Assessment of the Zygote and Cleaved Stages Frozen-Thawed Human Embryos by Vitrification and their Survival to Blastocysts

Abdel-Wahab El-Ghareeb[1,]*; Hamida Hamdi*, Abdel-Latif Sweilam**, Niveen Ahmed***

*Department of Zoology, Faculty of Science, Cairo University
Egypt

**Department of Gynecology, Faculty of Medicine, Cairo University
Egypt

***Farah IVF Center, Cairo
Egypt

[1]*Corresponding author's email: drelghareeb [AT] yahoo.com*

ABSTRACT--- ***Cryopreservation of human gametes and embryos is now considered as an important tool in assisted reproduction treatments as it increases the cumulative pregnancy rates while decreasing cost. Cryopreservation procedures are such unphysiological circumstances mainly on account of severe temperature and osmotic alterations. Since the early 1990s, two common methods of cryopreservation have been used. Both of these methods have finally depended on the freezing and solidification of cell or tissue. Recently, the one known as vitrification has been claimed as the future of cryopreservation because of increased survival and success rates. However, this method is a non-equilibrium technique of cryopreservation that shows critical requirements of much higher concentration of permeable cryoprotectants and rate of cooling. Thus, it is a more vigorous mean of all possible cell damage except the formation of intracellular ice crystals that is totally prevented by vitrification. Nevertheless, there is no adequate cumulative data on the outcomes of vitrification performed at different stages of human embryos.***

The aim of this study a) is to assess whether vitrification at different early stages (zygotes and cleavage) of human embryos alters the outcomes of vitrification. b) if so, to predict the preferable stage between the two stages of vitrification of human embryos to attain the highest yields. It has been well demonstrated that vitrification of human gametes and embryos have resulted in different success rates according to the developmental stage of the cell. Then, the most preferable stage of vitrification will be identified on the basis of blastocystformation obtaining from extended culture conditions of the thawed embryos till day 5, using non-transferable embryos.

Keywords---Human zygote, cleaved stages, vitrification

1. INTRODUCTION

Cryopreservation of supernumerary embryos produced during human IVF provides an opportunity for patients to have repeated attempts at conception following a single drug stimulation cycle, preventing wastage of valuable genetic material and improving cumulative pregnancy rates. This approach may have several advantages for the patients. Firstly, it provides an opportunity to limit the number of embryos transferred while maximizing the usable embryo per oocyte retrieval cycle ratio at each stimulation attempts, a procedure that is costly and potentially difficult for patients. Secondly, the number of drug stimulation cycles in order to obtain oocytes can be decreased; consequently, the potential risk to the patient from exposure to anaesthesia and the possible development of hyperstimulation syndrome can be reduced. In addition, storage of embryos from a cycle allows the patient to space the timing of sibling pregnancies, and improve their potential to achieve a pregnancy at an advanced maternal age, since the eggs were retrieved when the patient was younger (Kuwayama et al.; 2005).

Currently, there are two methods used to cryopreserve mammalian oocytes and embryos: slow-rate freezing and vitrification. Independent of the methodology used for cryopreservation, effects on oocyte and embryonic cellular functions can compromise abilities to develop normally following the cryopreservation process.

Taking into consideration that slow freezing is more time consuming and requires an expensive programmable freezing machine with much unreliable result in oocyte cryopreservation (Kuwayama, et a., 2005& 2007 and Kuwayama, 2009).

Vitrification, was assumed to lead to improved viability and survival rates of cells because of the prevention of intra-cellular ice crystallization. Also, only one embryologist without the use of any costly equipment can perform this method within a few minutes. More recently, it has been addressed as the future of cryopreservation of human gametes and embryos due to improved outcomes regarding the rates of survival and pregnancy (Ozmen et al., 2007).

Vitrification in principle is a simple technology, that is potentially faster to apply, and relatively inexpensive; furthermore, it is becoming clinically established, and is seemingly more reliable and consistent than conventional cryopreservation when carried out appropriately (Tucker et al., 2003; Liebermann & Tucker, 2004).

