Article

Aseptic vitrification of blastocysts from infertile patients, egg donors and after IVM

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Abstract

During embryo vitrification, it is advisable that cooling and storage should occur in a carrier device in which there is complete separation of the embryos from liquid nitrogen to ensure asepsis. The consequence of a reduction in the cooling rate resulting from the heat-insulating barrier aseptic devices has to be counteracted by gradually increasing intracellular concentrations of cryoprotectants without inducing a toxic effect. Blastocysts originating from couples with male and/or female factor infertility (group 1) or from oocyte donors (group 2) or from in-vitro matured oocytes (group 3) were gradually exposed to increasing concentrations of dimethylsulphoxide/ethylene glycol (5/5%, 10/10% and 20/20%) before aseptic vitrification using a specially designed carrier (VitriSafe), a modification of the open hemi-straw plug device. A total of 120 aseptic vitrification/warming cycles were performed in group 1, 91 in group 2 and 22 in group 3. Survival rates before embryo transfer, ongoing pregnancy and implantation rates were as follows: for group 1, 73, 43 and 26%; for group 2, 88, 53 and 34%; and for group 3, 69, 50 and 38%, respectively. In spite of reduced cooling rates due to aseptic vitrification conditions, a three-step exposure to cryoprotectant solutions protects the embryos effectively from cryo-injuries and guaranties high survival rates.

Keywords: *egg donation, embryo carrier device, human blastocyst, in-vitro maturation*

Introduction

Vitrification is a cryopreservation procedure by which solutions are converted into a glass-like amorphous solid, free of any crystalline structures. After the initial application of vitrification to cleaving embryos (Mukaida *et al.*, 1998) or blastocysts in closed 0.25 ml insemination straws (Yokota *et al.*, 2001; Vanderzwalmen *et al.*, 2002), the trend was to increase significantly the cooling and warming rates from <2000°C/min to >20,000°C/min in order to reduce the likelihood of lethal ice-crystal formation in the crystalline phase (Lane *et al.*, 1999). Several embryo carrier devices (open systems) were therefore designed (Vajta and Nagy, 2006) in a way to allow a direct contact of the biological sample with liquid nitrogen in order to permit cooling of embryos instantaneously below the glass transition temperature where cells are captured in an amorphous state. Under such ultra-rapid cooling conditions, a vitrified state is obtained even if embryos are exposed for only a very short period of time to high concentrations of the cryoprotectant solutions. This allows the extraction of intracellular water while limiting the amount of cryoprotectant permeating into the cells. During the warming process, the open devices allow for instantaneous release of cryopreserved

embryos into a diluting solution. In this way, extremely high warming rates (>20,000°C/min) can be achieved and the recrystallization phenomenon avoided. Most publications on the clinical application of ultra-rapid vitrification have mainly focused on the blastocyst stage (Lane *et al.*, 1999; Son *et al.*, 2002; Mukaida *et al.*, 2003; Vanderzwalmen *et al.*, 2003; Hiraoka *et al.*, 2004; Huang *et al.*, 2005; Kuwayama *et al.*, 2005; Stehlik *et al.*, 2005; Takahashi *et al.*, 2005; Liebermann and Tucker, 2006; Balaban *et al.*, 2008).

Although satisfactory results were reported, one major drawback to the ultra-rapid cooling process is the possible risk of bacterial as well as viral contamination of the biological sample during the cooling process as well as during long-term storage (Bielanski, 2005; Morris, 2005). However, the question of contamination during storage in liquid nitrogen remains debatable (Kyuwa *et al.*, 2003). Nevertheless, a European Parliament directive on tissues and cells (European Union, 2004), and the revised version (European Union, 2006), has defined medical safety requirements for the cryopreservation of human cells. In view of the issues raised by this directive, it was imperative to revise this study centre's ultra-rapid vitrification procedure (Vanderzwalmen *et al.*, 2002, 2003) and to develop a new vitrification technique ensuring total protection and isolation of the sample from the liquid nitrogen during the cooling procedure as well as during long-term storage.

