

First delivery of healthy offspring after freezing and thawing of oocytes in Switzerland

Maria De Geyter, Sabine Steimann, Wolfgang Holzgreve, Christian De Geyter

Woman's Hospital, University of Basel, Switzerland

Summary

The interest in long-term storage of unispermated oocytes through cryopreservation has seen a recent upsurge, because it provides the potential to assist young women to postpone childbirth after having overcome a malignant disease or delaying childbirth until after management of a professional career. The low fertilisation rate of frozen/thawed oocytes in earlier feasibility trials can now be improved by using intracytoplasmic sperm injection (ICSI) for assisting the penetration of the spermatozoon through the oocyte's hardened zona pellucida. Another reason for the reported low success rates of oocyte cryopreservation in earlier studies may have been the low developmental potential of spare oocytes, which were available for experimental cryopreservation. Oocytes retrieved from supernumerary follicles in women treated with gonadotropins for ovulation

induction and intrauterine insemination can be used for the optimisation of cryostorage of unispermated oocytes. We intended to investigate to what extent the well-established and successful cryopreservation protocols for pronucleate oocytes are also applicable for the cryopreservation of unispermated oocytes. We herewith report the first successful pregnancy and delivery of frozen/thawed oocytes in Switzerland, which were inseminated with ICSI. In unbiased treatment groups the freezing and thawing of unispermated oocytes and pronucleate oocytes give comparable results, if the additional manipulation during ICSI was taken into account.

Key words: cryopreservation; oocyte; assisted reproduction; reproductive storage; pronucleate oocyte

Introduction

The treatment of both male and female infertility nowadays strongly relies on assisted reproductive technology, most notably in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI). However, consistently satisfying pregnancy rates can only be achieved within the context of controlled ovarian stimulation, particularly if performed by administering gonadotropins throughout the follicular phase of the menstrual cycle. Through this hormonal pretreatment the reproductive biologist usually has access to more oocytes or embryos than can be subsequently replaced in a responsible way. In order to reduce the degree of multiple pregnancies and to enable the patient to have repeated embryo transfers in sequential menstrual cycles without having to undergo repeated ovarian stimulation, various strategies for the cryopreservation of supernumerary reproductive material have been developed over time. Not only the male and the female gonadal tissue can be cryopreserved (eg stored frozen), but also semen, unispermated oocytes, oocytes at the pronucleate stage and embryos at various developmental stages. The freezing and

thawing of unispermated oocytes was actually one of the first techniques to be introduced successfully [1], but has found little application, mainly due to its low efficacy leading to inconsistent results and due to the availability of other, more efficient technologies, such as cryopreservation of embryos. In Switzerland, a restrictive legislation, as stipulated by the so-called "Fortpflanzungsmedizingesetz", has banned all freezing of embryos (unless in an emergency situation) since January 2001. Only a maximum of three embryos can be cultured and all viable embryos have to be transferred [2]. Similar restrictive laws were issued in Germany and in Italy, the latter even banning the cryopreservation of oocytes at their pronucleate stage. Cryopreservation of embryos is being performed in most other countries worldwide. In Switzerland methods for freezing and thawing of oocytes in the pronucleate stage have been adopted successfully [3]. Various studies have demonstrated that freezing and thawing of oocytes at the pronucleate stage is efficient and leads to acceptable pregnancy rates [4, 5].

The discrepancy between the low efficacy of

cryopreservation of oocytes and the much higher pregnancy rates after freezing and thawing of pronucleate oocytes is striking, because from the cryobiological point of view these structures are very similar. Both are developmentally separated by only a few hours, have the same size, a similar membrane and a similar cytoplasmic watery appearance. The difference between both structures relates to the status of the nucleus and the organelles associated with it, the meiotic spindle in particular. Having a well-established and successful cryostorage protocol for pronucleate oocyte at our disposal, we decided to evaluate the efficacy of freezing and thawing of unseminated oocytes using our established freezing and thawing protocol for pronucleate oocytes.

When compared with pronucleate oocytes, the freezing and thawing of unseminated oocytes have various potential advantages. Oocyte freezing could be used in women at risk of losing their reproductive potential, and in men in whom a malignant disease has been diagnosed and who will have to undergo either chemotherapy or radiation [6]. In addition to this, the current trend of

delaying family planning in women aiming at a professional career also entails the risk of losing most of their reproductive potential later, when a pregnancy would be feasible. Finally, prolonged cryostorage of unseminated oocytes does not carry the burden of the many ethical concerns associated with the cryostorage of embryos or even of pronucleate oocytes.

