Article

Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination

Masashige Kuwayama (PhD) is currently the Scientific Director of Kato Ladies' Clinic (Tokyo, Japan), the world's largest IVF unit. In 1986, he began work in the field of embryology with Dr Hanada. They developed assisted reproduction techniques (IVM, IVF, vitrification, embryo culture, ES cell) and established a bovine embryo mass production system as the leader of a National Project in Japan in 1990. He obtained the first calves after oocyte vitrification, IVF, in-vitro culture and blastocyst transfer in 1992. He moved to human IVF in 1999, developed the Cryotop vitrification method for human oocytes and established the first human oocyte bank in 2001. The first babies following oocyte vitrification in USA and Japan were obtained by his group using the Cryotop method. He is also interested in rejuvenescence of old defective oocytes, and obtained the first calf from old infertile cattle with germinal vesicle transfer in 2002.

Dr Masashige Kuwayama

Masashige Kuwayama[1,3], Gábor Vajta[2], Shoko Ieda[1], Osamu Kato[1]
[1]Kato Ladies Clinic, 7–20–3, Nishishinjuku, Shinjuku, Tokyo 160–0023, Japan; [2]Population Genetics and Embryology, Department of Genetics and Biotechnology, Danish Institute of Agricultural Sciences, Research Centre Foulum, DK-8830 Tjele, Denmark
[3]Correspondence: Tel: +81 3 33663777; Fax: +81 3 53327373; e-mail: masaabc@bekkoame.ne.jp

Abstract

Survival and development of human embryos was compared following slow cooling versus vitrification involving more than 13,000 vitrified embryos. In addition, the efficacy of an open system, the Cryotop, and a closed vitrification system, the CryoTip™, were compared using human blastocysts. One hundred percent of vitrified human pronuclear stage embryos survived and 52% developed to blastocysts as compared with 89% survival and 41% blastocyst development after slow cooling. Similar survival rates were seen with vitrification of 4-cell embryos (98%) as compared with slow cooling (91%). Furthermore, 90% of vitrified blastocysts survived and resulted in a 53% pregnancy rate following transfer, as compared with 84% survival and 51% pregnancy rates following slow cooling. All corresponding values were significantly different. When the closed and open vitrification systems were compared, no difference was found with regard to supporting blastocyst survival (93 and 97% for CryoTip and Cryotop respectively), pregnancies (51 versus 59% respectively) and deliveries (48 versus 51% respectively). Vitrification is a simple, efficient and cost-effective way to improve cumulative pregnancy rates per cycle. The use of the closed CryoTip system eliminates the potential for embryo contamination during cryopreservation and storage without compromising survival and developmental rates *in vitro* and *in vivo*.

Keywords: *blastocyst, cryopreservation, pregnancy, vitrification, zygote*

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 patient (Veeck, 2003; Anderson, 2005). Firstly, it provides an opportunity to limit the numbers of embryos transferred while maximizing the usable embryo per oocyte retrieval cycle ratio at each stimulation attempt, 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.

Successful cryopreservation of human embryos was first reported in 1983 by Trounson and Mohr with multicellular embryos that had been slow-cooled using dimethyl sulphoxide (DMSO). Subsequent modifications of the technique, introducing 1,2 propanediol and sucrose as cryoprotectants (Lassale *et al.*, 1985)

and slow cooling to –30°C prior to plunging into liquid nitrogen, resulted in the introduction of cryopreservation as a standard method offered by virtually every full-service IVF programme world-wide (Anderson *et al.*, 2003).

The primary disadvantages to slow cooling for human embryo cryopreservation are the requirement for an expensive programmable freezing machine and the time-consuming procedure. The introduction of a technique that could be performed without the use of costly equipment and could be completed by one cryopreservation specialist within minutes would provide significant benefits for any busy IVF programme.

Vitrification of embryos and oocytes may offer a solution for this problem. Vitrification can be defined as an extreme elevation in viscosity, i.e. solidification of solutions without ice crystal formation at low temperature (Liebermann *et al.*, 2002a; Fuller and Paynter, 2004; Kasai, 2004; Liebermann and Tucker, 2004). This phenomenon can be induced by either applying an extreme cooling rate or by using high concentrations of cryoprotectant solutions. Methods developed for vitrification in embryology use a combination of these two possibilities (Liebermann *et al.*, 2003). The advantages of vitrification in embryology may be considerable. Oocytes and embryos are sensitive to ice crystal formation; consequently, the elimination of this type of injury may increase their chances for survival. The required high cooling rate can be achieved by simple methods including, for example, direct plunging into liquid nitrogen, thus the need for expensive equipment is eliminated. Additionally, the time required for equilibration and cooling is considerably reduced. On the other hand, disadvantages of vitrification are the required high cryoprotectant concentration, and consequently the increased risk of toxic and osmotic damage, and the need to use special tools permitting high cooling rate by reducing radically the volume of solutions containing the embryos.

