Article

Cryotop vitrification of human oocytes results in high survival rate and healthy deliveries

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Abstract

Vitrification, an ultra-rapid cooling technique, offers a new perspective in attempts to develop an optimal cryopreservation procedure for human oocytes and embryos. To further evaluate this method for human oocytes, 796 mature oocytes (metaphase II) were collected from 120 volunteers. Since Italian legislation allows the fertilization of a maximum of only three oocytes per woman, there were 463 supernumerary oocytes; instead of being discarded, they were vitrified. When, in subsequent cycles, these oocytes were utilized, 328 out of 330 (99.4%) oocytes survived the warming procedure. The fertilization rate, pregnancy rate and implantation rate per embryo were 92.9, 32.5 and 13.2% respectively. Thus, as already reported in the literature, the vitrification procedure seems to be highly effective, safe (since healthy babies have been born) and easy to apply. In situations where embryo cryopreservation is not permitted (as in Italy), there is now good indication for routine application of the method, once further standardization is achieved.

Keywords: *cryopreservation, Cryotop, human oocytes, pregnancy rate, survival rate, vitrification*

Introduction

Cryopreservation of human oocytes, embryos and blastocysts has progressed to become a useful tool in human IVF–embryo transfer programmes (Yoon *et al.*, 2003). The first pregnancy from frozen–thawed human embryos was reported in 1983 (Trounson and Mohr, 1983) and the first successful pregnancy resulting in a delivery was reported in 1984 (Zeilmaker *et al.*, 1984). Since then, simplifications and improvements in slow freezing protocols have resulted in a wide application of the method for human embryos at early cleavage stages as well as for blastocysts (Lassalle *et al.*, 1985). The majority of assisted reproduction laboratories are using slow-cooling methods for human conceptuses based on the original work of Testard *et al.* (1986), while most of the human blastocyst freezing protocols have developed from the work of the group led by Menezo *et al.* (1992).

Because of legal, moral and religious problems arising from the cryo-storage of human embryos, scientific efforts have been extended to the cryopreservation of female gametes. Cryo-storage of embryos is forbidden or thoroughly restricted by law in many countries, including Italy, which prohibited it in 2004 (Ragni *et al.*, 2005); oocyte freezing might therefore become a necessity in assisted reproduction procedures in certain countries and a preferred option for many couples. The wide application of this method would not only bypass the above-mentioned restrictions, but would also broaden the range of fertility options for women likely to loose ovarian function prematurely for medical or biological reasons, or to those willing to postpone having a family to a later time (Boldt *et al.*, 2003; Mandelbaum *et al.*, 2004).

There are several unique biological characteristics of human oocytes that might be susceptible to damage during the cryopreservation procedure such as the meiotic spindle, the cytoskeletal elements and the cortical granules (Boiso *et al.*, 2002; Mandelbaum *et al.*, 2004; Koutlaki-Kourti *et al.*, 2006). The exposure time to the cryoprotectant solutions, the concentration of the cryoprotectant, as well as the extra- and

intracellular ice formation are critical factors affecting the viability of the oocyte (Fabbri *et al*., 2001).

The technical difficulties of the method were highlighted in a series of reports published after the first successful attempts and were conducted as early as the mid-1980s; they include a low pregnancy rate and an increased percentage of aneuploidy after gamete exposure to cryoprotectants and the freezing–thawing process (Al-Hasani *et al*., 1987; Paynter, 2000).

Although optimism has been expressed regarding the viability of cryopreservation when performed not only on mature but also on immature oocytes in appropriate circumstances (Chen *et al*., 2004), there is a clear need to improve its effectiveness.

Vitrification, an ultra-rapid cooling technique, offers a new perspective in attempts to develop an optimal cryopreservation procedure for human oocytes and embryos. The method produces a glass-like solidification of cells, completely avoiding intracellular ice crystallization during the cooling and warming process (Kuwayama *et al*., 2005a). Furthermore, the severe cytotoxicity resulting from the high concentration of cryoprotectants required by present techniques can be bypassed, thanks to the introduction of cryoprotectants with higher membrane permeability and lower toxicity, together with an appropriate concentration of non-permeable cryoprotectants (Kuleshova and Lopata, 2002; Katayama *et al*., 2003). This new technique could be introduced without the use of expensive equipment and could be completed by one embryologist within a few minutes, providing significant benefits for any busy IVF programme (Kuwayama *et al*., 2005a).

The purpose of the present study was to assess the efficacy of the protocol published by Kuwayama *et al*. (2005b) for human oocyte vitrification using Cryotops, in the context of further standardization of the method

Materials and methods

Ovarian stimulation, oocyte retrieval and culture protocol

The original group consisted of 251 women (mean age 33.5 ± 3.74 years) seeking pregnancy through IVF, from October 2004 to June 2006, who had agreed that their surplus oocytes (any more than three, as laid down by the current legislation for fertilization) would be cryopreserved utilizing the new vitrification technique described by Kuwayama *et al*. (2005). All women included in the study were informed about the procedure, and written consent was obtained from each.