Hermetically closed containers (closed systems) have already been developed to vitrify mice and human oocytes and embryos (Kuleshova and Shaw, 2000; Walker *et al.*, 2004; Isachenko *et al.*, 2005, 2007; Kuwayama *et al.*, 2005; Larman *et al.*, 2006; Stachecki *et al.*, 2008). Kuwayama *et al.* (2005) developed the Cryotip device, allowing isolation of biological material from liquid nitrogen. In the first report, Kuwayama *et al.* (2005) showed similar pregnancy rates after vitrification of blastocysts in an open (53% with the Cryotop device) and in a closed system (51% with the Cryotip device). Nevertheless, in a more recent paper, the superiority of the open Cryotop system was recognized (Kuwayama, 2007). At present, the clinical application of the Cryotip device remains sporadic and ultra-rapid vitrification with the Cryotop device is still the method of choice in the majority of assisted reproduction laboratories. Recently, Stachecki *et al.* (2008) reported encouraging results after vitrification of human blastocysts in closed 0.25 ml straws. After warming, a survival rate of 89% was obtained. Out of 43 transfers, clinical pregnancy and implantation rates of 60% and 45% were recorded, respectively.

Therefore, to achieve maximal survival rates with hermetically sealed and thus aseptic embryo carriers, the problem relating to the heat-insulating barrier, which dramatically reduces the speed of cooling from >20,000°C/min to <2000°C/min, has to be solved.

Since the probability of fixing the intracellular parts into a glass-like state depends on the cooling–rewarming speeds and the concentration of cryoprotectant (Yavin and Arav, 2007), it is obvious that the risk of ice-crystal formation will increase if the cells have not been exposed long enough to cryoprotectant solutions. Consequently, the decrease in the speed of cooling and rewarming, as observed with aseptic devices, has to be compensated by higher intracellular concentrations of cryoprotectant. As a consequence, aseptic cooling, by which the embryos are shielded from liquid nitrogen, may have an adverse effect on embryo survival after warming if the embryos are not exposed sufficiently to the cryoprotectants at the beginning of the procedure.

The initial aim of this study was to construct an aseptic embryo carrier device easy to handle and guaranteeing medical safety of vitrified human embryos. With the aim of achieving adequate intracellular vitrified conditions before aseptic cooling without inducing cell toxicity, two parameters regarding the optimum cryoprotectant concentration and the adequate exposure time to the different cryoprotectants were studied. Finally, this study reports further on the results of the clinical application of aseptic vitrification of blastocysts originating from couples with male and/or female factor infertility or from oocyte donors and also after in-vitro maturation (IVM).

Materials and methods

The development of an aseptic vitrification technique was performed after informed consent on supernumary human blastocysts that were not eligible for a fresh embryo transfer or for ultra-rapid vitrification.

The embryo carrier device: the VitriSafe plug

The VitriSafe (VitriMed, Austria) is an aseptic embryo carrier device specifically designed for the study centre, based on a modification of the open hemi-straw plug device (Vanderzwalmen *et al.*, 2003). It consists of a large gutter in which a small quantity of cryoprotectant (<1 µl) containing the blastocysts can be deposited (**Figure 1**). Before plunging the biological material into liquid nitrogen, the VitriSafe is inserted into a high-security 0.3 ml straw (Cryo Bio System, France). Welding of both edges of the outer protective straw took place before being plunged into liquid nitrogen. This ensures a hermetic isolation of the sample. Under these conditions, the cooling rate decreased from above 20,000°C/min to less than 1300°C/min. Measurement of the cooling rate was carried out between −20 and −120°C with a very thin thermocouple (Digital thermometer GMH 3230; Greisinger Electronic, Germany) introduced in a small drop located in a 0.3 ml straw (Cryo Bio System).

For warming, the top of the outer straw is cut and the VitriSafe device is removed without contact with the liquid nitrogen. In order to achieve an ultra-rapid warming rate of >20,000°C/min, the tip of the gutter containing the biological material is instantaneously immersed into the dilution solution.

Solutions used for vitrification and dilution after warming

Three non-vitrifying solutions (NVS), 2.5/2.5, 5/5 and 10/10 NVS and one vitrification solution (20/20 VS) were