Successful vitrification of mammalian embryos was first reported by Rall and Fahy in 1985. Since then, a number of cryoprotectant solutions have been investigated for human use including the use of DMSO, glycerol, ethylene glycol, propanediol and sugars in various combinations (Chen *et al.*, 2000; Mandelbaum, 2000; Shaw *et al.*, 2000; Wright *et al.*, 2004). In addition, numerous carrier systems have been tried, including electron microscope grids, open pulled straws, denuding pipettes, open hemi-straws, and cryoloops (Martino *et al.*, 1996; Arav and Zeron, 1997; Vajta *et al.*, 1998a,b; Lane *et al.*, 1999; Kuleshova and Shaw, 2000; Park *et al.*, 2000; Vanderzwalmen *et al.*, 2000; Vandervorst *et al.*, 2001; Yeoman *et al.*, 2001; Liebermann and Tucker, 2002; Liebermann *et al.*, 2002a,b; Mukaida *et al.*, 2002, 2003; Selman and El-Danasouri, 2002; Vanderzwalment *et al.*, 2002, 2003; Isachenko *et al.*, 2003; Son *et al.*, 2003, 2005; Cremades *et al.*, 2004; Walker *et al.*, 2004). While all of these systems have their advantages and disadvantages, the primary concern for many authorities and scientists is the potential risk of contamination for patients. As the rapid cooling in all these systems requires direct contact of the embryo containing solution and liquid nitrogen, there is a potential risk of disease transmission through contaminated liquid nitrogen during cooling and storage (Bielanski *et al.*, 2000).

The purposes of this study were: (i) to compare the efficacy of vitrification versus traditional slow cooling for cryopreservation of human embryos, and (ii) to investigate the possibility of replacing an earlier open system (Cryotop) with a newly introduced closed method (Cryotip™) to eliminate the potential danger of contamination.

Materials and methods

Patient treatment and embryo culture

Experiments were conducted in patients following informed consent and IRB approval. Patients were stimulated during an IVF cycle by the use of clomiphene citrate (Clomid; Shionogi Co. Ltd, Osaka, Japan). Clomiphene administration (50 mg/day) was initiated on day 3 of the cycle and continued until the rise in LH, caused by the nasal administration of 300 µg of gonadotrophin-releasing hormone agonist (GnRHa) (Suprecur; Mochida Pharmaceutical Co. Ltd, Tokyo, Japan). Human menopausal gonadotrophin (HMG) (Humegon; Organon Co. Ltd, Netherlands) was initiated on day 8 (150 IU) and continued every other day until the administration of GnRHa.

Oocytes were recovered by using a transvaginal ultrasound-guided device (GM07M05V110; Mochida Pharmaceutical). After retrieval, oocytes were cultured in TCM199 medium buffered with 11 mmol/l Hepes, 9 mmol/l Na-Hepes and 5 mmol/l $NaHCO_3$ (referred further as TCM 199) supplemented with 10% Synthetic Serum Substitute (SSS; Irvine Scientific, Santa Ana, CA, USA) in 5% CO_2 in air at 37°C. After 2 h, insemination was performed by intracytoplasmic sperm injection (ICSI). The following day, fertilized oocytes with 2 pronuclei and two polar bodies were transferred into 0.5 ml droplets of Quinn's Advantage Cleavage Medium (Sage BioPharma, USA) supplemented with 10% SSS and cultured further as described above. Embryos that developed beyond the 4-cell stage on day 5 were transferred into 100 µl droplets of blastocyst medium (Irvine Scientific) and cultured under identical conditions to day 6 after ICSI.

