

Embryo development and gestation using fresh and vitrified oocytes

Carlos Gilberto Almodin*, Vania Cibele Minguetti-Camara, Cassia Lopes Paixao, and Priscila Cardoso Pereira

Materbaby Human Reproduction - Human Reproduction, Av XV de Novembro, 1232, Maringá, Paraná 87013230, Brazil

*Correspondence address. Tel: +55-44-32243992; Fax: +55-44-32251162; E-mail: almodin@materbaby.com.br

Submitted on September 16, 2009; resubmitted on January 18, 2010; accepted on January 28, 2010

BACKGROUND: The objective of this study was to compare the gestational results obtained with vitrified/thawed oocytes by a novel vitrification method (Vitri-ingá) to results obtained with fresh oocytes.

METHODS: A total of 125 IVF-ET procedures carried out over 2008 were analysed, in which 79 patients received embryos from fresh oocytes (Group 1), and 46 patients received embryos from vitrified/thawed oocytes using Vitri-ingá (Group 2). Fresh and vitrified/thawed oocytes were fertilized and embryos were transferred. Fertilization, pregnancy and implantation rates were compared.

RESULTS: Vitrified oocytes presented a survival rate of 84.9%. Fertilization, pregnancy and implantation rates showed no statistically significant differences between fresh and cryopreserved/thawed groups (81.3, 51.9, 21.3% and 80.8, 45.6 and 14.9%, respectively). Only the average number of blastomeres in Group 1 (6.86 ± 2.07) was significantly higher than in Group 2 (6.35 ± 2.26 ; $P = 0.010$).

CONCLUSIONS: Despite some differences in the patient groups, the clinical results of this study demonstrated that the Vitri-ingá method preserves the potential of human vitrified oocytes for fertilization and further development.

Key words: oocyte cryopreservation / vitrification / embryo development / pregnancy rates

Introduction

Oocyte cryopreservation solves the legal and ethical problems associated with the cryopreservation of embryos in patients undergoing *in vitro* fertilization procedures. In addition to that, it may also offer the possibility of extending the reproductive capability of young women with malignant diseases in cases where the treatment may compromise the ovarian reserve. Moreover, it may also offer alternatives for infertile patients who are subject to ovarian hyper-stimulation syndrome or premature ovarian failure or who require oocyte donation. The creation of banks for donated cryopreserved oocytes avoids the need for cycle synchronization or the formation of an over-supply of embryos destined for cryopreservation.

Nevertheless, oocytes present different characteristics from embryos that compromise their survival. Oocytes in metaphase II (MII) have a formed fuse that is sensitive to thermal changes (Chen and Yang, 2009). In addition to that, the low permeability coefficient of the plasmatic membrane of oocytes makes the penetration of cryoprotectant substances more difficult, although at the same time their intra-cytoplasmatic lipids make them more sensitive to freezing than embryos (Ruffing *et al.*, 1993). After fertilization, however, the increase of free calcium increases the plasmatic membrane

permeability, improving cryoprotectant transit and decreasing ice crystal formation in embryos (Gook *et al.*, 1993; Fabbri *et al.*, 2000).

Two techniques have been used for the cryopreservation of oocytes, slow freezing and vitrification. Both techniques have been employed quite successfully with human embryos. However, when the oocyte is the cell in question, the results have been conflicting with non-reproducible results (Kuleshova and Lopata, 2002; Kuwayama *et al.*, 2005; de Santis *et al.*, 2007; Cao *et al.*, 2009; Check, 2008, 2009; Parmegiani *et al.*, 2009). Since Chen (1986) published the first pregnancy using cryopreserved oocytes using the slow freezing technique, this method has been used with varying success (Yang *et al.*, 2002; Borini *et al.*, 2006; Levi Setti *et al.*, 2006). A recent meta-analysis on slow freezing revealed that the clinical pregnancy rate per transfer with this method was just 6%, and 2.3% per oocyte thawed (Oktay *et al.*, 2006).