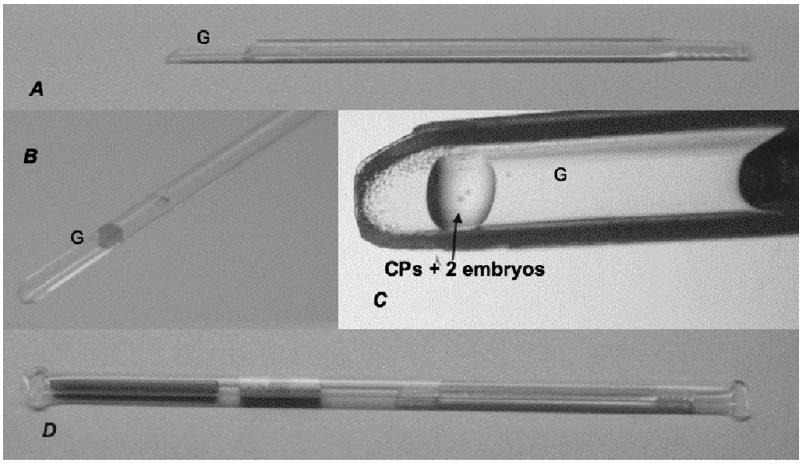

Figure 1. **(A)** The VitriSafe embryo carrier, showing **(B)** the tip of the VitriSafe, **(C)** the tip with a drop of cryoprotectant and two embryos and **(D)** a hermetically closed straw before plunging in liquid nitrogen. G = gutter.

prepared for the development of the aseptic vitrification protocol. The 2.5/2.5 and 5/5 NVS were prepared by diluting the 10/10 NVS (Fertipro, Bernem, Belgium), which contained (v/v) 10% dimethylsulphoxide (DMSO) and 10% ethylene glycol (EG). The vitrification solution (Fertipro) was composed of 20% DMSO, 20% EG, 25 µmol/l (10 mg/ml) Ficoll (70,000 MW) and 0.75 mol/l sucrose.

The solutions for warming were made of 1, 0.5, 0.25 and 0.125 mol/l sucrose in phosphate-buffered saline with 20% human serum albumin (HSA) or Global HEPES with 20% HSA (LifeGlobal, Ontario, Canada).

Improvement of exposure conditions for blastocysts to cryoprotectants in reduced cooling conditions

Different experiments were designed with the aim to define appropriate conditions for blastocysts before cooling them aseptically. The effect of exposing embryos to different combinations of cryoprotectants was analysed after comparing the survival capacity of human blastocysts immediately after warming and after 24 h of in-vitro culture.

Effect of a direct exposure to 10/10 NVS for shorter or longer time periods before vitrification in aseptic conditions

Blastocysts were exposed directly to 10/10 NVS either for a period of 2–4 min or for a prolonged period of 7–8 min. After this step, the embryos were exposed to the 20/20 VS solution before aseptic cooling.

Effect of gradual exposure of blastocysts to different non-vitrifying cryoprotectants

In the next experiment, before placing the blastocysts in the 10/10 NVS solution for a period of 3–4 min, they were first exposed to increasing concentrations of cryoprotectant. One group of blastocysts were first exposed sequentially to 2.5/2.5 NVS for 5 min and then equilibrated for 10 min to 5/5 NVS. In the other group, blastocysts were first exposed to 5/5 NVS for either 5 min, 7.5 min or 10 min. After exposure to 10/10 NVS for 3–4 min the blastocysts were placed in contact with 20/20 VS for a period of 40–60 s.

Effect of the exposure time in 20/20 VS on embryos

The time needed from placing embryos into the 20/20 VS up to the final step of plunging the closed system into liquid nitrogen ranges from 40 to 60 s. However, for different reasons, manipulation problems could occur, increasing the exposure time in this potentially toxic solution. Therefore, the effect of the exposure time in 20/20 VS on the capacity of the embryos to further develop was analysed. The blastocysts were exposed to the NVS followed by exposure to 20/20 VS for two different durations of 60 s and 90 s before being plunged into liquid nitrogen in an aseptic way.

Warming procedure

After cutting the upper part of the protective straw, the tip of the VitriSafe was immediately immersed in 1 ml of 1 mol/l sucrose. After 1–2 min, the blastocysts were put into 0.5 mol/l sucrose for 2–3 min followed by 0.25 mol/l and 0.125 mol/l sucrose at intervals of 4 min. All procedures were performed at room temperature. The blastocysts were then washed several times in Global medium supplemented with 7.5% HSA and cultured for an additional period of 24 h.

Clinical application of aseptic vitrification of blastocysts

Aseptic vitrification was carried out on blastocysts originating from three groups of patients. The first group included couples entering the IVF programme for different types of female (tubal, endometriosis, ovarian hyperstimulation syndrome, idiopathic) and/or male factor infertility (oligoasthenoteratozoospermia with freshly ejaculated sperm

only). In a second group, the blastocysts originated from egg donation cycles. The third group consisted of polycystic ovary syndrome (PCOS) patients whose oocytes underwent IVM and further culture to the blastocyst stage after intracytoplasmic sperm injection.