The poor outcome of oocyte freezing and thawing has been partially attributed to the selective use of low quality oocytes in many of earlier feasibility trials, and to hardening of the zona pellucida during the freezing process, reducing its penetrability during subsequent *in vitro* fertilisation [7]. Whereas the zona hardening can now be overcome with ICSI, we decided to use oocytes retrieved from infertile women previously stimulated with exogenous gonadotropins for intrauterine insemination, in whom supernumerary oocytes had to be removed in order to prevent multiple pregnancies [8]. Herewith, we report the first pregnancy and delivery in Switzerland after freezing and thawing of unseminated oocytes.

Material and methods

The pregnancy and delivery described in this report arose within the context of a prospective study, which was presented to and accepted by our local ethics committee (EKBB 141/04). This prospective study consisted of offering to infertile couples treated with gonadotropins for ovulation induction for IUI the option of freezing oocytes from supernumerary follicles, which were aspirated through transvaginal ultrasound-guided puncture, as described earlier [8]. All couples signed an informed consent form prior to the cryopreservation of their oocytes. The cryopreservation protocol was identical to the protocol used for storage of pronucleate oocytes, which has been successfully used in our laboratory for many years. This protocol consisted of the stepwise addition of an increasingly concentrated cryoprotectant solution (0.25 M, 0.5 M, 0.75 M, 1.0 M, 1.25 M until the final concentration of 1.5 M each five minutes) containing both dimethylsulfoxide (DMSO) and propanediol (PROH) to the oocytes at room temperature. Over a period of 5.5 hours the cryoprotective solution containing the oocytes were then slowly cooled down using a computer-guided cooling system equipped with automatic seeding (CTE 920 Kryotechnik, Erlangen, Germany). Subsequently, the cryopreserved oocytes in the sealed straws were stored in liquid nitrogen.

If no pregnancy arose after IUI, the frozen oocytes were taken from the container and thawed rapidly at room temperature. The cryoprotectant was removed stepwise by transferring the oocytes into the cryoprotective solutions, as described above but in reverse order. Then, the oocytes were treated with ICSI and all fertilised oocytes with two pronuclei were replaced at the embryonic cleaving stage into the uterine cavity. The embryos were transferred to the recipient either during a natural menstrual cycle or after induction of follicular development with clomiphene citrate followed by ovulation induction with HCG.