Vitrification, on the other hand, avoids the formation of intra and extra-cellular ice crystals, which are responsible for damaging cell membranes and organelles. This process is determined by the use of cryoprotectants in high concentrations and ultrarapid cooling and thawing procedures (Papadopoulos *et al.*, 2002). Results obtained with vitrification have varied considerably with several different

methods being adopted (Fuku *et al.*, 1995; Vajta *et al.*, 1998; Hamawaki *et al.*, 1999; Lane *et al.*, 1999; Mukaida *et al.*, 2001, 2003a, b; Kuwayama *et al.*, 2005; Vajta and Nagy, 2006). However, no relevant studies comparing these methods have been performed.

In order to achieve ultrarapid cooling rates, several techniques using small volumes of vitrification solution have been developed, thus improving conventional methods such as the open pulled straw (OPS) (Vajta *et al.*, 1997). A recent proposal for vitrification using minimum volume involves a device named Cryotop (Kitazato Supply Co., Fujinomiya, Japan), which consists of a hard plastic handle and a fine, thin film strip over which an oocyte is vitrified (Kuwayama *et al.*, 2005).

More recently, a novel vitrification method (Vitri-Ingá), was tested in a study with bovine oocytes, which presented a survival rate of 86% after vitrification/thawing of oocytes matured *in vitro* (Almodin *et al.*, 2008). Additionally in another study, this time using human oocytes, the Vitri-Ingá method again demonstrated good results with survival, fertilization and pregnancy rates of 80.5, 76.6 and 24.5%, respectively (Fachini *et al.*, 2008). Despite those promising results obtained with Vitri-Ingá, no studies comparing the use of fresh and vitrified/thawed oocytes have been performed. Therefore, the objective of this study was to compare the gestational results obtained with oocytes vitrified and thawed using the Vitri-Ingá method to that from fresh oocytes in a series of IVF-ET procedures carried out during 2008.

Materials and Methods

Patients

This study was approved by the Hospital Almodin Ethics Committee. Patients that were scheduled to undergo *in vitro* fertilization treatment during 2008 at a human reproduction centre (Materbaby, Reprodução Humana e Genética, Maringá, Brazil) were included in the study after routine assessment and a duly signed informed consent to disclose their data.

The patients' ages ranged from 30 to 40 years. A total of 125 procedures were carried out: 79 cycles for patients who underwent *in vitro* fertilization treatment using fresh oocytes (Group 1), while 46 procedures involved patients who received the same treatment using oocytes which had previously been vitrified and stored in an oocyte bank (Group 2). In Group 1, 68 patients were undergoing their first IVF treatment attempt. Among the 11 patients who had previously been through the treatment, seven patients did not get pregnant, two patients had had abortions and two patients returned for a possible second pregnancy. None of those 11 patients had excess oocytes after their first treatment and had to perform a new attempt using fresh oocytes. In Group 2, all the patients had failed to achieve an ongoing pregnancy in their previous treatments and returned for a new attempt using their own frozen oocytes. There were 33 patients who had just had one previous fresh cycle, 11 patients who had undergone two fresh cycles and two patients who had undergone three fresh cycles. Table 1 illustrates the reasons for the patients' infertility in both groups. All the procedures for both groups were strictly performed under the same laboratory conditions and using the same culture medium.

Oocyte recovery

Ovarian hyper-stimulation was performed using the long protocol. In the mid-luteal phase, the use of leuprolide acetate (Lupron Abbott, Brazil) was

Table 1 Infertility causes of patients who received fresh or vitrified/thawed oocytes.

	Fresh oocytes (Group 1)	Vitrified/thawed oocytes (Group 2)
No. of cycles	79	46
Male infertility	19 (24.1%)	8 (17.4%)
Female causes	30 (38.0%)	20 (43.5%)
Unapparent	11 (13.9%)	7 (15.2%)
Multiple causes	19 (24.1%)	11 (23.9%)

initiated with subcutaneous applications of 0.15 ml/day. On the second day of menstruation, the leuprolide acetate dose was reduced to 0.05 ml/day and stimulation with rFSH (Gonal, Serono, Brazil) was initiated. Follicular growth was monitored by endovaginal ultrasonography only, and human chorionic gonadotrophin (HCG 10,000 IU, Choriomom, Meiszer, Brazil) was administered when at least three follicles with 20 mm of diameter were observed.