For the first two groups, standard procedures were applied to stimulate the ovaries as published elsewhere (Prapas *et al.*, 2005; Zech *et al.*, 2007). PCOS patients from the third group received a priming of 150 IU of rFSH (Gonal–F; Serono, Italy) on days 4, 5 and 6 of the cycle. On day 8, they received 10,000 IU of human chorionic gonadotrophin (Ovidrel; Serono). Oocyte retrieval was performed between days 9 and 10, when follicle diameter and endometrial thickness were at least 10 mm and 9 mm, respectively. Immature oocytes were cultured in maturation medium (IVM media; Sage, USA) supplemented with 75 IU of FSH (HMG 75; Instituto Massone, Buenos Aires, Argentina) and 75 IU of LH during a period of 24–30 h.

For all groups, intracytoplasmic sperm injection was performed on metaphase II oocytes. From day 1 to day 5, embryo culture was carried out in four-well multidishes (Nunc), each well containing 800 µl of non-sequential Global medium supplemented with 7.5% HSA or 50 µl drops of sequential medium (Sage media) at 37°C in a humidified atmosphere of 6% CO_2 in air or 6% CO_2 with O_2 concentration reduced to 5%. For groups 1 and 3, good and moderate-quality blastocysts were vitrified and, for group 2, only good-quality blastocysts were selected for vitrification. They were classified as good, moderate or poor quality according to the degree of expansion, the quality of the inner cell mass and the trophectoderm as defined by Gardner *et al.* (2000). According to the conclusions from the different trials, the blastocysts were exposed to 5/5 NVS for 5–10 min and to 10/10 NVS for 4 min at room temperature. They were then placed in the 20/20 VS. On average it took 40–60 s from the time of placing embryos into the 20/20 VS up to finally plunging the closed straw harbouring the embryos into liquid nitrogen. The warming procedure as describe above in the laboratory study was applied.

Before the transfer of warmed embryos, hormone replacement therapy was administered to each group of patients. After confirming steroid down-regulation, oestrogen therapy was started consisting of increasing doses of oestradiol valerate (Progynova; Schering, Spain) until appropriate endometrial thickness (>8 mm). From that day on, intramuscular or intravaginal progesterone was administered until week 16.

Ongoing pregnancy was defined by observing one or more gestational sacs with visualization of fetal heart beat using ultrasound scan at 6 weeks after the last menstrual period and was used to calculate the pregnancy rate (PR). The implantation rate (IR) represents the percentage of gestational sacs with fetal heart beat divided by the total number of embryos transferred.

Statistical analysis

The chi-squared test was used to compare the outcome of blastocyst development according to the different incubation times in the cryoprotectants. *P* values below 0.05 were considered as statistically significant.

Results

Development of an aseptic vitrification protocol

Table 1 illustrates the development of human blastocysts exposed directly in 10/10 NVS for a shorter or longer time interval before applying aseptic vitrification. Although not statistically different, an increase in the survival rate up to 71% was noticed after extending the exposure time in 10/10 NVS from 2–4 min to 7–8 min. Although only 55% of those embryos re-expanded after 24 h of culture, this still represented a two-fold increase compared with the short exposure protocol.

Table 2 illustrates the development of human blastocysts exposed progressively to increased concentrations of NVS. Survival rates of more than 90% post warming and of approximately 80% after 24 h of additional culture were observed when embryos were first pre-incubated in less concentrated cryoprotectants. No significant difference in survival rates was observed regardless of whether or not the blastocysts were exposed to 5/5 NVS for 5–10 min. Although not significantly different, there was a tendency towards higher survival rates after stepwise addition (four steps) of cryoprotectants starting with 2.5/2.5% DMSO/EG.

Table 1. In-vitro development of human blastocysts exposed to 10/10 non-vitrifying solution for different time periods before vitrification in aseptic conditions.

No. of blastocysts	*Exposure time*		*Survival rate % (n)*	
	10/10 NVS (min)	*VS (s)*	*t = 0 h*	*t = 24 h*
10	2–4	40–60	40 (4)	20 (2)
31	7–8	40–60	71 (22)	55 (17)

NVS = non-vitrifying solution [10/10 NVS = 10% dimethylsulphoxide (DMSO), 10% ethylene glycol (EG) (v/v)]; VS = vitrifying solution [20% DMSO, 20% EG, 25 µmol/l (10 mg/ml) Ficoll (70,000 MW) and 0.75 mol/l sucrose].
There were no statistically significant differences between the two groups.

