Oocyte Vitrification

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Abstract

Oocyte cryopreservation represents a great alternative for many women, becoming a promising choice especially in cases of patients suffering from cancer malignancy. In these women, the option to reliably cryopreserve oocytes and store them with the intention to safeguarding their fertility would be quite welcome. In addition, oocyte cryopreservation is the most valuable option for women who wish to delay their motherhood, a choice that is increasingly frequent, especially in developed countries, raising the proportion of women at advanced ages that desire to get pregnant. Oocyte cryostorage results very useful to overcome the most common drawbacks involved with ovum donation as currently applied, such as synchronization between donors and recipients, long-waiting lists subject to the availability of a suitable donor, and, the most important, the absence of a quarantine period. Oocyte cryostorage brings additional advantages to ART programs being helpful to solve different clinical situations as in low-responder patients, unpredictable unavailability of semen sample collection from the male partner, risk of suffering ovarian hyperstimulation syndrome, or some other cases in which the embryo transfer is not advisable.

Keywords

Oocyte cryopreservation • Oocyte vitrification • Cryostorage of oocytes • Fertility in cancer malignancy • Cryostorage of oocytes • Vitrification of oocytes

The essential role of cryopreservation in ART has become obvious since the commencement of the infertility treatment, becoming a more flexible and efficient practice. Semen and embryo cryopreservation has been a successful strategy, routinely applied in IVF procedure for a long time. However, in spite of numerous studies conducted over the last 20 years, the reliability of oocyte cryopreservation is just being confirmed currently. All of the efforts that have been made are clearly justified, mostly because an efficient oocyte cryopreservation program would be quite welcome and widely applied in ART, since there are many other indications for oocytes cryopreservation different of the abovementioned egg banking for ovum donation. Potential beneficiaries of this technology would be cancer patients who need an option for fertility preservation before undergoing the potential sterilizing treatment [1], or women who wish to delay their motherhood due to a variety of reasons [2], government restrictions on IVF [3, 4], ethical reasons against embryo cryopreservation, and practical reasons such us unavailability of the male gamete the day of pick up, due to a variety of reasons [5, 6].

Despite all of this wide diversity of potential applications, egg banking has not been a routine procedure until very recently; in fact, there are still very few centers worldwide that routinely apply oocyte cryopreservation in their clinical routine. This fact can be explained because the methodology to cryopreserve human oocytes has been disappointing, with results that have not always been reproducible. Lately, vitrification has proven to be a very efficient method for successful

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oocyte cryostorage. Some fundamental principles of cryobiology would be helpful to understand why it has been extremely difficult to reach the goal of safely cryopreserved human oocytes.

Cryobiology Background

There are some reasons that could explain the low rate of successes that has been traditionally observed. Some of them include the size, shape, and cell number regarding oocytes. These gametes are the largest cells of the human body, and this could explain, at least in part, the great differences in cryotolerance between them and, for example, the spermatozoa. In addition, the spherical shape of the oocyte could disturb the uniform distribution of cryoprotectants (CPAs). Finally, considering the oocytes as a unique cell, they have only one chance of success, and there are few possibilities to restore from a serious damage; this situation is completely different from the one observed in tissues composed by millions of cells, in which the damage could be compensated in different proportions.

Other major factors responsible for the high oocyte sensitivity to cryopreservation include chilling injury and intracellular ice crystal formation. These factors are related to the cryopreservation method. There are two main strategies in cryobiology: slow cooling and vitrification. During the former, the cells are gradually dehydrated in the presence of CPAs, and the temperature is lowered at a very slow cooling rate (−0.3°C) [7]. In this way, the cells are exposed to low temperatures during an extended period of time, which can lead to what is known as chilling injury [8]. At the final stage, water solidifies into ice crystals. Chilling injury can be defined as the irreversible damage following exposure of cells to low temperatures, from +15 to −5°C before the nucleation of ice [9]. This detrimental event affects mainly the cytoskeleton [10] and cell membranes [11]. The ice crystal formation within the cytoplasm must be avoided at all cost in order to guarantee the survival and integrity of the cells when they are later thawed. Chilling injury can be minimized during vitrification by use of high cooling rates. Moreover, as the sample vitrifies, ice crystal formation is avoided [12].