Vitrification of embryos

Equilibration, vitrification, thawing, dilution and washing solutions were equivalents to those in Vit Kit (Irvine Scientific). At the time of vitrification embryos were transferred into equilibration solution (ES) consisting of 7.5% (v/v) ethylene glycol and 7.5% (v/v) DMSO dissolved in TCM199 supplemented with 20% SSS at 27°C for 5–15 min. After an initial shrinkage, embryos regained their original volume, and were transferred into three 20 µl drops of vitrification solution (VS) consisting of 15% (v/v) EG and 15%(v/v) DMSO and 0.5 mol/l sucrose dissolved in TCM199 supplemented with 20% SSS, After incubation for 20 s in each drop respectively, embryos were loaded into CryoTips™ (Irvine Scientific) or on Cryotops and plunged into liquid nitrogen.

CryoTip consists of a plastic straw with a thin part (250 µm inner diameter, 20 µm wall thickness and 3 cm length) connected to a thick part (2000 µm inner diameter and 150 µm wall thickness, 4.5 cm length) and equipped with a movable protective metal sleeve (**Figure 1**). Embryos were loaded in approximately 1 µl solution into the narrow part of the CryoTips without any air bubbles by aspiration of medium, embryo and medium, and medium by a connected syringe. Subsequently, the straw was heat-sealed at both ends, the protective sleeve was pulled over the narrow part and the device was plunged into liquid nitrogen. The time required for loading, sealing, adjustment of the sleeve

and plunging did not exceed 90 s.

For warming, CryoTip was removed from liquid nitrogen, plunged into a 37°C water bath for 3 s, wiped with 70% ethanol and a paper towel and the sealed ends were cut with a sterile scissor. By using a syringe adjusted to the thick end of the straw, the contents were expelled onto a sterile Petri dish. Solutions used for further manipulations were kept at 27°C. A 1 µl aliquot of thawing solution (TS) consisting of 1.0 mol/l sucrose in TCM199 plus 20% SSS was placed adjacent to the expelled drop and merged subsequently with the other drop containing the embryos. After 1 min, embryos were retrieved and transferred to a second drop of TS for 1 min, then transferred to two 20 µl drops of dilution solution (DS) consisting of 0.5 mol/l sucrose in TCM199 plus 20% SSS for 2 min each. After three subsequent washings through three successive 20 µl drops of washing solution (WS; TCM199 supplemented with 20% SSS) for 3 min each, embryos were transferred into 100 µl droplets of Blastocyst medium (Irvine Scientific) and cultured under conditions described earlier.

Cryotop (Kitazato Supply Co., Fujinomiya, Japan), consists of a 0.4 mm wide × 20 mm long × 0.1 mm thick polypropylene strips attached to a plastic handle and equipped with a cover straw (**Figure 2**; Kuwayama *et al*., 2005). When using the Cryotops, after equilibration as described above, individual embryos

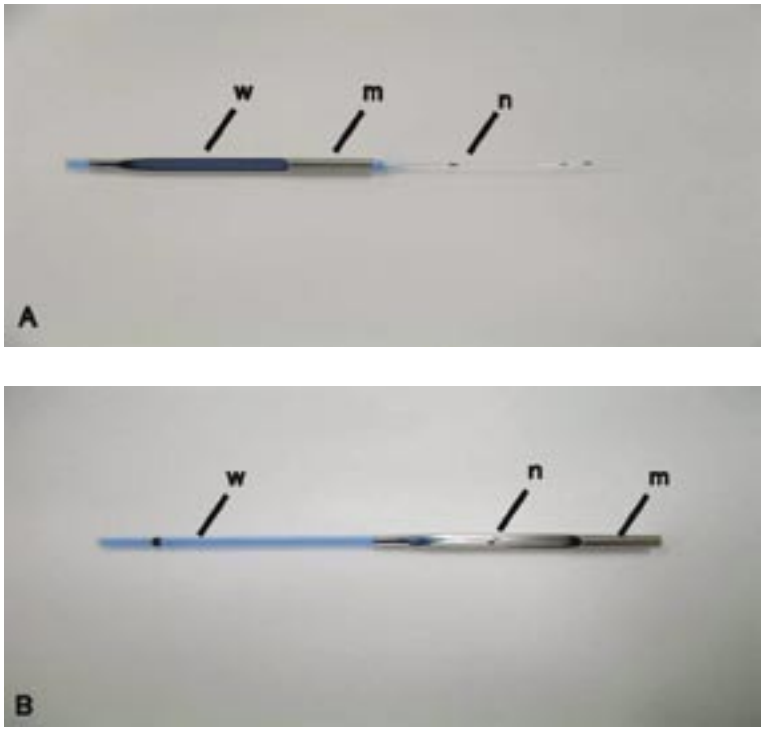

Figure 1. The CryoTip™ vitrification tool. (**A**) For loading, sealing, warming and expelling, the metal sleeve (m) is positioned over the wide part (w) of the straw. (**B**) For safe storage in liquid nitrogen, the sleeve is pulled over the narrow part (n) of the straw.