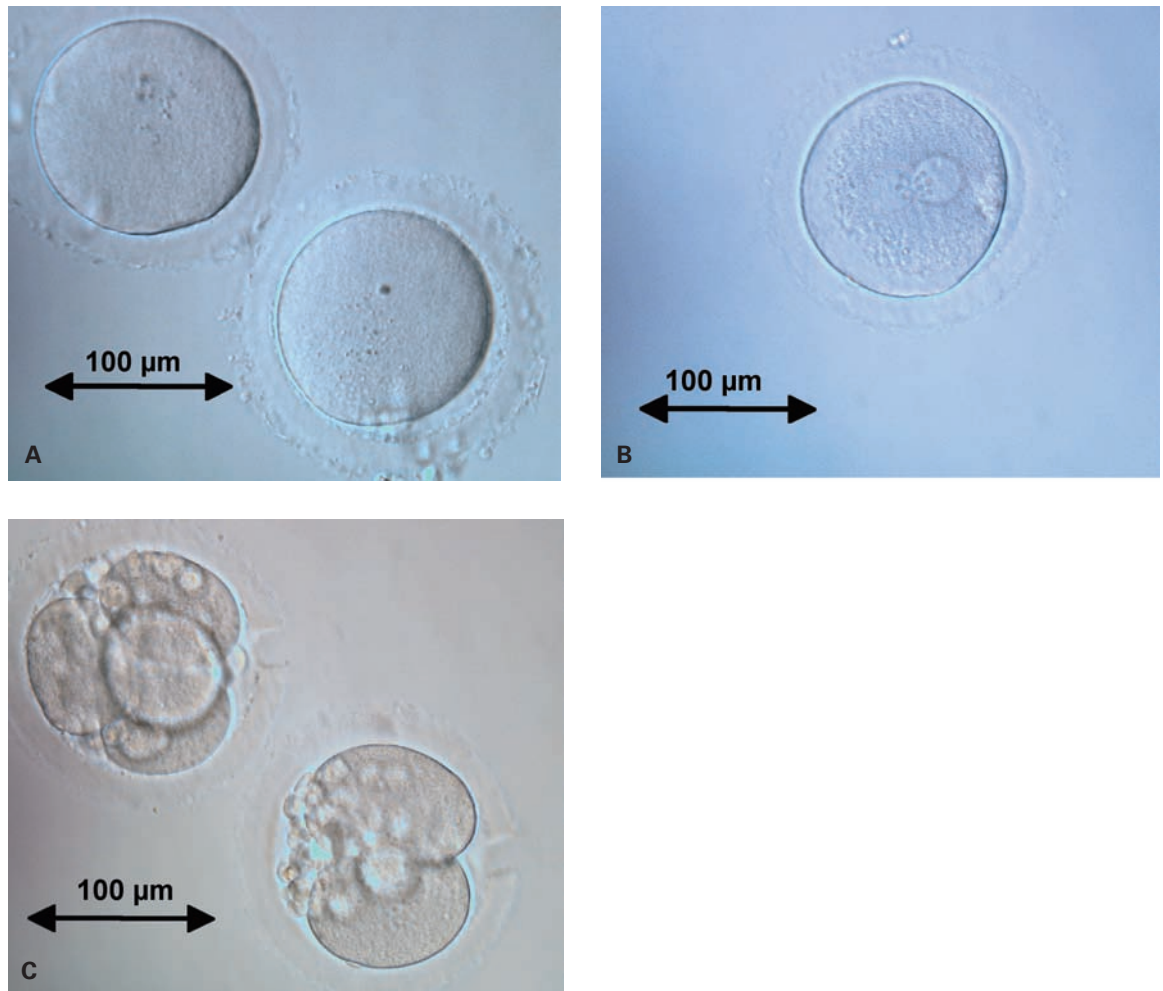
The aim of this study was to examine both the feasibility and the efficacy of freezing and thawing of unseminated oocytes under the conditions described above. The results of freezing and thawing of oocytes at the pronucleate stage of infertile women treated with either IVF or ICSI during the same time period, but not achieving pregnancy in their initial fresh treatment cycle, were selected as controls.

Case

A 29 year old patient was diagnosed in our service to have chronic anovulatory infertility due to the polycystic ovary syndrome as indicated by both elevated testosterone levels and the typical appearance of the ovaries during ultrasound scanning. Her husband had normal semen parameters according to the WHO-guidelines (1999) and the results of his physical exam were normal. The couple had a five year old daughter, but no other pregnancies. At the moment of the present treatment the patient had already undergone three treatments with clomiphene citrate and one ovulation induction with recombinant gonadotropins (Gonal F, Serono, Zug, Switzerland) following the chronic low dose protocol, but none of these treatments led to pregnancy. During the second treatment trial with recombinant gonadotropins a supernumerary cohort of mature ovarian follicles developed. Selective aspiration of some of these follicles had to be performed in order to prevent multiple pregnancy [8]. A total of seven oocytes were aspirated through transvaginal, ultrasound-guided puncture of the follicles. These oocytes were cryopreserved following our cryostorage protocol for pronucleate oocytes (figure 1). This protocol consists of a slow cooling process of the denuded oocytes in a cryoprotectant solution containing DMSO and PROH.

Figure 1

View of the two un-inseminated oocytes (A), which after thawing were treated with ICSI. One of the resulting pronucleate oocytes (B) and the two embryos (C) were replaced approximately 48 hours after thawing. One of these embryos led to the pregnancy and delivery, reported in this communication.