The purpose of this study is to compare between vitrification at the zygote stage (2PN stage) and the early cleaved embryo stage (4-cell stage) of human embryos if they can alter the outcome of vitrification. If so, to predict the most preferable stage for vitrification between the two stages to attain the highest yields. The most preferable stage will be identified on the basis of ability to survive after thawing and to resume the meiotic division to reach blastocyst stage in controlled culture conditions.

2. MATERIALS AND METHODS

The source of zygotes and embryos

Candidates for embryo cryopreservation were recruited from couples underwent ART programs with excess embryos that they did not want to freeze. The age of the female partner is between 25 to 35 years old.

Ovarian stimulation

Each woman underwent the proper regulation and desensitization of controlled ovarian stimulation (COS) with various protocols of Gonadotropin releasing hormone (GnRH) agonist and Follicle stimulating hormone (FSH) during luteal phase. The follicular growth was monitored by using vaginal ultrasonography and detection of serum estradiol (E2) levels.

Oocyte retrieval, ICSI and embryo culture

Retrieval of oocytes is carried out by ultrasound-guided trans-vaginal aspiration, 36 hours after HCG administration. Follicular fluid is examined under microscope equipped with a heated stage for proper handling of oocytes at 37ºC. The oocytes retrieved are in the form of oocyte-corona-cumulus complexes.

The identified oocyte-corona-complex are selected and transferred to 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES)-buffered culture medium in order to maintain the appropriate PH. Preparation of the retrieved mature oocytes should be carried out under conditions of constant PH of 7.3 and stable temperature of 37ºC. After collection, oocytes are denuded enzymatically by brief exposure and continuous pipetting on hyaluronidase enzyme. The corona cells are completely removed by pipetting through micropipettes. The mature metaphase II oocytes (MII) are then determined by the presence of the first polar body. ICSI is performed on mature oocytes using fresh sperms. Most of the procedures are performed with CO2-equilibrated culture media under paraffin/ mineral oil that prevents the evaporation of the media and minimize the fluctuations of both the PH and the temperature. After ICSI, the injected oocytes are cultured in 20µl drops of culture media under paraffin/ mineral oil in 6.0% CO2 incubator at 37ºC.

Fertilization Check

Fertilization is assessed on day one, approximately 17-19 hours after sperm micro-injection, and the normal fertilization is determined by the presence of two pronuclei and two polar bodies.

Normally fertilized oocytes should be spherical and have two polar bodies and two pronuclei (2PN). PNs should be juxtaposed, approximately the same size, centrally positioned in the cytoplasm with two distinctly clear, visible membranes (Tesarik and Greco, 1999; Tesarik et al., 2000; Scott, 2003).

The defined normal zygotes are grouped into two categories:

Group (1): Contains a number of normal zygotes that will be cryopreserved using the vitrification technique.

Group (2): Zygotes of this group are allowed to resume their divisions in the same culture conditions 44hours micro-injection (day-2) forming 4-cell embryo with equal blastomeres and no fragmentations [small portions of cytoplasm enclosed by a cell memberane but usually not containing DNA are often formed during cell division. Fragmentation is therefore is defined as the presence of anucleate structures of blastomeric origin (Keltz et al., 2006)] before the embryos are cryopreserved using the same vitrification technique.

The vitrification equipments

The cryocarrier system

The Cryotop method is used which was developed by Kuwayama in Advanced medical Research Institute of kato ladies clinic.

The vitrification solutions

The full vitrification protocol involves just two steps: the vitrification and the warming processes. The vitrification/warming protocol was performed according to the method described previously (Kuwayama et al., 2005a). The PN zygotes and/or embryos were incubated in equilibrium solution (ES) comprising 7.5% ethylene glycol (EG) (Sigma-Aldrich, Steinem, Germany) and 7.5% dimethyl sulphoxide (DMSO) (Sigma-Aldrich) in Ham's F-10 media supplemented with 20% patient serum for 5-15 minutes (depending on the time needed for re-expansion of the cell) at room temperature. After an initial shrinkage and recovery, they were then aspirated and placed into the vitrification solution (VS) (15% EG, 15% DMSO, 0.5 M sucrose) (Merck, Darmstadt, Germany) in Ham's F-10 medium supplemented with 20% patient serum for 50-60 seconds at room temperature. After having observed cellular shrinkage, zygotes and/or embryos were aspirated and placed on the tip of the Cryotop (Kitazato, Japan). No more than two zygotes and/or embryos were placed on each Cryotop. Cooling of the zygotes and/or embryos was done by direct contact with liquid nitrogen. The Cryotops were stored in liquid nitrogen for at least two months.