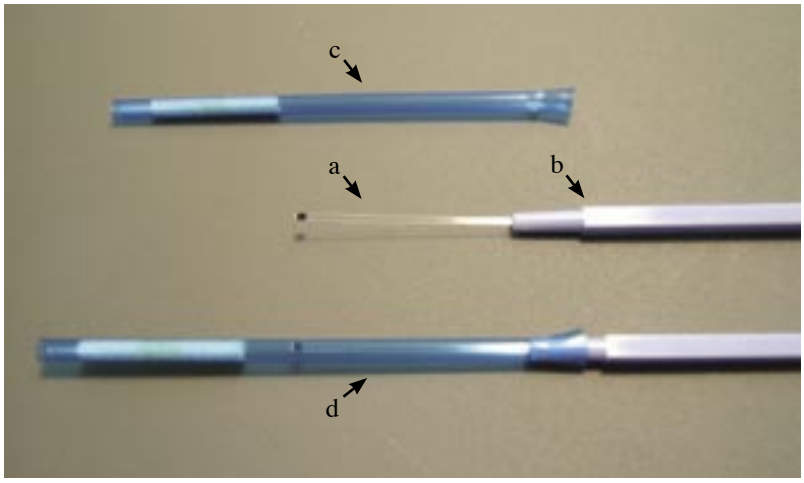

Figure 2. The Cryotop vitrification tool. The polypropylene strip (**a**) is attached to a hard plastic handle (**b**). After vitrification, a hard plastic cover (**c**) is attached to protect the strip during storage in liquid nitrogen (**d**). From: Kuwayama *et al*. (2005), by permission of the Editor.

were picked up in an extremely small volume (<0.1 μl) of VS, placed on the strip, and submerged into filtered liquid nitrogen. Subsequently, under the liquid nitrogen the plastic cover was placed over the strip to provide protecting during storage.

For warming of the Cryotop, the protective cover was removed in liquid nitrogen and the end of the polypropylene strip was immersed directly into 4.5 ml of 37°C TS for 1 min. Embryos were then transferred into 4.5 ml of 37°C DS for 3 min, then washed twice in WS for 5 min each, and cultured as described previously.

Measurement of cooling and warming rates at vitrification

An ultra-fine thermocouple probe (0.1 mm diameter; Chino Ltd, Tokyo, Japan) was introduced into the small droplet on Cryotop or the liquid column of CryoTip. Subsequently, the tools were submerged directly into liquid nitrogen, held briefly inside, and then warmed in a 37°C water bath. Temperature changes were measured with an electronic thermometer (Model EB22005, Chino Ltd). For each method, the time required for the temperature to drop from –20 to –100°C (or the reverse) was measured during cooling and during warming of five replicates each, and the average cooling and warming rates were calculated.

Slow cooling

For comparison with standard methods of cryopreservation, human embryos were frozen in 1.5 mol/l propylene glycol plus 0.1 mol/l sucrose dissolved in Hepes-buffered P1 medium. Embryos were exposed to the cryoprotectant solutions, then aspirated into 0.25 ml straws and cooled to –6°C. After seeding, further cooling was performed in a programmable freezer (Planer Co. Ltd, UK) at 0.3°C/min to –35°C before plunging straws into liquid nitrogen. Frozen straws were thawed rapidly, and propylene glycol was removed by stepwise dilution (Lassale *et al*., 1985).

Evaluation methods

The efficacy of cryopreservation was assessed *in vitro* by survival (i.e. normal response to osmotic changes during the dilution process) and developmental rates (for embryos cryopreserved at 2PN and 4-cell stage, respectively) and *in vivo* after embryo transfer by pregnancy rates and delivery rates. In-vitro development of 2PN stage embryos was assessed on day 6 after ICSI. Embryos that developed to the blastocyst stage were transferred. Surviving cryopreserved and thawed blastocysts were cultured *in vitro* for 3–4 h before transfer.

Embryo transfer and pregnancy detection

All embryo transfers were performed non-surgically with ultrasound guide. Pregnancy was diagnosed by the formation of the gestational sacs by ultrasound observations at 6 weeks after the last menstrual period.