Follicular puncture was performed 36 h after HCG administration using transvaginal ultrasonography. After recovery, oocytes were transferred to an incubator (Forma Los Angeles, CA, USA) with 5.5% CO₂ at 37°C. One to two hours after follicular puncture, oocytes were placed in hyaluronidase (Irvine Scientific, Santa Ana, CA, USA) for removing the cumulus cells. Six fresh oocytes in MII were routinely separated to be submitted to ICSI, while the remaining oocytes in MII were vitrified.

Vitrification equipment and materials

All the equipment and materials used for the vitrification/thawing procedures were supplied by Ingámed Ltda. (Perobal, PR, Brazil); these included the Vitri-Equip, the vitrification solutions VI-1 and VI-2, the thawing solutions DV1, DV2 and DV3, and the vitrification strips Vitri-Ingá and their plastic sheaths.

Vitri-Equip consists of an expanded polystyrene box (44.0 × 19.5 × 15.0 cm) containing two stainless-steel containers of different sizes (18.5 × 6.0 × 8.8 and 5.3 × 5.3 × 10.0 cm) for liquid nitrogen. In the larger container there is a stainless-steel rack (Fig. 1). Equilibrium solution VI-1 consists of 7.5% ethylene glycol and 7.5% dimethylsulfoxide (DMSO), although the vitrification solution VI-2 is composed by 15% ethylene glycol, 15% DMSO and sucrose 0.5 M. The thawing solution DV-1 contains 1M sucrose, DV-2 contains 0.5 M sucrose and DV-3 contains buffer solution. The Vitri-Ingá vitrification strip is an apparatus that consists of a fine, very thin polypropylene strip (0.7 mm thick) with a specially designed round tip, in which there is a minute hole to receive the oocyte; the strip is connected to a hard and thicker plastic handle (Fig. 2). Vitri-Ingá plastic sheaths are 0.5 ml semen straws cut in the middle.

Vitrification

When the vitrification solutions reached room temperature (25°C), oocytes were denuded, separated for vitrification and transferred to VI-1 solution, in which they were equilibrated for 5–15 min. After this period, each oocyte was individually immersed into three consecutive 20 µl drops of VI-2 vitrification solution and placed into the hole in the tip of a Vitri-Ingá strip with a minimum amount of vitrification solution, and then immediately immersed into liquid nitrogen. When more than



Figure 1 Vitri-Equip: expanded polypropylene box with two stainless-steel containers for cooling and vitrification.

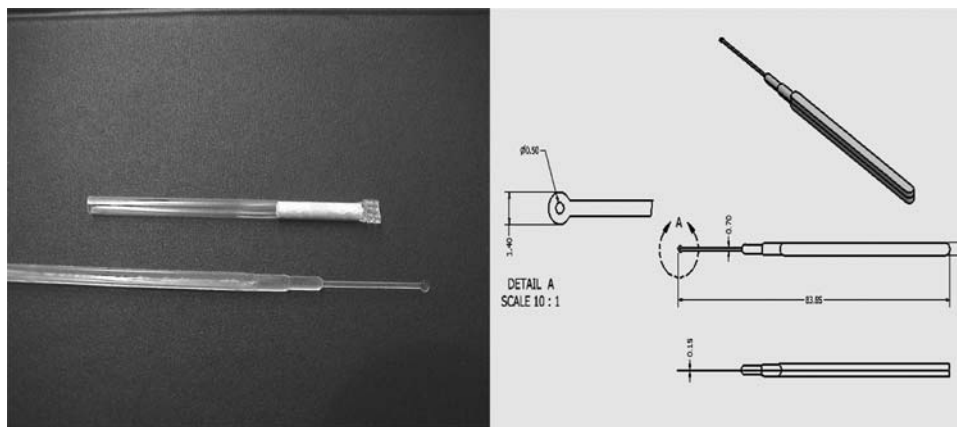


Figure 2 Vitri-Ingá vitrification strip and protective sheath.

0.1 μl of solution was placed with the oocyte on the strip, excess liquid was aspirated. The total time from when the oocyte was placed into the vitrification solution till its immersion into liquid nitrogen was between 50 and 60 s.

The plastic sheaths, which had been previously cooled for at least 2 min in liquid nitrogen vapour on the metal rack inside in the Vitri-Equip, were vertically immersed into liquid nitrogen. The Vitri-Ingá strip with the vitrified oocyte was then inserted into the plastic sheaths for safe storage, and transferred to a liquid nitrogen tank.