Ovarian stimulation was achieved using a combination of a gonadotrophin-releasing hormone superagonist analogue (GnRHa) (Decapeptyl; Ipsen, Italy) and menotropins (Menogon human menopausal gonadotrophin, HMG; Ferring, Italy) 75 IU per day, and Gonal F (rFSH; Serono, Italy) 75 IU per day. Follicular growth was monitored by serum 17β-oestradiol measurements and ovarian ultrasonography. Ovulation was induced using 10,000 IU of human chorionic gonadotrophin (HCG; Gonasi HP; Amsa, Italy) when at least three follicles of diameter ≥17 mm were observed and with 17β-oestradiol concentrations corresponding to the number of follicles. Transvaginal ultrasound guided oocyte retrieval was performed 34–36 h later. After retrieval, three of the recovered oocytes were transferred to dishes containing culture medium (MediCult, Italy), and were submitted to the intracytoplasmic sperm injection (ICSI) procedure. A total of 1755 metaphase II (MII) oocytes were recovered and 1029 of them were vitrified.

Out of the 251 women, 71 became pregnant at the first attempt; 60, failed to become pregnant and did not come for a second attempt; and 120 did not become pregnant at the first attempt, expressed the desire to be given a second chance and were therefore included in the present study. Their mean age was 33.3 ± 3.87 years; they sought help because of infertility due to male factor, tubal factor, or because of unexplained infertility. From this group, 463 vitrified mature human oocytes became available for assessment of the vitrification procedure.

For the study group, preparation of the endometrium for the second embryo transfer was performed by the administration of GnRHa (starting from day 21 of the previous cycle) and oestradiol valerate (Schering, Italy) (2 mg, three times a day, starting from day 3 of the next cycle). An endometrial thickness of 8 mm was considered to be optimal for performing an embryo transfer. Progesterone (Prontogest, Amsa) administration (50 mg daily) was started 72 h prior to the embryo transfer.

Cryobanking

Oocyte cryostorage was provided in 2-year-old, number-labelled, liquid nitrogen tanks (Mod.HC35; Taylor-Wharton, Thoeodore, AL, USA) in which samples were immersed. Each tank accommodates 10 canisters (with liquid drain openings), in which a maximum of five patients/canister are allocated.

Since the clinical protocol includes infectivity screening for all patients undergoing IVF (including: Vdrl, ToRCH-related antibodies, HbSAg, HCV, HIV), special liquid nitrogen containers are devoted to storage of specimens from infective subjects, thus preventing any possible contamination of healthy biological material.

Daily checking of liquid nitrogen level was performed using a graduated dipstick introduced to the tank bottom. Manual filling was provided when the nitrogen tank level was below 30 cm. Oocytes involved in the present study were stored for a median time of 8 months (range 3–13 months).

Vitrification/warming protocol

The vitrification/warming protocol was the same as that reported by Kuwayama *et al*. (2005b). For vitrification, the denuded MII oocytes were incubated in equilibration solution (ES) containing 7.5% ethylene glycol (EG) (Sigma-Aldrich, Steinheim, Germany) and 7.5% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Steinheim, Germany)

in Ham's F-10 medium supplemented with 20% serum substitute supplement (SSS; Irvine Scientific, USA) for 5–15 min (according to the time needed for re-expansion of the vitrified material) at room temperature. After initial shrinkage and recovery, they were aspirated and placed into the vitrification solution (VS) (15% EG, 15% DMSO and 0.5 mol/l sucrose) (Merck, Darmstadt, Germany) for <60 s at room temperature. After having observed that cellular shrinkage had taken place, oocytes were aspirated and placed on the tip of the Cryotop (Kitazato, Japan). No more than two oocytes were placed on each Cryotop. Cooling of the oocytes was done by direct contact with the liquid nitrogen.

Samples to be warmed were moved from the tank after their identification (tank number, canister number, patient name on the cane/visotube). The visotube, containing Cryotop carriers, held by metallic grab, was detached from the cane and quickly immersed in a polystyrene box previously filled with liquid nitrogen; with a second grab, the Cryotop was pulled out, the cap was removed and the cryo-carrier was left on the box bottom ready for the thawing procedure. Warming of oocytes was performed by placing the Cryotop in thawing solution (TS) (1 mol/l sucrose) for <60 s at 37°C and then into dilution solution (DS) (0.5 mol/l sucrose) for 3 min, followed by another DS of 0.25 mol/l sucrose for 3 min. The warmed oocytes were placed 4–5 times into washing solution (WS) (Ham's F-10 + 20% serum) and then placed into the incubator.

The ICSI procedure was performed 2 h later for the survived oocytes. The embryo quality was scored according to Steer *et al*. (1992).