Warming of zygotes and/or embryos was performed by placing the Cryotop in thawing solution (TS) (1 M sucrose) for 50-60 seconds at room temperature and then into dilution solution (DS) (0.5 M sucrose) for 3 minutes, followed by another dilution solution of 0.25 M sucrose for 3 minutes, both at room temperature. The warmed zygotes and/or embryos were placed 4-5 times into washing solution (WS) (Ham's F-10 + 20% serum) before incubation (Al-Hasani et al., 2007).

Morphological evaluation is done under inverted microscope with different magnifications. Zygotes are assessed to be normal and vital if they have two centrally located PN, normal cytoplasm, zonapellucida and perivitelline space of normal size. Morphologically damaged zygotes are those with dark granulated or fragmented cytoplasm and those that have irregular shape. Morphologically normal zygotes are only selected and incubated.

Embryo survival can be defined as an embryo with evenly sized blastomeres with no fragmentation or dark granulation. Those embryos are selected and incubated.

Culturing of thawed zygotes and embryos

The vital zygotes and embryos are then transferred into culture dish with 20µl drops of pre-equilibrated culture media under paraffin/mineral oil and incubated at 37ºC in 6.0% CO2 (the same culture conditions before vitrification).

The zygotes and embyos are cultured till blastocyst formation, depending on the cultured stage (zygote or embryo). The stage that could resume the embryonic development till the blastocyst formation is recorded and the final data is collected.

Statistics

Data were analyzed by the Mann-whitney U test.

3. RESULTS

Table (1) presents results for human zygotes and embryos vitrified at the 4-cell stage. The data represents consecutive vitrification-warming cycles performed over a 1.5 year interval. A total of 408 zygotes and embryos were vitrified and warmed from 99 couples and were grouped into:

Group (1): includes 225 zygotes that were vitrified 17-19 hours post-ICSI.

Group (2): includes 183 embryos in the 4-cell stage that were vitrified on the second day of injection.

Morphological survival of the thawed zygotes and embryos

In the first series of the experiment, the survival and developmental rates for pronuclear stage was lower than the 4-cell embryo stage.

The morphological survival of the zygotes included in group (1) was 88.88% (200/225), while the survival rate of the embryos of group (2) was 91.25% (167/183).

The highest rate of morphological survival was shown in group (2) (P>0.05).

Blastocyst formation

In the second series of the experiment, blastocyst formation was detected after extended culture of the survived zygotes and embryos in suitable culture conditions till the fifth day of micro-injection.

It was found that 58.03% (213/236) of warmed zygotes and embryos of both groups showed signs of compaction and blastulationby the time of day 5 of culture. Predominantly early stage blastocysts were noted during the early morning observation but by the late afternoon, expansion was clearly evident in high quality embryos. The timely post-warming development of vitrified zygotes and embryos appeared to be an excellent indicator of subsequent developmental potential.

Group (1) showed a blastocyst formation rate of 53.50% (107/200).

Group (2) showed a percentage of 63.47% (106/167).

A significantly higher proportion of blastocyst formation was seen in group (2) (P<0.05).