Thawing

Immediately after removing the Vitri-Ingá strip from the protecting plastic sheath, the thin part of the strip was totally immersed into DV-I thawing solution at 37°C for 1 min. The oocyte was then transferred to DV-II diluting sucrose solution for 3 min at room temperature and rinsed in a buffer solution twice, for 5 min each.

After thawing, a survival assessment was carried out using morphological criterion. Oocyte survival was defined by its expansion, intact cellular membrane, normal ooplasm and zona pelucida and normal-sized perivitelline space. ICSI was performed on the surviving oocytes 2–3 h after thawing.

Embryo culture and transfer

In oocyte warmed cycles, the obtained embryos were all transferred in the course of natural cycles. When the mean diameter of a leading follicle was 17–18 mm and the endometrial thickness was >7 mm with a triple line pattern, ovulation was triggered by human chorionic gonadotrophin (HCG 10,000 IU, Choriomon, Meizler, Brazil). Then 36–40 h later, the oocytes were warmed and submitted to ICSI. Fertilization of embryos from both fresh and vitrified/thawed oocytes was assessed 17–19 h after ICSI. Pre-embryos were kept in culture in GV culture media (Ingamed-Perobal-Brazil) until Day-3 post-fertilization, when the quality of the embryos was assessed. Good quality embryos were defined as those with more than 6 cells, similarly-shaped blastomeres and no fragmentation (Grade I) or those with more than 6 cells and similarly-shaped blastomeres with less than 20% fragmentation (Grade II). Before transfer, an assessment of the total number of embryos was performed, and embryos that were not selected for transfer were vitrified and stored into a nitrogen tank. Then, guided embryo transfer was performed using ultrasonography.

The luteal phase was supplemented with 50 mg/day of injectable progesterone, initiated on the day of follicular puncture for Group 1, and on the thawing day for Group 2. Chorionic Gonadotrophin

(β -hCG) was assessed on Day-14 after embryo transfer. Implantation and pregnancy was defined only after heart beating was present at Week-13 after transfer.

Statistical analysis

Statistical significance was estimated using χ^2 test. It was adopted with a level of significance of 0.05% ($\alpha = 5\%$). Descriptive levels (P) under this value were considered to be significant.

Results

The survival rate of vitrified/thawed oocytes, as well as the fertilization, pregnancy and implantation rates from fresh and vitrified/thawed oocytes are shown on Table II.

There were 79 patients who had fresh oocytes injected. From 413 oocytes injected, 336 (81.3%) developed into embryos. Between one and five embryos were transferred per patient, with the average number of embryos transferred being 3.57 ± 1.03 , which resulted in 41 (51.9%) pregnant patients. There were 61 sacs observed by ultrasound examination with an implantation rate of 21.3% (61/286) and an ongoing implantation rate of 19.2% (55/286). The rate of twin pregnancies was 20.25% (16/79) and triplets was 2.53% (2/79).

During the same period of study, 46 patients received embryos from vitrified oocytes. Out of 252 thawed oocytes, 214 (84.9%) presented morphological signs of survival with normal cytoplasm for ICSI. After fertilization, 173 (80.8%) of these oocytes developed into embryos. Again, between one to five embryos were transferred, with the average number of embryos transferred being 3.46 ± 1.34 , which resulted in 21 (45.6%) pregnant patients. There were 24 sacs observed by ultrasound examination with an implantation rate of 14.9% (24/161) and an ongoing implantation rate of 13.7% (22/161). The rate of twin pregnancies was 4.35% (2/46) and triplets was 2.17% (1/46).

In Group 1, 158 (47.0%) embryos were Grade I or II on Day-3, whereas in Group 2, 69 (39.9%) embryos were Grade I or II on

Day-3. The average number of blastomeres in Group 1 was 6.86 ± 2.07 , and in Group 2 it was 6.35 ± 2.26 .

Discussion

The current study compares the gestational results obtained with vitrified/thawed oocytes to the results obtained from fresh oocytes in a series of 125 IVF-ET procedures carried out during 2008 in a Human Reproduction Centre in Brazil. Embryo development as well as the clinical results obtained in this study demonstrated the potential of Vitri-ingá method.