Survival evaluation

Morphological comparison of oocytes before vitrification and after thawing was carried out by image recording and archival software (OCTAX Eyeware™) connected to an inverted microscope with Hoffmann modulation contrast (×200 magnification; Nikon Dhiafot 300, Tokyo, Japan) (**Figure 1a,b**).

Warmed oocytes were considered 'morphologically survived' in the absence of negative characteristics (Martino *et al*., 1996; Kathy *et al*., 2000): dark or contracted ooplasm, vacuolization, cracked zona pellucida, polar body alteration or loss of cytoplasm portion (**Figure 2a,b**).

Statistical analysis

A comparison between fresh and vitrified/warmed groups in terms of fertilization, cleavage, pregnancy and abortion rates was performed. Proportions were compared by the binomial test. *P*-values less than or equal to 0.05 were regarded as significant. The 95% confidence interval (CI) was calculated by the Wilson method.

Results

Results obtained from fresh and vitrified/warmed groups are shown in **Table 1**.

Outcome from fresh oocytes

From a total of 1755 MII recovered oocytes, 726 were inseminated. The fertilization and cleavage rates were respectively 96.7 (702/726) and 97.6% (685/702).

Out of the original 251 patients, a total of 248 received embryo transfers, with an average of 2.8 embryos transferred per patient. Three patients did not receive embryo transfers because of fertilization failure and/or cleavage arrest. There were 71 clinical pregnancies (implantation rate of 10.3% per embryo transferred, pregnancy rate of 28.6%) of which 13 aborted and 58 delivered.

Outcome from vitrified–warmed oocytes

From a total of 463 MII oocytes vitrified, 330 were warmed for a second attempt (133 are still frozen). Of these, 328 survived the vitrification/warming process, representing a survival rate of 99.4% (95% CI 97.8–99.8). Of the survived oocytes, 305 showed evidence of fertilization (2PN) 18 h after the ICSI procedure, giving a fertilization rate of 93.0% (95% CI 89.7–95.3). After 24 h in culture, 295 zygotes divided to a 2-4 cell stage embryo, with a cleavage rate of 96.7% (95% CI 94.1–98.2). At 72 h after warming, all 295 embryos (6–8 cells) were replaced in the 120 patients returning for their second attempt (2.45 embryos/transfer). A positive HCG assay was obtained in 39 patients at 14 days post-embryo transfer; in all these subjects, 5 weeks later, a fetal heartbeat was detected, giving a pregnancy rate of 32.5% (95% CI 24.8–41.3) and an implantation rate per embryo of 13.2% (95% CI 9.8–17.6). Subsequently, eight of these 39 patients (20.5%; 95% CI 10.8–35.5) had spontaneous abortions, whereas the remaining 28 have an ongoing pregnancy. At the time of writing, three healthy babies have been born (in October 2005, January 2006 and June 2006).

Discussion

Oocyte cryopreservation with the new Cryotop vitrification technique resulted in high post-warming survival, fertilization, embryo development and pregnancy rates. Although properly designed, comparative studies are still required to determine which cryopreservation method for human oocytes is the most effective, it can now be stated that oocyte freezing can be performed on a routine basis (Fabbri *et al*., 2001).

Porcu *et al*. (1999) were the first to freeze mature MII oocytes by the slow-cooling method, with apparently good results: 54.1% survival, 57.7% fertilization and 91.2% cleavage rates. Sixteen pregnancies were achieved and nine of them resulted in the delivery of 11 healthy newborns. Fabbri *et al*. (2001) reported an 82% survival rate (183 out of 224 MII oocytes); Fosas *et al*. (2003), by adopting the same protocol, obtained 90% survival (79 out of 88 MII oocytes), 73% fertilization and 57% pregnancy rates. Boldt *et al*. (2003), using a Na^+-depleted culture medium for freezing and thawing, reported a 74.4% survival rate, 36.4% pregnancy rate and 15.6% implantation rate per transfer, obtaining with frozen oocytes similar results to those previously shown when thawing embryos: 33.3% pregnancy rate and 14% implantation rate, thus suggesting that oocyte cryopreservation can be an effective alternative to embryo freezing. Recently, the