Vitrification, in particular by means of methodologies that use a minimum volume, is altering this situation by producing results that have not been achieved with other approaches [5]. The physical phenomenon of vitrification takes place when the solidification of the solution occurs not by ice crystallization but by extreme elevation in viscosity, which is achieved by using high cooling rates from −15,000 to −30,000°C per minute, therefore avoiding the risk of chilling injury [12]. This ice-free cryopreservation method has undergone modifications in order to optimize results. One such modification has been to reduce the volume of the vitrification solution containing oocytes, which allows the CPA concentration and, consequently, the cytotoxicity to be decreased [13, 14]. As abovementioned, this procedure circumvents the two major limiting factors for achieving optimal cryopreservation: chilling injury [13] and ice formation [15]. Chilling injury can be minimized during vitrification by use of high cooling rates [12]. The velocity of the process is dependent on the volume of the vitrification solution. Thus, the smaller the volume of the sample, the higher the cooling rate. On the other hand, direct contact with liquid nitrogen also contributes to increase the cooling rate. To avoid ice formation, the vitrification technique makes use of high CPA concentrations [12] despite the fact that such high concentrations are considered toxic to cells [16]. Nonetheless, an appropriate, phased composition of CPA could mitigate the toxic and osmotic consequences of highly concentrated CPAs [5]. In this way, a combination of two or three of these agents can decrease the individual specific toxicity. The most common mixture employed for this purpose consists of EG, DMSO, and sucrose [5]. To optimize the results, in addition to an appropriate selection of CPAs, it is also helpful to use these agents at lower concentration while maintaining the necessary concentration to achieve vitrification. By dramatically increasing the cooling rate, the CPA concentration could be reduced. As a result, a high cooling rate avoids chilling injury and allows the reduction of the concentration of CPA, thereby preserving the cells at nontoxic concentrations of CPA. Several approaches fit these conditions. The “minimum drop vitrification” method was proposed by Arav, using a very small volume of vitrification solution placing the samples on a specific device which must be cooled very quickly [17]. High cooling rates are achieved when samples are loaded in minimum volume that are directly immersed in LN. These methods are also known as open systems. Such high rates are impossible to achieve with hermetically closed vials, which are used in closed systems. Nevertheless, it is worth mentioning that the direct contact of samples with LN has raised some concerns due to the theoretical risk of cross-contamination mainly because the LN itself has been considered as a potential source of pathogen agents. Even though, no any case of cross-contamination has ever been reported in ART in many years of practice, highlighting that such eventuality could be extremely unlikely.

On the other hand, the limit imposed by the cooling rate could be restricted to oocytes since satisfactory results have been obtained after the vitrification of embryos at different developmental stages using closed systems. In any case, there are no formal comparative studies which make possible to draw definitive conclusions. A wide variety of open approaches have been reported in the literature [18–23] as well as closed ones [24, 25].

Clinical Outcomes

A review on human oocyte cryostorage based on all reports on vitrification MII oocytes and providing data regarding implantation and pregnancy outcome depicts the situation of the clinical application of human oocyte cryopreservation [26]. It is worth mentioning that nearly all of these studies assessed employed open systems for vitrification, highlighting the advantage of these systems with regard to oocyte cryopreservation and its most wide application for clinical practice in humans. Within this session, we will focus on our clinical experience using the Cryotop method for vitrification [27]. This device consists of a fine transparent polypropylene film attached to a plastic handle and equipped with a cover straw, into which can be loaded very small volumes of oocytes (up to ~0.1 μL), 10 times lower than the capacity of other minimum volume devices [27].

Our first approach with the vitrification of donor oocytes using the Cryotop method was carried out through the simultaneous comparison of the outcome of both vitrified and fresh oocytes from the same ovarian stimulated cycle; we confirmed the potential of vitrification of oocytes using this methodology [28]. We obtained a 96.7% survival rate and detected no significant difference in fertilization rates (76.3 and 82.2%), day 2 (94.2 and 97.8%) or day 3 embryo cleavage rates (77.6 and 84.6%), or blastocyst formation rates (48.7 and 47.5%) for vitrified and fresh oocytes, respectively. In that study, either vitrified or fresh oocytes belonging to the same cohort were simultaneously inseminated using the same semen sample. In this way, we were able to analyze the potential of vitrified and fresh oocytes under exactly the same conditions. The ratios of good quality embryos on day 3 and at blastocyst stage were similar in both groups. Pregnancy, implantation, miscarriage, and ongoing pregnancy rates (OPRs) per transfer were 65.2, 40.8, 20, and 47.8%, respectively. The potential use of this approach for fertility preservation in cancer patients as well as in other clinical situation in ART has also been highlighted [1, 29].