**Results****Results of prospective cohort study**

Between January 2005 and July 2007 20 cryopreservations of 102 uninseminated oocytes were performed originating from 19 women, whose oocytes were collected after treatments with gonadotropins for IUI in need of preovulatory aspiration of supernumerary follicles in order to prevent polyovulation and thereby multiple pregnancy [8]. Of those women with unsuccessful IUI, 15 thawings of their frozen oocytes were performed (table 1). Of the 73 oocytes thawed, 33 survived (60.8%) and were treated with ICSI. 24 of the 33 injected oocytes were fertilised with two visible pronuclei, developed to the cleaving embryo stage (72.7%) and replaced into the uterine cavity of nine recipients (transfer rate per oocyte freezing: 45.0%) leading to three pregnancies (pregnancy rate per freezing: 15.0%). One pregnancy ended as an early miscarriage and another pregnancy proved to be a vital tubal pregnancy as evidenced both by ultrasound and laparoscopy. A third pregnancy proved to be viable and is presented here as the first delivery after cryopreservation of an uninseminated oocyte in Switzerland.

Case

As the second ovulation induction with recombinant gonadotropins followed by IUI failed to produce a pregnancy, we offered the couple thawing of the frozen oocytes and insemination of the thawed oocytes with ICSI followed by transfer of the developing embryos into the uterine cavity. The couple agreed to participate in the trial and menstruation was induced with dydrogesteron (20 mg daily over 10 days, Duphaston, Solvay, Berne). The endometrium was then brought to proliferation by inducing follicular growth with clomiphene citrate (50 mg daily over five days starting on day five after menstruation, Serophene, Serono) and the ovulation of a 20 mm follicle was induced with human chorionic gonadotropins (10'000 international units of HCG, Pregnyl, Organon, Pfäffikon). Five days prior to ovulation induction the couple was instructed to refrain from sexual intercourse. After having confirmed ovulation with ultrasound the thawing of all seven oocytes was started in batches of up to three oocytes. Finally, all seven thawed oocytes were treated with ICSI, resulting in two embryos, which were replaced into the uterine cavity under transabdominal transvesical ultrasound guidance

Table 1

Results from freezing and thawing of uninseminated oocytes, retrieved from supernumerary follicles after ovulation induction with gonadotropins. This data collection started in January 2005 and ended in October 2006. The data were compared with the results of pronucleate oocyte freezing and thawing in the same time period using the same cryostorage protocol. However, for the purpose of comparison we excluded those treatments with IVF and ICSI, which led to a pregnancy in the original stimulated cycle.

Freezing and thawing of oocytes	uninseminated	pronucleate	sign. ¹
No. of patients:	19	118	
Age (years, mean \pm SD)	34 \pm 5.1	34 \pm 4.1	
No. of freezing cycles:	20	120	
No. of cryopreserved oocytes:	102	600	
No. of thawings (% per freezing):	15 (75.0)	65 (54.2)	
No. of thawed oocytes:	73	281	
No. of surviving oocytes (% per thawed oocyte):	33 (60.8)	–	
No. of fertilized oocytes (% per thawed oocyte):	24 (72.7)	–	
No. of cleaving embryos (% per thawed oocyte):	21 (28.8) ²	176 (62.6) ²	p <0.0001
No. of implanted embryos (% per replaced embryo):	3 (14.3)	54 (30.7)	n.s.
No. of cycles with embryo transfer (% per cycle):	9 (45.0)	55 (84.6)	n.s.
No. of recorded pregnancies (% per freezing):	3 (15.0) ³	19 (15.8)	n.s.
No. of deliveries (% per freezing):	1 (5.0)	8 (6.7)	n.s.

¹ Chi-squared test with Yates' correction

² As stipulated by Swiss legislation all cleaving embryos were transferred.

³ One pregnancy ended in an early miscarriage, another pregnancy presented as a tubal pregnancy with embryonic cardiac activity, and one pregnancy proved to be intact leading to the delivery of a healthy child.

(figure 1). Six days after embryo transfer the luteal phase was supported with one single injection of 5000 international units of HCG (Pregnyl). An intact intrauterine singleton pregnancy was confirmed by serial measurements of human chorionic gonadotropin serum levels and vaginal ultrasound. The pregnancy evolved without complica-

tions and the patient delivered a healthy boy in August 2006 in the University Women's Hospital of Basel. His neonatal birth weight was 3225 g with a length of 47 cm, and no malformations were detected. The child continued to develop well.

Discussion

Although the patient ovulated naturally during the replacement cycle, it is very likely that she conceived from the frozen-thawed oocyte, because she was asked to refrain from intercourse around the time of ovulation. This delivery is the first to be reported after freezing and thawing of an uninseminated, nonfertilised oocyte in Switzerland and fits well to the current renewed interest in oocyte cryopreservation [7].