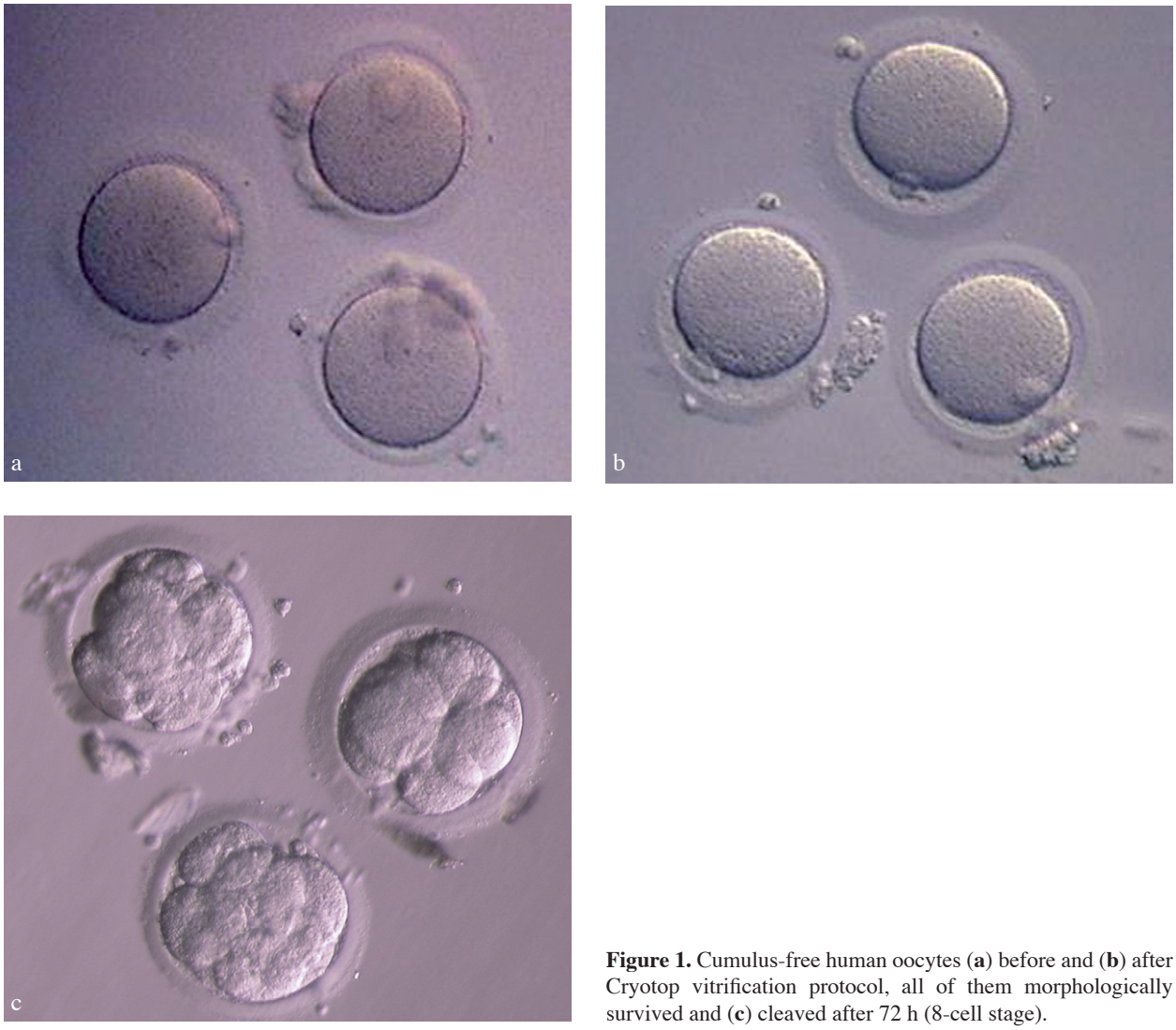

Figure 1. Cumulus-free human oocytes (**a**) before and (**b**) after Cryotop vitrification protocol, all of them morphologically survived and (**c**) cleaved after 72 h (8-cell stage).

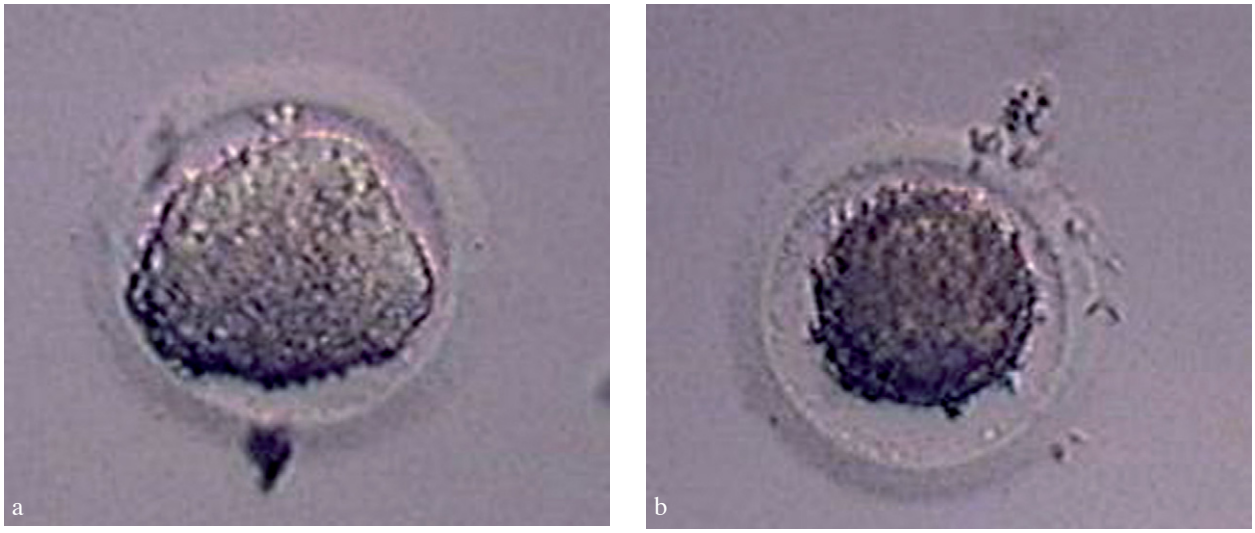

Figure 2. (**a**) and (**b**) Damaged human oocytes after warming.