	Zygotes Group (1) (N=225)	**Embryos Group (2) (N=183)**	
No. of survived after thawing	200 (89%)	167 (91%)	**(P>0.05)**
No. of blastocysts formed	107 (48%)	106 (58%)	**(P<0.05)**

4. DISCUSSION

It has been acknowledged without doubt that the routine use of a good, reliable, and safe cryopreservation programme leads to improved cumulative clinical outcomes of IVF and ICSI cycles (Koutlaki et al., 2006; Vajta and Nagy, 2006). The cost of a live birth could also be reduced by both preventing the repetitive use of expensive induction agents and avoiding the expensive clinical follow-up of ovulation induction cycles. However, the possibility of cell damage caused by cryopreservation is a major issue. Thus, several strategies used to prevent cell damage have led to the introduction of different cryopreservation techniques over the past decades (Vajta et al., 1998).

The two most popular cryopreservation techniques of human embryos at different development stages are slow-rate freezing and vitrification. The slow-rate freezing method is also known as equilibrium freezing due to the exchange of fluids between the extra- and intracellular spaces that results in safe freezing without serious osmotic and deformation effects to cells (Mazur, 1990). It was accepted to be a safe procedure because of the use of relatively low concentration of cryoprotectants that might not cause any serious toxic and osmotic damage. However, these low concentrations are insufficient to avoid the formation of ice crystals, which is one of the main sources of cell injury. Also, previously reported low survival and pregnancy rates, along with the high cost of cryopreservation, has not satisfied the experts (Boldt et al., 2003; Fosas et al., 2003). Although there are some recent data indicating better survival rates, cryopreservation by the slow freezing method has started to be discontinued (Fabbri et al., 2001).

In contrast, vitrification is a non-equilibrium method and may be regarded as a radical approach in which ice crystal formation is totally eliminated. Nevertheless, it requires an extremely high cooling rate alongside much higher

concentrations of cryoprotectants when compared with slow-rate freezing (Vajta and Nagy, 2006). Therefore, there is an increased probability of cell injury due to factors other than the formation of ice crystals. In 1985, vitrification was reported for the first time as an efficient method in mouse embryo cryopreservation (Rall and Fahy, 1985). Afterwards, Mukaida et al. (1998) reported successful vitrification of human 4-8 cell embryos using the method developed for mouse embryos and vitrification was then suggested as a viable and real alternative to slow-rate freezing.

Thereafter, vitrification of human embryos especially at early stages became a more popular alternative to the slow-rate freezing method due to reported comparable clinical and laboratory outcames (Saito et al., 2000; El-Danasouri and Selman, 2001; Kuleshova and Lopata, 2002; Liebermann and Tucker, 2002). Subsequently, El-Danasouri and Selman (2001) reported that survival rates following vitrification are positively correlated with the number of blastomeres in the cleavage-stage embryos.

More notable is the observation that vitrification , either at blastocyst stage or at cleavage and PN stage, along with subsequent embryo transfer either at day 3 or 5, was shown to result in almost similar pregnancy rates as fresh cycles (Huang et al., 2005; Kuwayama et al., 2005; Stehlik et al., 2005; Zheng et al., 2005). Also the rate of blastocyst formation after vitrification, either at PN or cleavage stage, was similar to fresh cycles and commonly above 40-50%. These findings suggest the advantage of the early-stage vitrification based on the similar survival rates of vitrification at different stages and high blastulation rates, comparable to fresh cycles. Consequently, vitrification now seems to be the future method of cryopreservation of human embryos at different development stages (Vajta and Nagy, 2006).

In Germany, most embryos are cryopreserved at the 2PN stage. Local embryo protection laws allow cryopreservation of cleaved embryos only in emergency cases. This situation creates a need to develop the protocol that is the most appropriate for vitrification of zygotes. Empirical experience with cryopreservation using slow cooling rate protocols and vitrification show that the various stages of early embryo development require different freezing protocols. Oocytes and zygotes are more sensitive to osmotic shock than are cleavage-stage embryos because they have lower permeability to cryoprotective additives (McWillams et al., 1995). During equilibration with cryoprotectants before cooling, zygotes can change in volume to approximately 20% of their original isotonic size. The rate of volume change and the temperature at which it occurs are more critical to zygote survival than is the extent of the change in volume.