The case described here is part of an ongoing prospective study, in which the feasibility of freezing and thawing of uninseminated oocytes is being examined systematically. It was thought that the low developmental quality of the oocytes used in previous cohort studies was a major determining factor in the unsatisfactory outcome of oocyte cryopreservation [7, 9]. The oocytes used in this study came from supernumerary ovarian follicles after ovarian stimulation with gonadotropins for scheduled treatments with intrauterine insemination [8]. The published high pregnancy rate with this mode of therapy [10], the high pregnancy rate achieved in treatments, originally intended for ovulation induction but then converted into IVF due to the ovaries' overreaction to the applied gonadotropin dose [11], and the results of cytogenetic analysis of oocytes retrieved from supernumerary follicles [12] suggest that these oocytes possess a high developmental potential. For the

cryopreservation of these good-quality oocytes we used the same protocol as for pronucleate oocytes, which has resulted in high pregnancy rates in our institution for many years.

In the present analysis we used the results of freezing and thawing of pronucleate oocytes collected from patients treated with IVF or ICSI during the same time period for control purposes. Because the thawings of the uninseminated oocytes were exclusively performed after unsuccessful IUI treatments, we excluded those thawings of pronucleate oocytes resulting from IVF or ICSI treatments, which had been successful in the fresh cycle.

Under those conditions we observed that the number of surviving oocytes after thawing (60.8%) was strikingly similar to the number of cleaving embryos resulting from thawing of frozen oocytes at the pronucleate stage (62.6%). However, the extra manipulation of the thawed oocytes with ICSI caused an additional progressive loss leading to significantly lower numbers of implanted embryos after thawing of uninseminated oocytes as compared to the thawed pronucleate oocytes (p <0.0001, table 1). Surprisingly, both the pregnancy rate and the delivery rate per freezing were not different among both groups, indicating the cryopreservation protocol *per se* is adequate for both cell types (table 1). Neverthe-

less, the fertilisation and pregnancy rates presented in this prospective study are somewhat lower than those published in recent meta-analyses [13, 14]. Despite the successful pregnancy presented in this communication, the ongoing results of our freezing and thawing protocol of unseminated oocytes using the protocols for pronucleate oocytes are somewhat disappointing.

The low success rate of freezing and thawing of unseminated oocytes is thought to be caused by the disruption of the meiotic spindle apparatus during the slow cooling phase of conventional cryoconservation leading to aneuploidy in the resulting embryos [15]. Better results have been published with conventional cryopreservation protocols based on other cryoprotective environments, particularly if sucrose is added to the cryoprotective solution [16, 17]. In recent years, much better results than with conventional cryopreservation were achieved with vitrification of unseminated oocytes [18–20]. Vitrification is a process of converting the oocyte and the cryoprotectant into a glass-like amorphous solid state, which is free of any crystalline structure. The formation of ice crystals is the major cause of cell damage through disruption of the membrane or of major organelles during conventional cryopreservation. Vitrification of unseminated oocytes is achieved by adding the cryoprotectant at room temperature and by extreme supercooling into liquid nitrogen at $-196\text{ }^{\circ}\text{C}$ [21, 22]. At present there is still some concern with the long-term risk for the offspring, as toxic cryoprotect-

ants are added to the oocytes at much higher concentrations (5 to 6 M) than in conventional slow-cooling cryopreservation protocols. Recent developments of vitrification, however, have enabled significant reductions of the freezing volume, which surrounds the oocytes, resulting in extremely rapid freezing rates [21, 22]. Speeding up freezing rates in vitrification allows reduction of the cryoprotectant concentrations resulting in similar freezing environments as in conventional freezing protocols, in which no malformations in the offspring have yet been described.

In conclusion, cryopreservation protocols, which have been shown to be adequate and successful for freezing and thawing of pronucleate oocytes, have now been shown to give rise to similar delivery rates with freezing and thawing of unseminated oocytes. However, our experience also indicates that implantation and pregnancy rates of this freezing and thawing protocol are insufficient for its future use in cancer patients or in women aiming at preserving some of their oocytes for future family planning.