Table 1. Comparison of survival, fertilization and pregnancy rates arising from non-vitrified oocytes and vitrified/warmed oocytes.

	Non-vitrified group	*Vitrified/warmed group*
No. of cycles	251	120
No. of recovered oocytes	1755	796
No. of vitrified oocytes	1029	463
No. of warmed oocytes	–	330
No. of surviving oocytes (%)	–	328 (99.4)
No. of injected oocytes	726	328
No. of fertilized oocytes (2PN) (%)	702 (96.7)[a]	305 (93.0)[a]
No. of cleaved oocytes (%)	685 (97.6)[b]	295 (96.7)[b]
No. of transfers	248	120
No. of transferred embryos	685	295
No. of embryos per transfer	2.8[c]	2.45[c]
No. of clinical pregnancies (%)	71 (28.6)[d]	39 (32.5)[d]
No. of ongoing pregnancies	–	28
No. of abortions (%)	13 (18.3)[e]	8 (20.5)[e]
No. of deliveries	58	3
IR per transferred embryo[g] %	10.3[f]	13.2[f]
IR per thawed oocyte[h] %	–	11.8

IR = implantation rate; PN = pronucleate.
[a]$P = 0.01$.
[b-f]$P > 0.05$ (not significant).
[g]No. of clinical pregnancies/no. of transferred embryos.
[h]No. of clinical pregnancies/no. of thawed oocytes.

same group, carrying on their previous study, showed a 60.4% survival rate, 62.0% fertilization rate and 13.3% implantation rate/embryos transferred (Boldt *et al.*, 2006). To date, although numerous studies have been conducted utilizing different slow-cooling procedures, results still remain contradictory: the figures showed by Borini *et al.* (2006) (43.4% survival rate; 19.2% pregnancy rate) considerably differ to those recently reported by Stachecki *et al.* (2006) who, adopting a choline based freezing solution, achieved a post-thawing oocyte survival rate up to 90%. These data suggest that the best slow freezing procedure has not yet been established, thus requiring further investigation.

Over the last few years, it has been suggested that the vitrification method may be a viable alternative to slow-cooling procedures (Kuleshova *et al.*, 1999; Kuleshova and Lopata, 2002; Yoon *et al.*, 2003), although a critical concentration of cryoprotectant is required for this process. Indeed, the high concentration of cryoprotectant used for vitrification and the known biological and physicochemical effects of cryoprotectants make the toxicity of these agents a key limiting factor in cryobiology (Yoon *et al.*, 2000). Therefore, a balance between maximizing the cooling rate and minimizing the cryoprotective concentration is important (Liebermann and Tucker, 2002; Liebermann *et al.*, 2002).

Reduction of cryoprotectant toxicity can be achieved by adding sugars (sucrose, glucose, fructose, sorbitol, saccharose, trehalose or raffinose) to the vitrifying solution, to withdraw water from the cells before cooling and so decrease the total concentration of penetrating cryoprotectant required to achieve the vitrification (Kuleshova *et al.*, 1999; Kuwayama *et al.*, 2000; Liebermann and Tucker, 2002; Lieberman *et al.*, 2002; Katayama *et al.*, 2003; Wright *et al.*, 2004).

In addition, the choice of cryoprotectant is strongly related to the success of vitrification procedures. Ethylene glycol (EG) has been widely used during the vitrification of human oocytes and embryos, due to its low toxicity and high permeability (Kuleshova *et al.*, 1999; Yoon *et al.*, 2000, 2003), while dimethylsulphoxide (DMSO) may cause spindle polymerization with an increased risk for polyploidy (Glenister *et al.*, 1987). It has also been reported that cryoprotectant mixtures might produce better results than solutions containing one permeable cryoprotectant (Vajta *et al.*, 1998; Mukaida *et al.*, 2003; Chian *et al.*, 2004; Isachenko *et al.*, 2006).