Cryopreservation of oocytes through vitrification may simplify the procedure, avoiding the need for the acquisition of freezing equipment, and reducing costs as well as embryologists' time. Furthermore, vitrification is a fast, simple method that provides high survival rates and high embryo quality.

Vitrification is a cryopreservation strategy in which cells are converted into an amorphous solid resembling crystal-free glass. This condition is achieved by a combination of high concentrations of cryoprotectants and ultrarapid cooling. However, the concentrations used to increase the solution's viscosity make protocols potentially hazardous and, therefore, the time that cells are exposed to cryoprotectants must be reduced as much as possible to increase cooling speed. Another important factor in obtaining good results with the vitrification methods is the use of a mixture of cryoprotectants in order to decrease toxicity levels during vitrification. Similarly, the use of ethylene glycol as one of the cryoprotectants is also important. Despite the low permeability of human oocytes in MII to ethylene glycol, when compared with propylene glycol and dimethyl sulphoxide, it prevents excessive osmotic stress and minimizes exposure to high concentrations of this substance, being less toxic (Mullen *et al.*, 2008).

Different devices have been employed in an attempt to decrease the volume of solution in which oocytes are vitrified and achieve the highest cooling speed possible. The first attempts in oocyte vitrification were performed with 0.25 ml straws, the same used for the

Table II Fertilization, pregnancy and implantation rates of fresh and vitrified/thawed human oocytes.

	Fresh oocytes (Group 1)	Vitrified/thawed oocytes (Group 2)	P
No. of cycles	79	46	
Patients age*	34.2 ± 4.5	35.4 ± 5.0	0.191
No. of fresh oocytes	906	–	
No. of thawed oocytes	–	252	
No. of surviving thawed oocytes (%)	–	214 (84.9%)	
No. microinjected oocytes	413	214	
No. of fertilized oocytes (%)	336 (81.3%)	173 (80.8%)	0.876
No. of transferred embryos*	3.57 ± 1.03	3.46 ± 1.34	0.318
No. of blastomeres*	6.86 ± 2.07	6.35 ± 2.26	0.010**
Total of grade I and II embryos (%)	158 (47.0%)	69 (39.9%)	0.125
Pregnancy rate per transfer (%)	41 (51.9%)	21 (45.6%)	0.500
Implantation rate (%)	21.3%	14.9%	0.089

*Data are presented as mean \pm SD.

**P < 0.05.

slow freezing protocol, without success. The first studies that resulted in oocyte survival and pregnancy used open pulled straws (OPS) that were pulled until they became extremely thin, in a technique used by Vajta et al. (1998) for bovine embryos (Kuleshova et al., 1999). Nowadays, there are several versions derived from the OPS used for vitrification, such as the super thin OPS (Isachenko et al., 2000), glass micropipettes (Kong et al., 2000), flexible denudation pipettes (Liebermann et al., 2002) and small diameter plastic micropipettes (Cremades et al., 2004; Hredzák et al., 2005). However, one of the great difficulties with using the OPS method and its derivatives is their handling, especially during thawing. The apparatus may cause some difficulties as the procedure must be rapidly performed due to the toxicity of the cryoprotectants.

Recent studies using Cryotop, a device that allows for a 10-fold reduction in the amount of vitrification solution in comparison to the OPS, demonstrated good results with survival of oocytes and pregnancy rates above 90 and 40%, respectively (Katayama et al., 2003; Kuwayama et al., 2005; Ruvalcaba et al., 2005; Antinori et al., 2007). In a study comparing the efficiency of OPS and Cryotop, porcine oocytes were vitrified, and 2 h after thawing they were parthenogenetically activated and cultivated *in vitro*. It was observed that cleavage and blastocyst formation rates were higher when Cryotop was used in comparison to OPS (Liu et al., 2008).

In the present study, the vitrification procedures were all carried out by a novel device (Vitri-ingá) similar to Cryotop. The main difference between the methods is the minute hole at the tip of Vitri-ingá's thin plastic film in which the oocyte is placed for vitrification. This method facilitates the removal of the excessive cryopreservation solution by aspiration, leaving just 0.1 µl around the oocyte. This permits cooling rates of 22 800°C/min, considerably greater those that obtained with OPS (16 340°C/min), which requires 1.0 µl of solution to fill the device (Kuwayama et al., 2005). The clinical results obtained using Vitri-ingá method showed that, along with a high oocyte survival rate (84.9%), no significant differences were observed in fertilization, pregnancy and implantation rates between fresh and vitrified/thawed oocytes.