Table 2. In-vitro development of human blastocysts exposed for different time periods to gradually increasing concentrations of dimethylsulphoxide/ethylene glycol before vitrification in aseptic conditions.

No. of blastocysts	*Exposure time (min) in NVS (%DMSO/%EG, v/v)*			*Exposure time (s) in VS*	*Survival rate % (n)*	
	2.5/2.5	5/5	10/10		*t = 0 h*	*t = 24 h*
31	–	5.0	3–4	40–60	94 (29)	84 (26)
12	–	7.5	3–4	40–60	92 (11)	75 (9)
23	–	10.0	3–4	40–60	96 (22)	87 (20)
21	5.0	10.0	3–4	40–60	95 (20)	95 (20)

DMSO = dimethylsulphoxide; EG = ethylene glycol; NVS = non-vitrifying solution; VS = vitrifying solution [20% DMSO, 20% EG, 25 µmol/l (10 mg/ml) Ficoll (70,000 MW) and 0.75 mol/l sucrose].
There were no statistically significant differences between the different exposure protocols.

The in-vitro survival in relation to different exposure time of blastocysts to the 20/20 VS is shown in **Table 3**. No adverse effect of prolonged exposure up to 90 s in the VS was observed.

Clinical outcomes of aseptic vitrification

Table 4 summarizes the data from the total of 233 aseptic vitrification–warming cycles that were performed between 2006 and 2008. Blastocysts originated from 103 couples with male and/or female factor infertility ($n = 120$), from 91 oocyte donors ($n = 91$) or after IVM of oocytes retrieved from 22 women with PCOS ($n = 22$). The overall cryo-survival rates after warming and after 24 h of further culture were 86% and 78%, respectively. An ongoing PR per vitrification cycle of 47% and an IR of 30% were achieved.

In the group of patients with male and/or female factor infertility where 348 blastocysts were warmed, 82% remained intact. At the time of embryo transfer, 73% had developed into good-quality blastocysts and 231 were selected for embryo transfer. The ongoing PR and IR per vitrification cycle were 43% and 26%, respectively.

From the 91 oocyte donation cycles, a total of 427 good-quality blastocysts were vitrified. After thawing of 218 blastocysts, 93% were intact 1–2 h after warming and 88% continued their development during 24 h of further culture. An average of 2.0 blastocysts were transferred resulting in an ongoing PR and IR per vitrification cycle of 53% and 34%, respectively.

In the IVM group, 64 blastocysts were vitrified and, after warming, 83% were intact, out of which 69% developed after 20–24 h of additional culture. After 21 transfers with 39 blastocysts, 15 pregnancies resulted. Four miscarriages (27%) were recorded. The ongoing PR and IR were 50% and 38%, respectively. The first patient received three blastocysts. A triplet pregnancy was achieved and three healthy babies were delivered recently at 35 weeks of gestation.

Discussion

In order to comply with the European Union's directive on tissues and cells storage (European Union, 2006), the objective of this work was to establish an aseptic vitrification method that guarantees good survival rates after the complete isolation of human blastocysts from liquid nitrogen during cooling, storage and warming. Satisfactory clinical outcomes are reported of 233 aseptic blastocyst vitrification–warming cycles despite reduced cooling rates to <2000°C/min due to thermo-isolation. It is shown that

Table 3. In-vitro development of human blastocysts after exposure to vitrifying solution for 60 s or 90 s before aseptic vitrification.

No. of blastocysts	*Exposure time (min) in NVS (%DMSO/%EG, v/v)*		*Exposure time (s) in VS*	*Survival rate*[a]	
	5/5	10/10		*t = 0 h*	*t = 24 h*
12	5′	3′–4′	60″	100 (12)	75 (9)
10	10′	3′–4′	60″	90 (9)	80 (8)
20	5′	3′–4′	90″	75 (15)	55 (11)
15	10′	3′–4′	90″	80 (12)	80 (12)

DMSO = dimethylsulphoxide; EG = ethylene glycol; NVS = non-vitrifying solution; VS = vitrifying solution [20% DMSO, 20% EG, 25 µmol/l (10 mg/ml) Ficoll (70,000 MW) and 0.75 mol/l sucrose].
[a]There were no statistically significant differences between the different exposure protocols.