Numerous carriers have been described in the literature with the aim of maximising cooling rates by reducing volume of the vitrification solution. These carriers include the open pulled straw (OPS; Vajta *et al.*, 1998; Chen *et al.*, 2000a,b; Hurtt *et al.*, 2000; Oberstein *et al.*, 2001), the Flexipet-denuding pipette (FDP; Liebermann and Tucker, 2002; Liebermann *et al.*, 2002), microdrops (Papis *et al.*, 2000), electron microscope copper grids (EM; Hong *et al.*, 1999; Chung *et al.*, 2000; Park *et al.*, 2000), and Cryotop (Katayama *et al.*, 2003, Kuwayama *et al.*, 2005). All of these are designed to be directly in contact with liquid nitrogen, raising the concern of a potential risk from

direct exposure to viral contaminants (Bielanski *et al.,* 2000). Attempts to eliminate any conceivable contamination were made by introducing special carriers capable of isolating the vitrified cells from direct contact with liquid nitrogen (Vajta *et al*., 1998; Kuwayama *et al*., 2005; Isachenko *et al.,* 2005).

Since the first pregnancy achieved with a vitrified/warmed human oocyte (Hong *et al*., 1999) and the first birth of a healthy baby (Yoon *et al*., 2000), vitrification results have improved significantly. Yoon *et al*. (2003) studied 474 vitrified/thawed human oocytes and reported 68.6% survival, 71.7% fertilization, 95% 2PN embryo cleavage rates and a 21.4% pregnancy rate per transfer. Cai *et al*. (2005) reported survival rates ranging between 79.1 and 82.3%, in a total of 2070 vitrified rabbit oocytes, using three different vitrification protocols. Recently, Oktay *et al*. (2006) compared the results obtained with different vitrification protocols applied before and after June 2005. In these two periods, fertilization, pregnancy and implantation rates increased from 70.6, 29.4 and 8.8% to 75.4, 51 and 20.5%, respectively.

The results obtained by vitrification with mixtures of cryoprotectants together with Cryotop cryo-carrier appear to be extremely encouraging. Chian *et al*. (2004) attained a 100% survival rate for both mature and immature human oocytes, by using a mixture including 15% EG + 15% PROH + 0.5 mmol/l sucrose. Kuwayama *et al*. (2005), using different proportions (7.5% EG + 7.5% DMSO + 0.5 mmol/l sucrose; Cryotop) also achieved a 100% survival rate, 93% cleavage rate, and 56% blastocyst formation rate with 5880 vitrified/warmed zygotes. Finally, Lucena *et al*. (2006), using 15% EG + 15% DMSO + 0.5 mmol/l sucrose and Cryotop, vitrified 159 oocytes and reported 96.7% survival, 89.2% viability, 87.2% fertilization and 94.3% cleavage rates.

Using the same protocol applied by Kuwayama *et al*. (2005b), on 330 vitrified/warmed MII oocytes, 99.4% (328/330) survival, 93.0% (305/328) fertilization and 96.7% (295/305) cleavage rates were achieved in this study. The high post-warming survival rate, even if evaluated only by morphological parameters, is confirmed by the total number of transferred embryos, pregnancies obtained and healthy deliveries that occurred. In any case, a small number of morphologically normal surviving oocytes still remained but with hidden inner damage resulting in a significant decrease of the fertilization rate compared with previous fresh cycles (92.9 versus 96.6%, $P = 0.01$). Cleavage, pregnancy and abortion rates between the non-vitrified and vitrified/warmed groups were not significantly different.

As to the possibility of repeating this protocol, the variability due to this manual procedure compared with a mechanical one must be carefully considered. The clinical application required a 5-month training period. A big part of it was devoted to avoiding damage to the oocytes immediately before plunging them into liquid nitrogen. Some accidents could arise during these early procedure steps: oocyte loss into the Petri dish, complete cell damage while pipetting or in cases of incorrect positioning of the oocytes on the Cryotop tip. Thus, it is clear that operator skill is crucial to guarantee the efficiency of this protocol. As to the possible contamination risk of cryo-stored samples by infectious liquid nitrogen or specimens, currently there is no evidence of infection transmission in patients involved in the present trial.

The results of oocyte vitrification published to date indicate that this procedure may be significantly better than traditional slow freezing methods (Kuleshova and Lopata, 2002; Kuwayama *et al*., 2005). In comparing the principles, procedures and results of slow cooling and vitrification protocols, Kuleshova and Lopata (2002) state that both methods resulted in the successful cryopreservation of human oocytes and embryos, but the slow cooling gives much lower success rates. Some years before, Kuleshova *et al*. (1999) highlighted the low pregnancy rate per thawed oocyte of this method, ranging from 1 and 2% (Porcu *et al*., 1998b; Tucker *et al*., 1998). Although in the last few years, slow freezing protocols have shown an increase in the survival rate even up to 90% (Stachecki *et al*., 2006), pregnancy rate per thawed oocyte (Borini *et al*., 2006 2.3% 16/705; Boldt *et al*., 2006 15/361 4.2%) remains poor, appearing to be much lower in comparison with vitrification (Kuwayama *et al*., 2005b: 11.2%, 12/107; Lucena *et al*., 2006: 8.2%, 13/159). These data are widely confirmed by the present study with 11.8% pregnancy rate per thawed oocyte (39/330; 95% CI 8.8–15.7).