Correspondence:

Prof. Dr. med. Christian De Geyter

Universitätsfrauenklinik

Spitalstrasse 12

CH-4031 Basel

Switzerland

E-Mail: cdegeyter@ubbs.ch

References

- Chen C. Pregnancy after human oocyte cryopreservation. *Lancet*. 1986;8486:884–6.
- Germond M, Senn A. A law affecting medically assisted procreation is on the way in Switzerland. *J Assist Reprod Genet*. 1999;16:341–3.
- Van den Bergh M, Hohl MK, De Geyter C, Stahlberg A, Limoni C. Ten years of Swiss IVF data. Are we making progress? *Reprod Biomed Online*. 2005;11:632–40.
- Horne G, Critchlow JD, Newman MC, Edozien L, Matson PL, Lieberman BA. A prospective evaluation of cryopreservation strategies in a two-embryo transfer programme. *Hum Reprod*. 1997;12:542–7.
- Senn A, Vozzi C, Chanson A, De Grandi P, Germond M. Prospective randomised study of two cryopreservation policies avoiding embryo selection: the pronucleate stage leads to a higher cumulative delivery rate than the early cleavage stage. *Fertil Steril*. 2000;74:946–52.
- Rovó A, Tichelli A, Passweg JR, Heim D, Meyer-Monard S, Holzgreve W, Gratwohl A, De Geyter C. Spermatogenesis in long-term survivors after allogeneic hematopoietic stem cells transplantation is associated with age, time interval since transplantation and absence of chronic graft versus host disease. *Blood*. 2006;108:1100–5.
- Porcu E. Oocyte freezing. *Semin Reprod Med*. 2001;19:221–30.
- De Geyter C, De Geyter M, Castro E, Bals-Pratsch M, Nieschlag E, Schneider HPG. Experience with transvaginal ultrasound-guided aspiration of supernumerary follicles for the prevention of multiple pregnancies after ovulation induction and intrauterine insemination. *Fertil Steril*. 1996;65:1163–8.
- Porcu E. Progress with oocyte cryopreservation. *Curr Opin Obstet Gynecol*. 2006;18:273–9.
- De Geyter C, De Geyter M, Nieschlag E. Low multiple pregnancy rates and reduced frequency of cancellation after ovulation induction with gonadotropins, if eventual supernumerary follicles are aspirated to prevent polyovulation. *J Assist Reprod Genet*. 1998;15:111–6.
- Nisker J, Tummon I, Daniel S, Kaplan B, Yuzpe A. Conversion of cycles involving ovarian hyperstimulation with intra-uterine insemination to in-vitro fertilization. *Hum Reprod*. 1994;9:406–8.
- Vollmer M, Wenzel F, De Geyter C, Zhang H, Holzgreve W, Miny P. Assessing the chromosome copy number in metaphase II oocytes by sequential fluorescence in situ hybridization (FISH). *J Assist Fertil Genet*. 2000;17:596–602.
- Oktay K, Pelin A, Bang H. Efficiency of oocyte cryopreservation: a meta-analysis. *Fertil Steril*. 2006;86:70–80.
- Jain JK, Paulson RJ. Oocyte cryopreservation. *Fertil Steril*. 2006;86(Suppl 3):1037–46.
- Boiso I, Marti M, Santalo J. A confocal microscopy analysis of the spindle and the chromosome configurations of human oocytes cryopreserved in the germinal vesicle and metaphase II stage. *Hum Reprod*. 2002;17:1885–91.
- 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. *RBM Online*. 2006;12:481–6.
- Bianchi V, Coticchio G, Distratis V, Di Giusto N, Flamigni C, Borini A. Differential sucrose concentration during dehydration (0.2 mol/l) and rehydration (0.3 mol/l) increases the implantation rate of frozen human oocytes. *RBM Online*. 2007;14:64–71.
- Kuleshova L, Gianaroli L, Magli C, Ferraretti A, Trounson A. Birth following vitrification of a small number of human oocytes. *Hum Reprod*. 1999;14:3077–9.
- Yoon TK, Kim TJ, Park Sem Hong SW, Ko JJ, Chung HM, Cha KY. Live births after vitrification of oocytes in a stimulated in vitro fertilization-embryo transfer program. *Fertil Steril*. 2003;79:1323–6.
- Kuwayama M. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. *RBM Online*. 2005;11:608–14.
- Vajta G, Kuwayama M. Improving cryopreservation systems. *The-riogenology*. 2006;65:236–44.
- Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. *Theriogenology*. 2007;67:73–80.