Although these evidences have been encouraging, the need of a controlled clinical trial to test the efficiency of oocyte vitrification was mandatory. A randomized, prospective, triple-blind, single-center, parallel group controlled clinical trial, including 600 recipients from our ovum donation program, aimed to compare the outcome of vitrified-banked oocytes to the gold standard procedure employing fresh oocytes that has been published by our group [30]. The study was designed to establish the superiority of the OPR of fresh oocytes over that of vitrified oocytes. The primary end point, the OPR per intention-to-treat (ITT) population, was 43.7% for vitrified oocytes, over 41.7% for fresh ones. As shown by the OR, we failed to prove the superiority of fresh oocytes over the vitrified oocytes, and moreover, we were able to assume the noninferiority of the vitrification group. Additionally, the proportion of top-quality embryos obtained either by inseminated oocyte (30.8% vs. 30.8% for day 2 and 36.1% vs. 37.7% for day 3, for vitrified and fresh oocytes, respectively) or by cleaved embryos (43.6% vs. 43.8% for day 2 and 58.4% vs. 60.7% for day 3, respectively) was similar between groups.

The outcome achieved in this controlled randomized clinical trial confirmed demonstrates that cryobanking by applying the vitrification technology can provide successful clinical outcome in oocyte donation programs helping to the validation of this strategy. With no doubt, it is of great importance to ovum donation procedures, as it allows traditional drawbacks associated with the use of fresh oocytes to be overcome. Supported by all this evidence, we have established an oocyte bank that currently operates to meet the basic needs of our donation program contributing greatly to improve the logistics of the program and, what is also relevant, allowing us to keep the quarantine period. To date, we have performed 1,856 ovum donation cycles involving 23,000 cryostored oocytes with an overall survival rate over 90%. A mean number of 1.6 ± 0.7 embryos have been replaced, and an OPR of 47.7% has been achieved. A 63.6% of the patients were able to vitrify surplus embryos; therefore, the cumulative OPR after cryotransfers has been over 60%. Additionally to ovum donation, we have applied oocyte cryopreservation in cases of infertile patients with own oocytes due to the risk of suffering ovarian hyperstimulation syndrome (OHSS), no available semen sample the day of ovum pick-up or many other situations in which the embryo transfer was not advisable. In nearly 450 cycles (mean age 47.4 ± 4.5), the overall survival rate was 84% with a clinical pregnancy rate of 41.5% and 32.8% of implantation rate. Oocyte vitrification has been applied for fertility preservation in cancer patients ($N = 70$; mean age 32.7 ± 5.7) and for social reasons ($N = 158$; 35.2 ± 5.8), however no warming procedures have been performed to date.

Other authors have confirmed the excellent profile of oocyte vitrification either with autologous or donated oocytes. Rienzi et al. showed in a prospective, randomized sibling study conducted with autologous oocytes that oocyte vitrification procedure is not inferior to fresh insemination procedure achieving comparable ongoing clinical pregnancy rates [31]. A very recent study has prospectively compared an open vs. closed system between fresh and sibling vitrified oocytes [32]. These authors have observed an impairment of embryo quality after employing the closed system. In contrast, the parameters analyzed were similar between fresh and sibling oocytes vitrified using the open system. Other authors have also endorsed the suitability of oocyte vitrification by the Cryotop method in an oocyte donation program [33]. These authors recommend this approach to be applied in infertile patients with different indications within a routine IVF program. All these evidences strongly support the efficiency of oocyte vitrification.

Practical Aspects of Oocyte Vitrification

Laboratory Procedure

- Aspirate the oocytes from the culture dish and keep them at the tip of the capillary or Pasteur pipette and transfer them within minimum drop volume to 20 μL of buffer solution (BS) supplemented with 20% serum substitute into a reproplate well (Kitazato, Biomedical Supply, Tokyo, Japan) (Fig. 57.1).
- Add 20 μL of equilibration solution (ES) containing 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) surrounding the previous drop containing the oocytes (Kitazato, Biomedical Supply, Tokyo, Japan). Wait for 3 min at room temperature (Fig. 57.1).
- Add another drop of 20 μL of ES in the same way as in the previous step and wait for 3 min more.
- Add 240 μL of ES slowly circling the previous drops and wait between 6 and 9 min. At the end of this step, the oocytes should be re-expanded and should recover their original appearance (Fig. 57.1).