Boldt *et al*. (2006) suggested that more oocytes should be thawed and fertilized, to increase the data on freezing outcome. This strategy appears to be unfeasible in Italy where reliable and highly effective protocols are required in order to avoid strict limitations imposed by the new legislation (Benagiano and Gianaroli, 2004).

In conclusion, the results confirm those recently reported by other authors, thus indicating that vitrification is a rapid, inexpensive and easily performed technique, which preserves the biological integrity of oocytes, resulting in high survival and pregnancy rates. In addition, to date, when considering the births of healthy babies from human oocytes fertilized after vitrification, the procedure seems to be safe.

The results obtained with the new technique are particularly important in Italy, where embryo cryopreservation is illegal and the only option is oocyte cryopreservation. Nevertheless, there is still a need for large-scale comparative studies of the vitrification procedure in humans before any final judgment can be made.

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References

Al-Hasani S, Diedrich K, van der Ven H *et al*. 1987 Cryopreservation of human oocytes. *Human Reproduction* **2**, 695–700.

Benagiano G, Gianaroli L 2004 The new Italian IVF legislation. *Reproductive BioMedicine Online* **9**, 117–125.

Bielanski A, Nadin-Davis S, Sappi T *et al*. 2000 Viral contamination of embryos cryopreserved in liquid nitrogen. *Cryobiology* **40**, 110–116.

Boiso I, Marti M, Santal J *et al*. 2002 A confocal microscopy analysis of the spindle and chromosome configurations of human oocytes cryopreserved at the germinal vesicle and metaphase II stage. *Human Reproduction* **17**, 1885–1891.

Boldt J, Tidswell N, Sayers A *et al*. 2006 Human oocyte cryopreservation: 5-year experience with a sodium-depleted slow freezing method. *Reproductive BioMedicine Online* **13**, 96–100.

Boldt J, Cline D, McLaughlin D 2003 Human oocyte cryopreservation as an adjunct to IVF-embryo transfer cycles. *Human Reproduction* **18**, 1250–1255.

Borini A, Lagalla C, Bonu M A et *al*. 2006 Cumulative pregnancy rates resulting from the use of fresh and frozen oocytes: 7 years' experience. *Reproductive BioMedicine Online* **12**, 481–486.

Cai XY, Chen GA, Lian Y *et al*. 2005 Cryoloop vitrification of rabbit oocytes. *Human Reproduction* **20**, 1969–1974.

Chen ZJ, Li M, Li Y, *et al*. 2004 Effects of sucrose concentration on the developmental potential of human frozen–thawed oocytes at different stages of maturity. *Human Reproduction* **19**, 2345–2349.

Chen SU, Lien YR, Chen HF *et al*. 2000a Open pulled straws for vitrification of mature mouse oocytes preserve patterns of meiotic spindles and chromosomes better than conventional straws. *Human Reproduction* **15**, 2598–2603.

Chen SU, Lien YR, Chao KH *et al*. 2000b Cryopreservation of mature human oocytes by vitrification with ethylene glycol in straws. *Fertility and Sterility* **74**, 804–808.

Chian RC, Kuwayama M, Tan L *et al*. 2004 High survival rate of bovine oocytes matured in vitro following vitrification. *Journal of Reproduction and Development* **50**, 685–696.

Chung HM, Hong SW, Lim JM *et al*. 2000 In vitro blastocyst formation of human oocytes obtained from unstimulated and stimulated cycles after vitrification at various maturational stages. *Fertility and Sterility* **73**, 545–551.

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 Reproduction* **18**, 1417–1421.

Glenister PH, Wood MJ, Kirby C, Whittingham DG 1987 Incidence of chromosome anomalies in first cleavage mouse embryos obtained from frozen thawed oocytes fertilized in vitro. *Gamete Research* **16**, 205–216.

Hong SW, Chung HM, Lim JM *et al*. 1999 Improved human oocytes development after vitrification: a comparison of thawing methods. *Fertility and Sterility* **72**, 142–146.

Hurtt AE, Landim-Alvarenga F, Seidel GE Jr, Squires EL 2000 Vitrification of immature and mature equine and bovine oocytes in an ethylene glycol, ficoll and sucrose solution using open-pulled straws. *Theriogenology* **54**, 119–128.

Isachenko V, Montag M, Isachenko E *et al*. 2006 Aseptic vitrification of human germinal vesicle oocytes using dimethyl sulfoxide as a cryoprotectant. *Fertility and Sterility* **85**, 741–747.

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

Katayama KP, Stehlik J, Kuwayama M *et al*. 2003 High survival rate of vitrified human oocytes results in clinical pregnancy. *Fertility and Sterility* **80**, 223–224.

Kathy L, Sharpe-Timms, Randall L. Zimmer 2000 Oocyte and Pre-Embryo Classification. In: Keel BA, May JV, De Jonge CJ (eds) *Handbook of the Assisted Reproduction Laboratory*. CRC Press LLC, USA, pp. 179–196.