Statistics

Data were analysed by the chi-squared test. *P*-values ≤0.05 were regarded as significant.

Results

In the first series of experiments, the efficiency of vitrification with slow rate freezing was compared by using human embryos at various stages of development. The vitrification procedure used in these trials was the Cryotop method.

The survival and developmental rates for pronuclear-stage human embryos cryopreserved by either slow cooling or vitrification are shown in **Table 1**. Vitrification resulted in higher survival, developmental rates than slow cooling. Vitrification was also more efficient than slow cooling for cryopreservation of 4-cell state embryos and blastocysts (**Tables 2** and **3** respectively). There was no difference in pregnancy and live birth rates between the two cryopreservation methods. On the other hand, pregnancy rates following blastocyst vitrification were higher than pregnancy rates of 4-cell embryos both after slow cooling and vitrification.

In the second series of experiments, the efficiency of the open and closed vitrification system, i.e. the Cryotop and CryoTip method, was compared. As shown in **Table 4**, there were no significant differences between the two with regard to survival, pregnancy rates, implantation rates, or delivery rates.

The average values of the cooling and warming rates were 12,000 and 24,000°C/min with the CryoTip, and 23,000 and 42,000°C/min with the Cryotop method.

Table 1. Survival and development rates of human pronuclear (PN) embryos cryopreserved by either slow cooling or vitrification using the Cryotop method.

	Slow cooling	*Vitrification*
Survived/cryopreserved rate (%)	1730/1944 (89)[a]	5881/5881 (100)[b]
Cleaved/surviving rate (%)	1557/1730 (90)[a]	5469/5881 (93)[b]
Blastocyst/cleaved rate (%)	796/1557 (51)[a]	3058/5469 (56)[b]
Blastocyst/cryopreserved rate (%)	796/1944 (41)[a]	3058/5881 (52)[b]

[a,b]Values within rows with different superscripts are significantly different ($P < 0.01$).

Table 2. Survival and pregnancy rates with human 4-cell embryos cryopreserved by either slow cooling or vitrification using the Cryotop method.

	Slow cooling	*Vitrification*
Survived/cryopreserved rate (%)	857/942 (91)[a]	879/897 (98)[b]
Pregnancy/transfer rate (%)	172/536 (32)[a]	136/504 (27)[a]

[a,b]Values within rows with different superscripts are significantly different ($P < 0.01$).

Table 3. Survival and pregnancy rates with human blastocysts cryopreserved by either slow cooling as compared with vitrification using the Cryotop method.

	Slow cooling	*Vitrification*
Survived/vitrified rate (%)	131/156 (84)[a]	5695/6328 (90)[b]
Number of blastocysts transferred	127	5659
Pregnancy/transfer rate (%)	50/98 (51)[a]	2516/4745 (53)[a]
Live birth/transfer rate (%)	40/98 (41)[a]	2138/4745 (45)[a]

[a,b]Values within rows with different superscripts are significantly different ($P < 0.05$).

Table 4. Survival, pregnancy and delivery rates after single embryo transfer of human blastocysts vitrified with either the Cryotop or the CryoTip method.

	Cryotop	*CryoTip*
Survived/vitrified rate (%)	221/227 (97)	82/88 (93)
Pregnancy/transfer rate (%)	131/221 (59)	42/82 (51)
Delivery/transfer rate (%)	113/221 (51)	39/82 (48)

No significant differences between corresponding values were found.

Discussion

Cryopreservation of embryos is a critical step in maximizing the efficiency of an IVF cycle for patients. Clinical success with cryopreservation seems to be highly variable from laboratory to laboratory, and may depend on many factors including patient age and stimulation protocol, quality of embryos selected for freezing, developmental stage at freezing, media formulation including type of cryoprotectants used, parameters of cooling and warming, and type and quality control of programmable freezing unit employed. The latter problem, however, occurs only at slow cooling, as vitrification does not require sophisticated equipment of questionable reliability at certain makes and types. Variations caused by different media and cryoprotectants can also be minimized by using commercially available kits, recently also produced for vitrification (by, for example, Irvine Scientific, Kitazato). On the other hand, according to previous publications, and also confirmed by the present work, results achieved by vitrification are at least equal or significantly better than those obtained with traditional slow cooling for cryopreservation of mammalian oocytes and embryos including humans (Kuleshowa and Lopata, 2002; Liebermann *et al.*, 2003; Smith and Silva, 2004; Walker *et al.*, 2004; Kuwayama *et al.*, 2005).