Table 4. Clinical outcomes of aseptic vitrification of blastocysts generated from different origins.

Parameter	*Origin of blastocysts*			*Total*
	Male and/or female factor infertility	*Egg donation*	*IVM*	
No. of patients	103	91	22	216
Mean female age ± SD (years)	34.5 ± 3.7	28.8 ± 3.1	32.3 ± 4.9	–
No. of vitrification–warming cycles	120	91	22	233
No. of vitrified blastocysts	348	427	64	839
No. of warmed blastocysts	348	218	64	630
No. of surviving blastocysts after warming (%)	285 (82)	203 (93)	53 (83)	541 (86)
No. of surviving blastocysts before transfer (%)	254 (73)	191 (88)	44 (69)	489 (78)
No. of embryo transfers	111	91	21	223
No. of blastocyst transfers (mean)	231 (2.1)	186 (2.0)	39 (1.9)	456 (2.0)
No. of pregnancies (%)	66 (55)	54 (59)	15 (68)	135 (58)
No. of miscarriages (%)	15 (23)	7 (13.0)	4 (27)	26 (19)
No. of ongoing pregnancies (%)	51 (43)	48 (53)	11 (50)	110 (47)
No. of FHB	60	64	15	139
Implantation rate	26	34	38	30
Deliveries	14	35	5	54

FHB = fetal heart beat; IVM = in-vitro maturation.

gradual exposure of blastocysts to increased concentrations of cryoprotectants before aseptic cooling has a beneficial effect on the outcome of embryo development.

The VitriSafe guarantees high warming rates of >20,000°C/min without neglecting aseptic conditions during extraction from the outer protective straw. It is well known that for any given concentration of cryoprotectants, the critical warming rates are much higher than the critical cooling rates (Scheffen *et al.*, 1986; Fahy, 1987; Fahy and Rall, 2007). Consequently, if the warming rate is lowered due to the use of devices that separate the drop containing the embryos, higher intracellular concentrations of cryoprotectant are needed in order to reduce the chance of recrystallization.

The protective straw, which is required to achieve aseptic vitrification conditions, inevitably leads to an important loss in the cooling rate, increasing the possibility of ice-crystal formation if the intracellular concentration of cryoprotectant is not well adapted to the need of the cells. As compared with ultra-rapid vitrification, a dramatic drop in the cooling rate to 1300°C/min is observed. Ali and Shelton (2007) and Vanderzwalmen *et al.* (2007) observed that a decrease in the speed of cooling reduced the survival rate of embryos and has to be compensated by higher intracellular concentrations of cryoprotectant. According to these observations, the simplest and most logical way to counteract the increased risk of ice-crystal formation consists of increasing the exposure time of human blastocysts to the non-vitrifying solution (10/10 NVS). With this, a two-fold increase in the survival rate was observed. However, less blastocysts re-expanded after 24 h of further culture. An explanation could be that the direct exposure of the embryos to higher concentrations cryoprotectants causes an osmotic shock due to a too rapid and too prolonged entry of cryoprotectants after an excessive dehydration. According to Paynter (2005), exposure of embryos to cryoprotectants can be damaging, even in the absence of freezing, if it is done incorrectly.

In order to keep the shrinking/swelling response of embryos to cryoprotectants within limits that are compatible with good survival rates post warming, blastocysts were gradually exposed to cryoprotectants. In view of the satisfactory in-vitro results, blastocysts are routinely exposed first to 5/5 NVS for 5–10 min, followed by a shorter exposure of 4 min to 10/10 NVS and 40–60 s to 20/20 VS. When embryos are placed into the 20/20 VS, an additional dehydration occurs, which also helps in further concentrating the cryoprotectants that have penetrated the embryos during the two initial steps. Only then it is possible to reach intracellular amorphous conditions before cooling. This study shows that high survival rates can be obtained when embryos are exposed to 20/20 VS for more than 1 min. This gives us more than enough time to handle the embryos before cooling, hence reducing the stress of the last step.