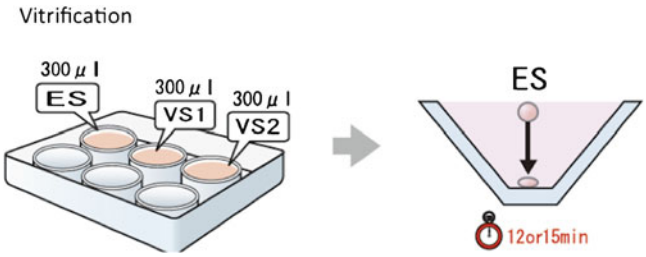

Fig. 57.1 Equilibration procedure

- After equilibrium, aspirate the oocytes at the tip of the pipette. Afterward, place them on the surface of the vitrification solution (VS) containing 15% EG plus 15% DMSO in BS solution (Fig. 57.1). Try to carry a minimum volume of ES solution. Note that the oocytes will float due to the high density of the VS media.
- Dispense 300 μL of VS solution into two well of the reproplate (Kitazato, Medical Supply, Tokyo Japan). Remove the ES medium just placed together with the oocytes (it will be noted clearly due to the difference in density) and wash the pipette outside the VS well. Keep removing continuously and keep on discarding the medium outside the plate. Repeat this operation as many times as possible within 30 s (Fig. 57.2).
- Aspirate the oocytes and bring them to the bottom of the plate (the floating will stop as soon as they start to equilibrate with the medium).
- Repeat the same procedure in the next VS well. Place the oocytes in the bottom of the plate and move them many times around the well, repeating the washing process during 30 more seconds.
- Aspirate the oocytes and maintain them at the tip of the pipette within minimum volume of VS.
- Place the Cryotop under the microscope.
- Proceed to load the oocytes onto the Cryotop within minimum volume.
- Aspirate excess medium to make sure they are contained within minimum volume.
- Immerse the Cryotop directly into the liquid nitrogen container (Fig. 57.2).
- Place the plastic protector Cryotop.
- Transfer the oocytes to the storage tank.