Moreover, a considerable number of publications reported normal in-vitro and in-vivo development following human oocyte, early embryo and blastocyst vitrification (Yokota *et al.*, 2000; Jelinkova *et al.*, 2002; Selman *et al.*, 2002; Isachenko *et al.*, 2003, 2004a,b; Katayama *et al.*, 2003; Liebermann *et al.*, 2003b; Son *et al.*, 2003; Huang *et al.*, 2004; Rienzi *et al.*, 2004; Smith *et al.*, 2004; Son *et al.*, 2005), as well as deliveries

of healthy births (El-Danasouri and Selman, 2001; Yokota *et al*., 2001a,b; Vanderzwalmen *et al*., 2002; Mukaida *et al*., 2003; Kuwayama *et al*., 2005). The extensive reports to date exhibit ongoing improvements in vitrification methods, and further support the overall efficacy of vitrification as a viable alternative to cryopreservation by slow cooling methods. The comprehensive results provided in this report further extend the efficacy and safety of vitrification for clinical human use.

However, one of the major unresolved issues associated with the vitrification process is the choice of vessel, vial or straw to hold the embryo during vitrification. As overviewed in the Introduction, numerous types of vessels have been described in the literature. While all of these devices have been shown to work as carriers for embryos during vitrification, a number of concerns have been raised regarding the potential risk to human embryos from exposure to contaminants already present in liquid nitrogen at the time of vitrification, or potentially introduced to the embryos during storage in open containers. While no studies have demonstrated unintentional uptake by a human or mammalian embryo of any pathogen during vitrification or storage, under experimental conditions such contamination may occur (Bielanski *et al*., 2000). Accordingly, a number of governing bodies worldwide have expressed concern about the potential risk. Previous attempts to eliminate this danger included cooling performed in liquid nitrogen filtered through a 0.2 μm pore-size filter and placing the carrier tool into a container that partially or completely isolates it from the liquid nitrogen during storage (Vajta *et al*., 1998; Kuwayama *et al*., 2005).

As proven by the present work, the newly developed CryoTip method may provide a simple and practical solution to the problem. After loading, the plastic straw can be heat-sealed on both ends, and consequently the solution containing the embryo and the liquid nitrogen is hermetical isolated during cooling and storage. On the other hand, although the isolation slightly decreased the rate of cooling, the values that could be obtained with the CryoTip were still high enough to obtain appropriate vitrification. Consequently, embryo survival, as well as in-vitro and in-vivo developmental data, was identical to those achieved by the open Cryotop vitrification system. An additional benefit of the CryoTip vitrification is that any embryologist familiar with the usual method of loading and sealing 0.25 ml cryo-straws will easily accommodate to using the smaller volume, but similar approach in handling, of the CryoTip. Moreover, the protective metal sleeve ensures safe storage of the CryoTip in a relatively small space, and the sleeve as well as the wide part of the plastic tube offers enough space for proper marking of straws for safe identification.

Recently, Isachenko *et al*. (2005) published a method for aseptic vitrification of human zygotes by using open pulled straws (Vajta *et al*., 1998) hermetically isolated from liquid nitrogen before cooling. According to the authors, the considerably decreased cooling rate achievable by this technique did not compromise survival and further development. This observation is in contrast to unpublished observations, and seems to contradict the principles of many previous ultrarapid vitrification methods (Martino *et al*., 1996; Vajta *et al*., 1998; Lane *et al*., 1999; Park *et al*., 2000; Vanderzwalmen *et al*., 2000; Liebermann and Tucker, 2002; Katayama *et al*., 2003; Mukaida *et al*., 2003; Kuwayama *et al*., 2005). Moreover, it may also be questioned if the physical phenomenon of vitrification could be obtained by using the applied cryoprotectant concentrations and the achievable cooling rate in the method of Isachenko *et al*. (2005). Based on these concerns, further comparative investigations performed by independent groups might be required to determine the difference in efficiency and practical value of the two approaches.

In conclusion, the present results, based on more than 16,000 human embryo cryopreservations, prove that vitrification is a simple, inexpensive and safe alternative of traditional slow cooing resulting in higher survival and in-vitro developmental rates for PN, multicellular and blastocyst stage human embryos. Pregnancy and live birth dates did not differ between the two methods. Additionally, the newly developed CryoTip™ technology eliminates the danger of contamination while maintaining the high efficiency of the procedure.

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