, Blohm P, et al. Oocyte cryopreservation/ a 3 year follow up of sixteen births. *Fertil Steril.* 2001;76(3, Suppl 1):S120. Abst P-28.
- Chen ZJ, Li M, Li Y, et al. Effects of sucrose concentration on the developmental potential of human frozen-thawed oocytes at different stages of maturity. *Hum Reprod.* 2004;19(10):2345–9.
- Bianchi V, Coticchio G, Distratis V, et al. Differential sucrose concentration during dehydration (0.2 mol/l) and rehydration (0.3 mol/l) increases the implantation rate of frozen human oocytes. *Reprod Biomed Online.* 2007;14(1):64–71.
- Borini A, Levi Setti PE, Anserini P, et al. Multicenter observational study on slow-cooling oocyte cryopreservation: clinical outcome. *Fertil Steril.* 2010;94(5):1662–8.
- Yang D, Winslow K, Blohm P, et al. Oocyte donation using cryopreserved donor oocytes. *Fertil Steril.* 2002;78(3, Suppl 1):S14. Abst O-37.
- Ebner T, Moser M, Sommergruber M, et al. Selection based on morphological assessment of oocytes and embryos at different stages of preimplantation development: a review. *Hum Reprod Update.* 2003;9(3):251–62.
- Hunter J, Bernard A, Fuller B, et al. Plasma membrane water permeabilities of human oocytes: the temperature dependence of water movement in individual cells. *J Cell Physiol.* 1992;150(1):175–9.
- Hunter JE, Bernard A, Fuller BJ, et al. Measurements of the membrane water permeability (L_p) and its temperature dependence (activation energy) in human fresh and failed-to-fertilize oocytes and mouse oocyte. *Cryobiology.* 1992;29(2):240–9.
- Newton H, Fisher J, Arnold JR, et al. Permeation of human ovarian tissue with cryoprotective agents in preparation for cryopreservation. *Hum Reprod.* 1998;13(2):376–80.
- Bourne H, Richings N, Harari O, et al. The use of intracytoplasmic sperm injection for the treatment of severe and extreme male infertility. *Reprod Fertil Dev.* 1995;7:237–45.
- Lawler C, Baker HW, Edgar DH. Relationships between timing of syngamy, female age and implantation potential in human in vitro-fertilised oocytes. *Reprod Fertil Dev.* 2007;19(3):482–7.
- Edgar DH, Bourne H, Speirs AL, et al. A quantitative analysis of the impact of cryopreservation on the implantation potential of human early cleavage stage embryos. *Hum Reprod.* 2000;15:175–9.
- Edgar DH, Karani J, Gook DA. Increasing dehydration of human cleavage-stage embryos prior to slow cooling significantly increases cryosurvival. *Reprod Biomed Online.* 2009;19(4):521–5.
- Gook DA, Hale L, Edgar DH. Live birth following transfer of a cryopreserved embryo generated from a cryopreserved oocyte and a cryopreserved sperm: case report. *J Assist Reprod Genet.* 2007;24(1):43–5.
- Borini A, Sciajno R, Bianchi V, et al. Clinical outcome of oocyte cryopreservation after slow cooling with a protocol utilizing a high sucrose concentration. *Hum Reprod.* 2006;21(2):512–7.
- Barritt J, Luna M, Duke M, et al. Report of four donor-recipient oocyte cryopreservation cycles resulting in high pregnancy and implantation rates. *Fertil Steril.* 2007;87(1):189.e13–189.e17.
- Ferraretti AP, Lappi M, Magli MC, et al. Factors affecting thawed oocyte viability suggest a customized policy of embryo transfer. *Fertil Steril.* 2010;94(4):1308–13.
- La Sala GB, Nicoli A, Villani MT, et al. Outcome of 518 salvage oocyte-cryopreservation cycles performed as a routine procedure in an in vitro fertilization program. *Fertil Steril.* 2006;86(5):1423–7.
- De Santis L, Cino I, Rabellotti E, et al. Oocyte cryopreservation: clinical outcome of slow-cooling protocols differing in sucrose concentration. *Reprod Biomed Online.* 2007;14(1):57–63.