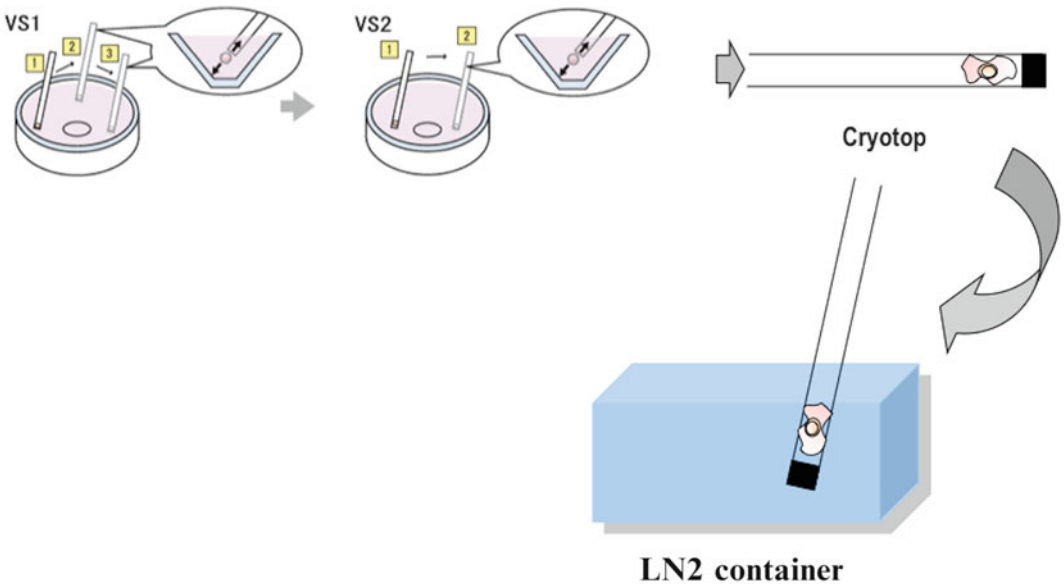

Fig. 57.2 Vitrification procedure

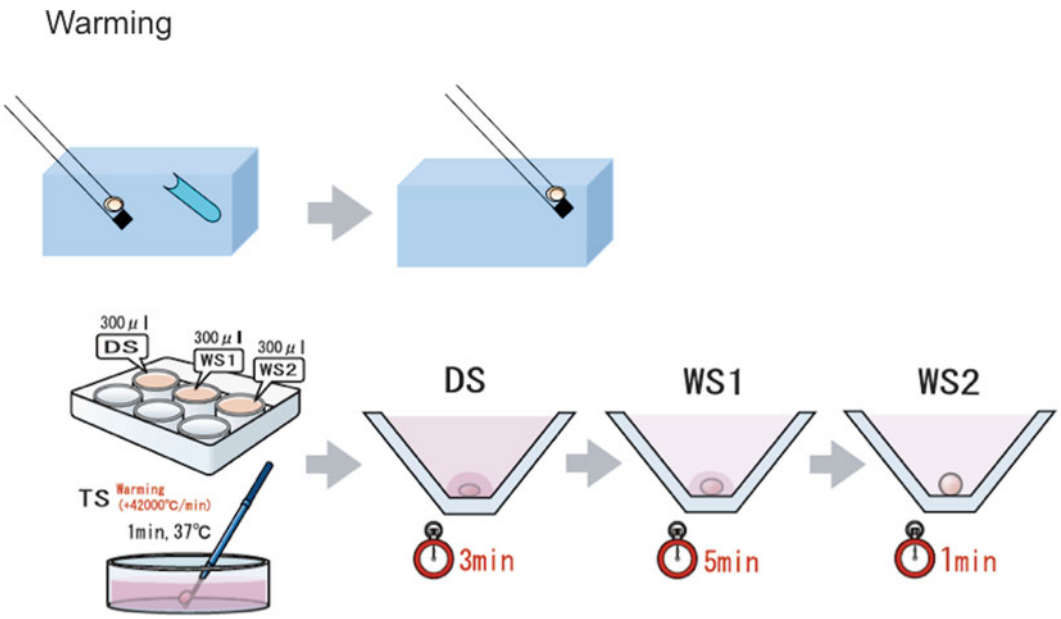

Fig. 57.3 Warming and dilution procedure

Warming Procedure

- Remove the Cryotops from the storage tank and handle them submerged into liquid nitrogen. Avoid temperature changes.
- Remove the protective straw maintaining the Cryotops immersed in liquid nitrogen.
- Remove Cryotop from the liquid nitrogen container and submerge it instantly (straight and quick movement) into 1.5 mL of a solution containing 1.0 M sucrose (TS) (Kitazato, Medical Supply, Tokyo, Japan) (Fig. 57.3).
- Visualize the oocytes and immediately start the countdown to 60 s.
- Do not manipulate the oocytes within the first 40 s.
- Retrieve the oocytes from the Cryotop with very gentle manipulations within a minute in TS solution.
- Transfer the oocytes to the DS well in the reproplate (Fig. 57.3). Aspirate the oocytes very gently and continue to aspirate TS until the column reaches approximately 2 mm in length (Fig. 57.3).
- Take the pipette to the DS well and immersed it to the bottom. Dispense the 2-mm column at first. The column of TS within DS will be clear due to the difference in density of both (Fig. 57.3). Leave the oocytes on the "top" of this column. Wait for 3 min.
- Transfer the oocytes to well WS (BS supplemented with 20% SSS) exactly as in the previous step. Wait for 5 min.
- Transfer the oocytes to the next well of WS well. Leave the oocytes on the surface of this media; they will drop down immediately due to the absence of differential density between media. Wait for 1 min.
- Transfer the oocytes to the regular culture dish and place them in the incubator at 37°C Wait for 2 h before ICSI.

References

1. Cobo A, Domingo J, Pérez S, Crespo J, Remohi J, Pellicer A. Vitrification, an effective new approach to oocyte banking in healthy women, could be applied in cancer patients to preserve their fertility. Clin Transl Oncol. 2008;10:268–73.
2. Homburg R, van der Veen F, Silber SJ. Oocyte vitrification—women's emancipation set in stone. Fertil Steril. 2008;91(4 Suppl): 1319–20.
3. Boggio A. Italy enacts new law on medically assisted reproduction. Hum Reprod. 2005;20:1153–7.
4. 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.
5. Vajta G, Nagy ZP. Are programmable freezers still needed in the embryo laboratory? Review on vitrification Reprod Biomed Online. 2006;12:779–96.
6. Cobo A, Bellver J, Domingo J, Pérez S, Crespo J, Pellicer A, Remohi J. New options in ART: the Cryotop method of oocyte vitrification. Case reports. Introduction. Reprod Biomed Online. 2008; 17(1):68–72.
7. Woods EJ, Benson JD, Agca Y, Critser JK. Fundamental cryobiology of reproductive cells and tissues. Cryobiology. 2004;48:146–56.
8. Mazur P. Principles of cryobiology. In: Fuller B, Lane M, Benson E, editors. Life in the Frozen State. New York: CRC Press; 2004. p. 4–55.
9. Watson PF, Morris GJ. Cold shock injury in animal cells. Symp Soc Exp Biol. 1987;41:311–40.
10. 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.
11. Ghetler Y, Yavin S, Shalgi R, Arav A. The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. Hum Reprod. 2005;20:3385–9.
12. Liebermann J, Dietl J, Vanderzwalmen P, Tucker MJ. Recent developments in human oocyte, embryo and blastocyst vitrification: where are we now? Reprod Biomed Online. 2003;7:623–33.