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.

Kuleshova L, Gianaroli L, Magli C *et al*. 1999 Birth following vitrification of a small number of human oocytes: case report. *Human Reproduction* **14**, 3077–3079.

Kuwayama M, Kato O 2000 Successful vitrification of human oocytes *Fertility and Sterility* **74** (suppl. 3) S49, Abstract 0–127.

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

Kuwayama M, Vajta G, Kato O, Leibo SP 2005b Highly efficient vitrification method for cryopreservation of human oocytes. *Reproductive BioMedicine Online* **3**, 300–308.

Lasalle B, Testard J, Renard JP 1985 Human embryo features that influence the success of cryopresevation with the use of 1,2 propanediol. *Fertility and Sterility* **44**, 645–651.

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 J, Tucker MJ, Graham J *et al*. 2002 Blastocyst development after vitrification of multipronuclear zygotes using the flexipet denuding pipette. *Reproductive BioMedicine Online* **4**, 146–150.

Lucena E, Bernal DP, Lucena C *et al*. 2006 Successful ongoing pregnancies after vitrification of oocytes. *Fertility and Sterility* **85**, 108–111.

Mandelbaum J, Anastasiou O, Levy R *et al*. 2004 Effects of cryopreservation on the meiotic spindle of human oocytes. *European Journal of Obstetrics and Gynecology and Reproductive Biology* **113S**, 17–23.

Martino A, Songsasen N, Leibo SP 1996 Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. *Biology of Reproduction* **54**, 1059–1069.

Menezo YJ, Nicollet B, Herbaut N, Andre D 1992 Freezing co-cultured human blastocysts. *Fertility and Sterility* **58**, 977–980.

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.

Oberstein N, O'Donovan MK, Bruemmer JE *et al*. 2001 Cryopreservation of equine embryos by open pulled straws, cryoloop, or conventional cooling methods *Theriogenology* **15**, 607–613.

Oktay K, Pelin Cil A, Bang H 2006 Efficiency of oocyte cryopreservation: a meta-analysis *Fertility and Sterility* **86**, 70-80.

Papis K, Shimizu M, Izaike Y 2000 Factors affecting the survivability of bovine oocytes vitrified in droplets. *Theriogenology* **15**, 651–658.

Park SP, Kim EY, Oh JH *et al*. 2000 Ultra-rapid freezing of human multipronuclear zygotes using electron microscope grids. *Human Reproduction* **15,** 1787–1790.

Paynter SJ 2000 Current status of the cryopreservation of human unfertilized oocytes. *Human Reproduction Update* **6**, 449–456.

Porcu E, Fabbri R, Giotti PM *et al*. 1999 Ongoing pregnancy after intracytoplasmic injection of testicular spermatozoa into cryopreserved human oocytes. *American Journal of Obstetrics and Gynecology* **180**, 1044–1045.

Ragni G, Allegra A, Anserini P *et al*. 2005 The 2004 Italian legislation regulating assisted reproduction technology: a multicentre survey on the results of IVF cycles. *Human Reproduction* **20**, 2224–2228.

Stachecki JJ, Cohen J, Garrisi J *et al*. 2006 Cryopreservation of unfertilized human oocytes. *Reproductive BioMedicine Online* **13**, 222–227.

Steer R, CJ Mills, SL Tan *et al*. 1992 The cumulative embryo score: a predictive embryo scoring technique to select the optimal number of embryos to transfer in an in-vitro fertilization and embryo transfer programme. *Human Reproduction* **7**, 117–119.

Testard J, Lassalle B, Belaisch-Allart J *et al*. 1986 High pregnancy rate after early human embryo freezing. *Fertility and Sterility* **46**, 268–272.

Trounson A, Mohr L 1983 Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature* **305**, 707–709.

Vajta G, Holm P, Kuwayama M *et al*. 1998 Open pulled straw (OPS) vitrification: a new way to reduce cryo-injuries of bovine

ova and embryos. *Molecular Reproduction and Development* **51**, 53–58.

Wright DL, Eroglu A, Toner M, Toth TL 2004 Use of sugars in cryopreserving human oocytes. *Reproductive BioMedicine Online* **9**, 179–186.

Yoon TK, Kim TJ, Park SE *et al*. 2003 Live birth after vitrification of oocytes in a stimulated in vitro fertilization embryo-transfer program. *Fertility and Sterility* **79**, 1323–1326.

Yoon TK, Chung HM, Lim JM *et al*. 2000 Pregnancy and delivery of healthy infants developed from vitrified oocytes in a stimulated in vitro fertilization embryo-transfer program. *Fertility and Sterility* **74**, 180–181.

Zeilmaker GH, Alberta AT, van Gent I 1984 Two pregnancies following transfer of intact frozen–thawed embryos. *Fertility and Sterility* **42**, 293–296.

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