Moreover, vitrified cells are more sensitive to hypotonic stress just after warming than are fresh cells. To reduce hypoosmotic stress, nonpermeating compounds are used as osmotic buffers during removal of permeating cryoprotectants from cells. The osmotic response of zygotes to sucrose and trehalose are similar (McWillams et al., 1995), but trehalose stabilizes membranes better than sucrose, leading to better survival and further development after osmotic shock during vitrification (Arav et al., 1997; Yokota et al., 2000). Trehalose has also been used for vitrification of 8- to 16-cell human embryos, resulting in deliveries (Saito et al., 2000).

In Germany, the Embryo Protection Law restricts cryopreservation to pronucleate eggs. This form of cryopreservation was advocated by Veeck et al. (1993) to raise cumulative pregnancy rates per retrieval cycle. This investigators showed how cryopreserved pronucleate eggs surviving freezing, thawing and progression through syngamy retained similar potentials for implantation and pregnancy to fresh embryos. However, a 20-40% rate of loss due to freezing-thawing limits success rates in comparison with fresh embryo transfer cycles. Survival was independent of maternal age, but not implantation rates, which declined significantly (<35 years versus 35-39 years: $p < 0.005$)(Veeck et al., 1993). Hence, Veeck et al. (1993) considered that post-thaw survival rates could not be used to evaluate cryopreservation protocols. Reports in the literature give post-thaw survival rates of pronucleate eggs of between 60 and 90%, as confirmed in this study (Al-Hasani et al., 1996; Horne et al., 1997; Queenan et al., 1997; Kattera et al., 1999).

Kattera et al., (1999) showed in a retrospective analysis of 140 women undergoing frozen-thawed embryo transfer (FET) with IVF embryos how multicellular embryos survived significantly better (73.9%) and produced more pregnancies (22.8%) than those frozen while pronucleate (64.4 and 14.8% respectively). Thawed cleaving embryos established by ICSI in 84 women also displayed significantly higher survival from cryopreservation (74.8%) versus pronucleate eggs (64.4%), although pregnancy outcomes were similar using either embryonic stage. Kattera et al. (1999) explained this difference by the greater ease of selecting the best cleaving embryos for transfer, and the opportunity to select the most robust for cryopreservation as compared with pronucleate stages.

In contrast, a prospective randomized study including 283 patients undergoing IVF/ICSI revealed how cryopreserved pronucleate stage embryos gave higher pregnancy rates than those in early cleavage stages (19.5 versus 10.9%, $P<0.02$) (Senn et al., 2000). Embryo transfers were cancelled more frequently as a consequence of the degeneration of all thawed embryos in cleavage stages than with pronucleate embryos (7.2 versus 1.8%, $P<0.003$). Senn et al. (2000), transferring a maximum of three unselected fresh embryos and preserving the rest as pronucleate eggs, reported significantly higher cumulative pregnancy rates with transfers of fresh and frozen-thawed embryos than with cryopreservation of supernumerary early cleavage stages (55.5 versus 38.6%, $P< 0.02$) (Senn et al., 2000).

Horne et al. (1997) reported on two randomly selected groups of patients. Only two pronucleate eggs were allowed to cleave in group 1, all other pronucleate eggs being cryopreserved (PN group). In group 2, the two best cleaving embryos were selected, and the remaining embryos were frozen (EG group). Using fresh embryos, group1 produced 19.4% clinical pregnancies, versus 30.6% in group 2. Rates of embryo survival following thawing were similar in both groups (group 1: 96/129, 74.4%; group 2: 79/102, 77.4%). Higher implantation and pregnancy rates using cryopreserved embryos were achieved in group 1 (14.9 versus 7.6% and 22.0 versus 13.0% respectively). Following three successive replacements using fresh embryos initially and then two sets of cryopreserved embryos, cumulative pregnancy rates were comparable in both groups (40.2 versus 41.1%). Such data imply that pronucleate egg strategy compromise fresh replacements but maximizes potential in frozen embryos.

Vitrification either at blastocyst stage or cleavage and PN stage of embryo seems to be favorable and efficient in view of an increased outcome such as survival and pregnancy rates (Kuwayama et al., 2005; Hiraoka et al., 2004; Isachenko et al., 2003).