Using the gradual exposure of cryoprotectants, this aseptic vitrification method seems very promising for cryopreservation of blastocysts. It is well appreciated that the quality of blastocysts is an important factor affecting the outcome in terms of survival and pregnancies. Therefore, application of aseptic vitrification on blastocysts originating from oocyte donors can be considered as the reference group to assess the efficiency of the vitrification technique. The oocytes are retrieved from young women (aged 28.8 ± 3.1 years; **Table 4**) and only good-quality blastocysts were selected for vitrification. One of the problems with oocyte donation is linked with synchronization between recipient and donors. The vitrification technique might help to solve such problems without compromising PR and IR.

An ongoing twin pregnancy after vitrification of blastocysts produced after IVM from a woman with PCOS has already been reported by Son *et al.* (2002). The application of the aseptic vitrification technique also seems very promising for cryopreservation of blastocysts produced from IVM oocytes retrieved from PCOS women. Successful ongoing pregnancy and deliveries can be achieved following vitrified embryo transfer, suggesting an adequate preparation of the endometrium before transfer. This highlights that endometrial quality or an inappropriate hormonal preparation of the selected PCOS population could negatively influence implantation.

Another significant advantage of this aseptic vitrification method is that it is not necessary to collapse the blastocoel. The approach described here ensures that sufficient cryoprotectant is present inside the cavity to prevent intracellular ice-crystal formation.

In spite of reduced cooling rates due to aseptic vitrification conditions, acceptable results are obtainable if the intracellular concentrations of cryoprotectants are well adapted to the needs of the cells. If such results are confirmed on a large scale, aseptic vitrification has the potential to become the standard cryopreservation technique not only for human blastocysts but also for early embryo developmental stages.

References

Ali J, Shelton J 2007 Development of vitrification solutions. In: Tucker M, Liebermann J, editors *Vitrification in Assisted Reproduction: A User's Manual and Trouble-shooting Guide*. London: Informa Healthcare; 2007. p. 3–44.

Balaban B, Urman B, Ata B *et al.* 2008 A randomized controlled study of human day 3 embryo cryopreservation by slow freezing or vitrification: vitrification is associated with higher survival, metabolism and blastocyst formation. *Human Reproduction* **23**, 1976–1982.

Bielanski A 2005 Non-transmission of bacterial and viral microbes to embryos and semen stored in the vapour phase of liquid nitrogen in dry shippers. *Cryobiology* **50**, 206–210.

European Union 2006 Directive 2006/17/CE de la Commission du 8 février 2006. *Journal officiel de l'Union européenne* **38**, 51. Available at http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:038:0040:0052:EN:PDF [accessed 9 September 2009].

European Union 2004 Directive 2004/23/CE du Parlement Européen et du Conseil. *Journal officiel de l'Union européenne* **102**, 48. Available at http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:102:0048:0058:EN:PDF [accessed 9 September 2009].

Fahy G 1987 Biological effects of vitrification and devitrification. In: Pegg DE, Karow Jr AM, editors *The Biophysics of Organ Cryopreservation*. New York: Plenum Press; 1987. p. 65–293.

Fahy G, Rall W 2007 Vitrification: an overview. In: Tucker M, Liebermann J, editors *Vitrification in Assisted Reproduction: A User's Manual and Trouble-shooting Guide*. London: Informa Healthcare; 2007.

Gardner D, Lane M, Stevens J *et al.* 2000 Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertility and Sterility* **73**, 1155–1158.

Hiraoka K, Kinutani M, Kinutani K 2004 Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. *Human Reproduction* **19**, 2884–2888.

Huang CC, Lee TH, Chen SU *et al.* 2005 Successful pregnancy following blastocyst cryopreservation using super-cooling ultra-rapid vitrification. *Human Reproduction* **20**, 122–128.

Isachenko V, Katkov I, Yakovenko S *et al.* 2007 Vitrification of human laser treated blastocysts within cut standard straws (CSS): novel aseptic packaging and reduced concentrations of cryoprotectants. *Cryobiology* **54**, 305–309.

Isachenko V, Montag M, Isachenko E *et al.* 2005 Vitrification of mouse pronuclear embryos after polar body biopsy without direct contact with liquid nitrogen. *Fertility and Sterility* **84**, 1011–1016.

Kuleshova L, Shaw J 2000 A strategy for rapid cooling of mouse embryos within a double straw to eliminate the risk of contamination during storage in liquid nitrogen. *Human Reproduction* **15**, 2604–2609.

Kuwayama M 2007 Highly efficient vitrification for cryopreservation of human oocytes and embryos: the cryotop method. *Theriogenology* **67**, 73–80.