A statistically significant difference between the groups was only observed in the number of blastomeres of Day-3 embryos ($P = 0.01$), with Group 1 presenting a significantly greater number than Group 2. This difference, however, did not seem to have a negative effect on pregnancy or implantation rates, which presented no statistically significant differences between groups. Nevertheless, the difference in the number of cells observed on Day-3 requires careful consideration. In a previous study comparing fresh and cryopreserved donor oocytes, vitrified by the cryotop method, embryos that presented poorer quality were observed on Day-2 in the vitrified oocytes group, but the difference did not persist on Day-3 (Cobo et al., 2008). Therefore, it is possible that in our study the number of cells might have become similar for both groups in the following days, rendering that initial difference clinically irrelevant. In addition to that, it has also been observed that fragmentation rates are related to implantation potential, but other parameters for embryo quality, such as the number of blastomeres on Day-3, do not seem to affect implantation (Della Ragione et al., 2007).

Although these results seem encouraging, it is important to keep in mind that, although all care was taken to guarantee that laboratorial conditions were exactly the same in all procedures, due to the

nature of this study, a number of differences inherent to each group of patients and difficult to avoid, were present. Besides the number of patients in each group being different (Group 1 = 79, Group 2 = 46), most of the patients in Group 1 were in their first IVF attempt whereas all the patients in Group 2 had previously failed to become pregnant in one or more previous cycles, which could favour patients in Group 1, who had not experienced previous pregnancies. In contrast, the fact that the patients in Group 1 received ovarian stimulation during the cycle, while the patients in Group 2 were in the course of natural cycles, may have favoured patients in Group 2, as the ovarian stimulation may have compromised the implantation potential (Reyftmann et al., 2007). Although these differences make comparison between the groups less than ideal, the results obtained are of significance as they reflect the actual clinical conditions.

Although not being the focus of this work, another interesting aspect observed in this study concerns the difference in the multiple pregnancy rates between groups. Despite the number of embryos transferred being the same for both groups, Group 1 presented a higher multiple pregnancy rate than Group 2. This difference seems to be directly related to the use of fresh or vitrified oocytes and deserves further investigation.

One of the possible criticisms of the vitrification technique using Vitri-ingá is the fact that it is an open system, i.e. the oocyte is placed into direct contact with liquid nitrogen during vitrification. This raises the issue concerning the theoretical possibility of contamination by liquid nitrogen. Although the risk of contamination by liquid nitrogen contact must be taken into consideration, no documented cases of diseases transmitted due to the transference of vitrified embryos have been reported. The only few cases known in the literature of liquid nitrogen contamination took place with frozen blood samples, which had volumes 103 to 104 times greater than the largest frozen samples used in embryology (Vajta and Nagy, 2006). In order to minimize the risk of contamination, it has been suggested that liquid nitrogen should be filtered in 0.22 filters, or even sterilized by ultra-violet radiation (Parmegiani et al., 2009). The development of a technique using a hermetically sealed vitrification device could eliminate the potential risks of contamination.

In conclusion, this study demonstrated that Vitri-ingá is an effective vitrification method for the cryopreservation of human oocytes. The developmental capacity of embryos obtained from vitrified oocytes using the Vitri-ingá method did seem not to be greatly affected by the vitrification procedure, as fertilization, embryo quality and clinical results were similar to those achieved with fresh oocytes, although the differences between the patient groups may have affected the results either way. Nevertheless, we are still in the early days of vitrification with different protocols and apparatuses being used. More studies are necessary for the eventual establishment of a universal protocol that can take on board all the knowledge accumulated so far and deal with the present limitations, similarly to what has happened in the last few years to the slow freeze technique for the cryopreservation of embryos.

Acknowledgements

The Vitri-ingá vitrification equipment used in this study was provided to the Federal University of São Paulo without charge from Ingámed Ltda. (Perobal, PR, Brazil).