On the other hand, more advanced pregnancy rates have been reported by vitrification at blastocyst stage. Since morphology alone of a vitrified and thawed embryo is not enough to assess viability, the possibility of culturing for a few more days before transfer. This is also advantageous as it was remarked that embryo transfer at day 5, in comparison to day 2 or 3, gives an increased pregnancy and implantation rate (Graham et al., 2007; Milki et al., 2000).

On conclusion, our results indicate that vitrification is a simple, inexpensive and safe technique for cryopreservation of human embryos and zygotes at any of their developmental stages. The survival rate after thawing is not dependent on the stage of the vitrified zygotes or embryos, but on the other hand, early-cleaved embryo stages gave the most successful outcome on the fifth day of culture.

5. REFERENCES

- Al-Hasani S, Ludwig M, Gagsteiger F et al. 1996 Comparison of cryopreservation of supernumerary pronuclear human oocytes obtained after intracytoplasmic sperm injection (ICSI) and after conventional in-vitro fertilization. Human Reproduction 11, 604–607.
- Arav A, Zeron Y.Vitrification of bovine oocytes using modified minimum drop size technique (MDS) is effected by the composition and the concentration of the vitrification solution and by the cooling condition. Theriogenology 1997;46:1.
- Boldt J, Cline D, McLaughlin D 2003 Human oocyte cryopreservation as an adjunct to IVF-embryo transfer cycles. Human Reproduction18, 1250–1255.
- El-Danasouri I, Selman H 2001 Successful pregnancies and deliveries after a simple vitrification protocol for day 3 human embryos. Fertility and Sterility 76, 400–402.
- Fabbri R, Porcu E, Marsella T et al. 2001 Human oocyte cryopreservation: new perspectives regarding oocyte survival. Human Reproduction 16, 411–416.
- Fosas N, Marina F, Torres PJ et al. 2003 The births of five Spanish babies from cryopreserved donated oocytes. Human Reproduction18, 1417–1421.
- Graham J, Han T, Porter R, Levy M,Stillman R, et al. Day 3 morphology is a poor predictor of blastocyst quality in extended culture. FertilSteril 2000;74:495–7.
- Hiraoka K, Kinutani M, Kinutani K. Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. Hum Reprod 2004;19(12):2884–8.
- Horne G, Critchlow JD, Newman MC et al. 1997 A prospective evaluation of cryopreservation strategies in a two-embryo transfer programme. Human Reproduction 12, 542–547.
- Huang CC, Lee TH, Chen HH et al. 2005 Successful pregnancy following blastocyst cryopreservation using super-cooling ultrarapidvitrification. Human Reproduction 20, 122–128.
- Isachenko V, Selman H, Isachenko E, et al. Modified vitrification of human pronuclear oocytes: efficacy and effect on ultrastructure. Reprod Biomed Online 2003;7:211–6.
- Kattera S, Shrivastav P, Craft I 1999 Comparison of pregnancy outcome of pronuclear- and multicellular-stage frozen–thawed embryo transfers. Journal of Assisted Reproduction and Genetics16, 358–362.
- Koutlaki N, Schoepper B, Maroulis G et al. 2006 Human oocyte cryopreservation: past, present and future. Reproductive BioMedicine Online 13, 427–436
- Kuleshova L, Lopata A 2002 Vitrification can be more favorable than slow cooling. Fertility and Sterility 78, 449–454.
- KuwayamaM, Vajta G, Ieda S, Kato O. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. Reprod Biomed Online 2005;11:608–14.
- Kuwayama, M.; Vajta, G.; Kato, O,; and Leibo, S. P. (2005): "Highly efficient vitrification method for cryopreservation of human oocytes," Reproductive BioMedicine Online, 11 (3): 300-308.