- Chamayou S, Alecci C, Ragolia C, et al. Comparison of in-vitro outcomes from cryopreserved oocytes and sibling fresh oocytes. *Reprod Biomed Online.* 2006;12(6):730–6.
- Magli MC, Lappi M, Ferraretti AP, et al. Impact of oocyte cryopreservation on embryo development. *Fertil Steril.* 2010;93(2):510–6.

38. Parmegiani L, Garello C, Granella F, et al. Long-term cryostorage does not adversely affect the outcome of oocyte thawing cycles. *Reprod Biomed Online*. 2009;19(3):374–9.
39. Leibo SP. Water permeability and its activation energy of fertilized and unfertilized mouse ova. *J Membr Biol*. 1980;53(3):179–88.
40. Grifo JA, Noyes N. Delivery rate using cryopreserved oocytes is comparable to conventional in vitro fertilization using fresh oocytes: potential fertility preservation for female cancer patients. *Fertil Steril*. 2009;93(2):391–6.
41. Gook DA, Osborn SM, Bourne H, et al. Fertilization of human oocytes following cryopreservation; normal karyotypes and absence of stray chromosomes. *Hum Reprod*. 1994;9(4):684–91.
42. Parmegiani L, Bertocci F, Garello C, et al. Efficiency of human oocyte slow freezing: results from five assisted reproduction centres. *Reprod Biomed Online*. 2009;18(3):352–9.
43. Ferraretti AP, Lappi M, Magli MC, et al. Factors affecting thawed oocyte viability suggest a customized policy of embryo transfer. *Fertil Steril*. 2010;94(4):1308–13.
44. Coticchio G, Distratis V, Bianchi V, et al. Fertilization and early developmental ability of cryopreserved human oocytes is not affected compared to sibling fresh oocytes. *Fertil Steril*. 2007;88 Suppl 1:P-700.
45. Cobo A, Romero JL, Perez S, et al. Storage of human oocytes in the vapor phase of nitrogen. *Fertil Steril*. 2010b.
46. Katayama KP, Stehlik J, Kuwayama M, et al. High survival rate of vitrified human oocytes results in clinical pregnancy. *Fertil Steril*. 2003;80(1):223–4.
47. Nagy ZP, Chang CC, Shapiro DB, et al. Clinical evaluation of the efficiency of an oocyte donation program using egg cryo-banking. *Fertil Steril*. 2009;92(2):520–6.
48. Garcia JJ, Noriega-Portella L, Noriega-Hoces L. Efficacy of oocyte vitrification combined with blastocyst stage transfer in an egg donation program. *Hum Reprod*. 2011;26:782–90.
49. Trokoudes KM, Pavlides C, Zhang X. Comparison outcome of fresh and vitrified donor oocytes in an egg-sharing donation program. *Fertil Steril*. 2011;95(6):1996–2000.
50. Schoolcraft WB, Keller JL, Schlenker T. Excellent embryo quality obtained from vitrified oocytes. *Reprod Biomed Online*. 2009;19(6):820–3.
51. Noyes N, Knopman J, Labella P, et al. Oocyte cryopreservation outcomes including pre-cryopreservation and post-thaw meiotic spindle evaluation following slow cooling and vitrification of human oocytes. *Fertil Steril*. 2010b.
52. Smith GD, Serafini PC, Fioravanti J, et al. Prospective randomized comparison of human oocyte cryopreservation with slow-rate freezing or vitrification. *Fertil Steril*. 2010;94(6):2088–95.
53. Bonetti A, Cervi M, Tomei F, et al. Ultrastructural evaluation of human metaphase II oocytes after vitrification: closed versus open devices. *Fertil Steril*. 2011;95(3):928–35.
54. McDonald CA, Valluzo L, Chuang L, et al. Nitrogen vapor shipment of vitrified oocytes: time for caution, in *Fertil Steril*. 2011: United States. p. 2628–30.
55. Vajta G, Nagy ZP. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reprod Biomed Online*. 2006;12(6):779–96.