References

- Almodin CG, Câmara VCM, Oliveira LM, Picada IE, Seko MB, Moron AF. Vitrificação em modelos bovinos: modelo de treinamento. *J Bras Reprod Assist* 2008;**12**:20–23.
- 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**:72–79.
- Borini A, Sciajino R, Bianchi V, Sereni E, Flamigni C, Cotichio G. Clinical outcome of oocyte cryopreservation after slow cooling with a protocol utilizing a high sucrose concentration. *Hum Reprod* 2006;**21**:512–517.
- Cao YX, Xing Q, Li L, Cong L, Zhang ZG, Wei ZL, Zhou P. Comparison of survival and embryonic development in human oocytes cryopreserved by slow-freezing and vitrification. *Fertil Steril*. 2009;**92**:1306–1311.
- Check JH. Advances in oocyte cryopreservation-part I: slow cool rapid thaw technique. *Clin. Exp. Obstet. Gynecol.* 2008;**35**:237–239.
- Check JH. Advances in oocyte cryopreservation-part II: rapid cooling using vitrification. *Clin. Exp. Obstet. Gynecol.* 2009;**36**:5–9.
- Chen C. Pregnancy after human oocyte cryopreservation. *Lancet* 1986;**1**:884–886.
- Chen SU, Yang YS. Slow freezing or vitrification of oocytes: their effects on survival and meiotic spindles, and the time schedule for clinical practice. *Obstet Gynecol* 2009;**48**:15–22.
- 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**:1657–1664.
- Cremades N, Sousa M, Silva J, Viana P, Sousa S, Oliveira C, Teixeira da Silva J, Barros A. Experimental vitrification of human compacted morulae and early blastocysts using fine diameter plastic micropipettes. *Hum Reprod* 2004;**19**:300–305.
- Della Ragione T, Verheeyen G, Papanikolaou EG, Landuyt LV, Devroey P, Steirteghem AV. Developmental stage on day-5 and fragmentation rate on day-3 can influence the implantation potential of top-quality blastocysts in IVF cycles with single embryo transfer. *Reprod Biol Endocrinol* 2007;**5**:2.
- De Santis L, Cino I, Rabellotti E, Papaleo E, Calzi F, Fusi FM, Brigante C, Ferrari A. Oocyte cryopreservation: clinical outcome of slow-cooling protocols differing in sucrose concentration. *Reprod. Biomed. Online.* 2007;**14**:57–63.
- Fabbri R, Porcu E, Marsella T, Primavera MR, Rocchetta G, Ciotti PM, Magrini O, Seracchioli R, Venturoli S, Flamigni C. Technical aspects of oocyte cryopreservation. *Mol Cell Endocrinol* 2000;**169**:39–42.
- Fachini FC, Almodin CG, Câmara VCM, Moron AF, Nakano R, Shimabukuru L. Vitri-íngá: Um novo protocolo de vitrificação. *J Bras Reprod Assist* 2008;**12**:16–19.
- 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**:177–185.
- Gook DA, Osborn SM, Johnston WIH. Cryopreservation of mouse and human oocytes using 1,2-propanediol and the configuration of the meiotic spindle. *Hum Reprod* 1993;**8**:1101–1109.
- Hamawaki A, Kuwayama M, Hamano S. Minimum volume cooling method for bovine blastocyst vitrification. *Theriogenology.* 1999;**51**:165.
- Hrdzák R, Ostró A, Zdilová V, Toporcerová S, Kacmárik J. Clinical experience with a modified method of human embryo vitrification. *Ceska Gynekol* 2005;**70**:99–103.
- Isachenko VV, Alabart JL, Isachenko EF, Bezugly ND, Michelmann HW. Ultra-rapid freezing and storage of rat embryos in an electric refrigerator at –130 degree C without liquid cryo-agents, with ultra-short exposure in the freezing medium and direct rehydration after thawing. *Cryo Letters* 2000;**21**:13–18.
- Katayama KP, Stehlik J, Kuwayama M, Kato O, Stehlik E. High survival rate of vitrified human oocytes results in clinical pregnancy. *Fertil Steril* 2003;**80**:223–224.
- Kong IK, Lee SI, Cho SG, Cho SK, Park CS. Comparison of open pulled straw (OPS) vs glass micropipette (GMP) vitrification in mouse blastocysts. *Theriogenology* 2000;**53**:1817–1826.