- Kuwayama, M. (2007): Highly efficient vitrification for cryopreservation of human oocytes and embryos: the cryotop method. Theriogenology, 67:73-80.
- Kuwayama, M. (2009): The human oocyte: Vitrification. In Gardner, D.K.; weissman, A.; Howles, C.M. and Shoham, Z. (3rd Eds.), textbook of assisted reproductive technologies: Laboratory and clinical perspectives. Informa UK Ltd,; 3(19): 267-247.
- Liebermann J, Tucker MJ 2002 Effect of carrier system on the yield of human oocytes and embryos as assessed by survival and developmental potential after vitrification. Reproduction 124, 483–489.
- Liebermann and Tucker (2004). Vitrifying and warming of human oocytes, embryos and blastocysts: vitrification procedures as an alternative to conventional cryopreservation methods MolBiol 254:345-364.
- Mazur P 1990 Equilibrium, quasi-equilibrium, and nonequilibrium freezing of mammalian embryos. Cell Biophysics 17, 53–92.
- McWillams RB, Gibbons WE, Leibo SP. Osmotic and physiological responses of mouse zygotes and human oocytes to mono- and disaccharides. Hum Reprod 1995;10:1163–71.
- Milki AA, Hinckley MD, Fisch JD, et al. Comparison of blastocyst transfer with day 3 embryo transfer in similar patient populations.FertilSteril 2000;73:126–9.
- Mukaida T, Wada S, Takahashi K et al. 1998 Vitrification of human embryos based on assessment of suitable conditions for 8-cell mouse embryos. Human Reproduction 13, 2874–2879.
- Ozmen B, Schultze-Mosgau A, Youssry M, Diedrich K, Al-Hasani S, Unlu C 2007. Current data on vitrification of human embryos: which one is the best; Zygote, cleavage or blastocyst stage? J Turkish-German Gynecol Assoc. 8, 218-226.
- Queenan JTJ, Ramey JW, Seltman HJ et al. 1997 Transfer of cryopreserved–thawed pre-embryos in a cycle using exogenous steroids without prior gonadotrophin-releasing hormone agonist suppression yields favourable pregnancy results. Human Reproduction 12, 1176–1180.
- Rall WF, Fahy GM 1985 Ice-free cryopreservation of mouse embryos at –196°C by vitrification. Nature 313, 573–575.
- Saito H, Ishida GM, Kaneko T, Kawachiya S, Ohta N, Takahashi T, et al. Application of vitrification to human embryo freezing. GynecolObstet Invest 2000;49:145–9.
- Senn A, Vozzi C, Chanson A et al. 2000 Prospective randomized study of two cryopreservation policies avoiding embryo selection: the pronucleate stage leads to a higher cumulative delivery rate than the early cleavage stage. Fertility and Sterility 74, 946–952.
- Stehlik E, Stehlik J, Katayama KP et al. 2005 Vitrification demonstrates signifi cant improvement versus slow freezing of human blastocysts. Reproductive BioMedicine Online 11, 53–57.
- Tucker et al. (2003).Cryopreservation protocols. In Color Atlas of Human Assisted Reproduction: Laboratory and Clinical Insights, eds. P Patrizio, V Guelman and MJ Tucker, pp 257-276, Lippincott, Williams and Wilkins, Philadelphia.
- Vajta G, Lewis IM, Kuwayama M et al. 1998 Sterile application of open pulled straw (OPS) vitrification method. Cryo-Letters 19, 389–392.
- Vajta G, Nagy ZP 2006 Are programmable freezers still needed in the embryo laboratory? Review on vitrification. Reproductive BioMedicine Online 12, 779–796.
- Veeck L, Admundson CH, Brothman LJ et al. 1993 Significantly enhanced pregnancy rates per cycle through cryopreservation and thaw of pronuclear stage oocytes. Fertility and Sterility 59, 1202–1207.
- Yokota Y, Sato S, Yokota M, Ishikawa Y, Makita M, Asada T, et al. Successful pregnancy following blastocyst vitrification: case report. Hum Reprod 2000;15:1802–3.
- Zheng WT, Zhuang GL, Zhou CQ et al. 2005 Comparison of the survival of human biopsied embryos after cryopreservation with four different methods using non-transferable embryos. Human Reproduction 20, 1615–1618.