13. Vajta G, Kuwayama M. Improving cryopreservation systems Theriogenology. 2006;65:236–44.
14. Kuwayama M, Cobo A, Vajta G. Vitrification of oocytes: general considerations and the use of Cryotop method. In: Liebermann J, Tucker M, editors. Vitrification in assisted reproduction. A user's manual and trouble shooting guide. London: Informa Health Care; 2007.
15. Mazur P. Freezing of living cells: mechanisms and implications. Am J Physiol. 1984;247:C125–42.
16. Fuller B, Paynter S. Fundamentals of cryobiology in reproductive medicine. Reprod Biomed Online. 2004;9:680–91.
17. Arav A. Vitrification of oocyte and embryos. In: Lauria AGF, Gandolfi F, editors. New trends in embryo transfer. Cambridge: Portland; 1992. p. 255–64.
18. Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol Reprod. 1996;54:1059–69.
19. Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H. Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol Reprod Dev. 1998;51:53–8.
20. Papis K, Shimizu M, Izaike Y. Factors affecting the survivability of bovine oocytes vitrified in droplets. Theriogenology. 2000;54:651–8.
21. Lane M, Gardner DK. Vitrification of mouse oocytes using a nylon loop. Mol Reprod Dev. 2001;58:342–7.
22. Matsumoto H, Jiang JY, Tanaka T, Sasada H, Sato E. Vitrification of large quantities of immature bovine oocytes using nylon mesh. Cryobiology. 2001;42:139–44.
23. Liebermann J, Nawroth F, Isachenko V, Isachenko E, Rahimi G, Tucker MJ. Potential importance of vitrification in reproductive medicine. Biol Reprod. 2002;67:1671–80.
24. Kuwayama M, Ieda S, Zhang J, Kato O. The CryoTip method: Aseptic vitrification of oocytes and embryos. Fertil Steril. 2005;84 Suppl 1:S187.
25. Vanderzwalmen P, Zech N, Prapas Y, Panagiotidis Y, Papatheodorou A, Lejeune B, Jareno D, Vanderzwalmen S, Ectors F. Closed carrier device: a reality to vitrify oocytes and embryos in aseptic conditions. Gynecol Obstet Fertil. 2010;38:541–6.
26. Cobo A, Vajta G, Remohi J. Vitrification of human mature oocytes in clinical practice. Reprod Biomed Online. 2009;19 Suppl 4:4385.
27. Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. Reprod Biomed Online. 2005;11:300–8.
28. Cobo A, Kuwayama M, Perez S, Ruiz A, Pellicer A, Remohi J. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. Fertil Steril. 2008;89:1657–64.
29. Cobo A, Bellver J, Domingo J, Perez S, Crespo J, Pellicer A, Remohi J. New options in assisted reproduction technology: the Cryotop method of oocyte vitrification. Reprod Biomed Online. 2008;17:68–72.
30. Cobo A, Meseguer M, Remohi J, Pellicer A. Use of cryo-banked oocytes in an ovum donation programme: a prospective, randomized, controlled, clinical trial. Hum Reprod. 2010;25:2239–46.
31. Rienzi L, Romano S, Albricci L, Maggiulli R, Capalbo A, Baroni E, Colamaria S, Sapienza F, Ubaldi F. Embryo development of fresh 'versus' vitrified metaphase II oocytes after ICSI: a prospective randomized sibling-oocyte study. Hum Reprod. 2010;25(1): 66–73.
32. Paffoni A, Guarneri C, Ferrari S, Restelli L, Nicolosi AE, Scarduelli C, Ragni G. Effects of two vitrification protocols on the developmental potential of human mature oocytes. Reprod Biomed Online. 2011;22(3):292–8.
33. Nagy ZP, Chang CC, Shapiro DB, Bernal DP, Elsner CW, Mitchell-Leef D, Toledo AA, Kort HI. Clinical evaluation of the efficiency of an oocyte donation program using egg cryo-banking. Fertil Steril. 2008;92(2):520–6.