- Kuleshova LL, Gianaroli L, Magli C, Trouson A. Birth following vitrification of a small number of human oocytes. *Hum Reprod* 1999;**14**:3077–3079.
- Kuleshova LL, Lopata A. Vitrification can be more favorable than slow cooling. *Fertil Steril.* 2002;**78**:449–454.
- Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod Biomed Online* 2005;**11**:300–308.
- Lane M, Bavister BD, Lyons EA, Forest KT. Containerless vitrification of mammalian oocytes and embryos. *Nat Biotechnol* 1999;**17**:1232–1236.
- Levi Setti PE, Albani E, Novara PV, Cesana A, Morreale G. Cryopreservation of supernumerary oocytes in IVF/ICSI cycles. *Hum Reprod* 2006;**21**:370–375.
- Liebermann J, Tucker MJ, Graham JR, Han T, Davis A, Levy MJ. Blastocyst development after vitrification of multipronuclear zygotes using the Flexipet denuding pipette. *Reprod Biomed Online* 2002;**4**:146–150.
- Liu Y, Du Y, Lin J, Kragh PM, Kuwayama M, Bolund L, Yang H, Vajta G. Comparison of efficiency of open pulled straw (OPS) and Cryotop vitrification for cryopreservation of in vitro matured pig oocytes. *Cryoletters* 2008;**29**:315–320.
- Mukaida T, Nakamura S, Tomiyama T, Wada S, Kasai M, Takahashi K. Successful birth after transfer of vitrified human blastocysts with use of a cryoloop containerless technique. *Fertil Steril* 2001;**76**:618–620.
- Mukaida T, Nakamura S, Tomiyama T, Wada S, Oka C, Kasai M, Takahashi K. Vitrification of human blastocysts using cryoloops: clinical outcome of 223 cycles. *Hum Reprod* 2003a;**18**:384–391.
- Mukaida T, Takahashi K, Kasai M. Blastocyst cryopreservation: ultrarapid vitrification using cryoloop technique. *Reprod Biomed Online* 2003b;**6**:221–225.
- Mullen SF, Li M, Li Y, Chen ZJ, Critser JK. Human oocyte vitrification: the permeability of metaphase II oocytes to water and ethylene glycol and the appliance toward vitrification. *Fertil Steril* 2008;**89**:1812–1825.
- Oktay K, Cil AP, Bang H. Efficiency of oocyte cryopreservation: a meta-analysis. *Fertil Steril* 2006;**86**:70–80.
- Papadopoulos S, Rizos D, Duffy P, Wade M, Quinn K, Boland MP, Lonergan P. Embryo survival and recipient pregnancy rates after transfer of fresh or vitrified, in vivo or in vitro produced ovine blastocysts. *Anim Reprod Sci* 2002;**74**:34–44.
- Parmegiani L, Bertocci F, Garelo C, Salvarani MC, Tambuscio G, Fabbri R. Efficiency of human oocyte slow freezing: results from five assisted reproduction centers. *Reprod Biomed Online* 2009;**18**:352–359.
- Reyftmann L, Déchaud H, Loup V, Anahory T, Brunet-Joyeux C, Lacroix N, Hamamah S, Hédon B. Natural cycle in vitro fertilization cycle in poor responders. *Gynecol Obstet Fertil* 2007;**35**:352–358.
- Ruffing NA, Steponkus PL, Pitt RE, Parks JE. Osmometric behavior, hydraulic conductivity, and incidence of intracellular ice formation in bovine oocytes at different developmental stages. *Cryobiology* 1993;**30**:562–580.
- Ruvalcaba L, Marínez R, Cuneo S, Chanona J, Beltrán M, Bermúdez A. Improving donor programs with na oocyte bank using vitrification. *Fertil Steril* 2005;**84**:S70.

- Vajta G, Nagy ZP. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reprod Biomed Online* 2006; **12**:779–796.
- Vajta G, Booth PJ, Holm P, Greve T, Callesen H. Successful vitrification of early stage bovine in vitro produced embryos with the open pulled straw (OPS) method. *Cryo-Letters* 1997; **18**:191–195.
- Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T. Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev* 1998; **51**:53–58.
- Yang DS, Blohm PL, Winslow KL, Brown SE, Nguyen YL, Brubaker C. Oocyte donation using cryopreserved donor oocytes (Abstract). *Fertil Steril* 2002; **78**:S14.