Kuwayama M, Vajta G, Leda S *et al.* 2005 Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. *Reproductive BioMedicine Online* **11**, 608–614.

Kyuwa S, Nishikawa T, Kaneko T *et al.* 2003 Experimental evaluation of cross-contamination between cryotubes containing mouse 2-cell embryos and murine pathogens in liquid nitrogen tanks. *Experimental Animal* **52**, 67–70.

Lane M, Schoolcraft WB, Gardner DK 1999 Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertility and Sterility* **72**, 1073–1078.

Larman M, Sheehan C, Gardner D 2006 Vitrification of mouse pronuclear oocytes with no direct liquid nitrogen contact. *Reproductive BioMedicine Online* **12**, 66–69.

Liebermann J, Tucker M 2006 Comparison of vitrification and conventional cryopreservation of day 5 and day 6 blastocysts during clinical application. *Fertility and Sterility* **86**, 20–26.

Morris G 2005 The origin, ultrastructure, and microbiology of the sediment accumulating in liquid nitrogen storage vessels. *Cryobiology* **50**, 231–238.

Mukaida T, Takahashi K, Kasai M 2003 Blastocyst cryopreservation: ultrarapid vitrification using cryoloop technique. *Reproductive BioMedicine Online* **6**, 221–225.

Mukaida T, Wada S, Takahashi K *et al.* 1998 Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. *Human Reproduction* **13**, 2874–2879.

Paynter S 2005 A rational approach to oocyte cryopreservation. *Reproductive BioMedicine Online* **5**, 578–586.

Prapas N, Prapas Y, Panagiotidis Y *et al.* 2005 GnRH agonist versus GnRH antagonist in oocyte donation cycles: a prospective randomized study. *Human Reproduction* **6**, 1516–1520.

Scheffen B, Vanderzwalmen P, Massip A 1986 A simple and efficient procedure for preservation of mouse embryos by vitrification. *Cryo Letters* **7**, 260–269.

Son WY, Yoon SH, Park SJ *et al.* 2002 Ongoing twin pregnancy after vitrification of blastocysts produced by in-vitro matured oocytes retrieved from a woman with polycystic ovary syndrome: case report. *Human Reproduction* **17**, 2963–2966.

Stachecki J, Garrisi J, Sabino S *et al.* 2008 A new safe, simple and successful vitrification method for bovine and human blastocysts. *Reproductive BioMedicine Online* **17**, 360–367.

Stehlik E, Stehlik J, Katayama KP *et al.* 2005 Vitrification demonstrates significant improvement versus slow freezing of human blastocysts. *Reproductive BioMedicine Online* **11**, 53–57.

Takahashi K, Mukaida T, Goto T *et al.* 2005 Perinatal outcome of blastocyst transfer with vitrification using cryoloop: a 4-year follow-up study. *Fertility and Sterility* **84**, 88–92.

Vanderzwalmen P, Ebner T, Zech N 2007 One decade of experience with vitrification of human embryos in straws, hemi-straws and high security vitrification straws. In: Tucker M, Liebermann J, editors *Vitrification in Assisted Reproduction: A User's Manual*

and Trouble-shooting Guide. London: Informa Healthcare; 2007. p. 195–217.

Vanderzwalmen P, Bertin G, Debauche CH *et al.* 2003 Vitrification of human blastocysts with the hemi-straw carrier: application of assisted hatching after thawing. *Human Reproduction* **18**, 1504–1511.

Vanderzwalmen P, Bertin G, Debauche CH *et al.* 2002 Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoelic cavity before vitrification. *Human Reproduction* **17**, 744–751.

Vajta G, Nagy Z 2006 Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reproductive BioMedicine Online* **12**, 779–796.

Walker D, Tummon IS, Hammitt DG *et al.* 2004 Vitrification versus programmable rate freezing of late stage murine embryos: a randomized comparison prior to application in clinical IVF. *Reproductive BioMedicine Online* **8**, 558–568.

Yavin S, Arav A 2007 Measurement of essential physical properties of vitrification solutions. *Theriogenology* **67**, 81–89.

Yokota Y, Sato S, Yokota M *et al.* 2001 Birth of a healthy baby following vitrification of human blastocysts. *Fertility and Sterility* **75**, 1027–1029.

Zech N, Lejeune B, Puissant F *et al.* 2007 Prospective evaluation of the optimal time for selecting a single embryo for transfer: day 3 versus day 5. *Fertility and Sterility* **88**, 244–246.

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