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Embryo development of fresh 'versus' vitrified metaphase II oocytes after ICSI: a prospective randomized sibling-oocyte study

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BACKGROUND: A successful oocyte cryopreservation programme is of utmost importance where a limited number of oocytes can be inseminated per cycle, to overcome legal and ethical issues related to embryo storage, for oocyte donation programmes and for fertility preservation (especially for cancer patients). Vitrification has been recently proposed as an effective procedure for this purpose.

METHODS: In order to validate the effectiveness of oocyte vitrification a non-inferiority trial was started on sibling metaphase II (MII) oocytes. To demonstrate the non-inferiority based on an absolute difference of 17% in the fertilization rate per sibling oocyte, a minimum of 222 oocytes were required. After oocyte denudation, MII oocytes with normal morphology were randomly allocated to fresh ICSI insemination or to vitrification procedure. If pregnancy was not obtained a subsequent ICSI cycle was performed with warmed oocytes of the same cohort. In both groups, three oocytes were inseminated per cycle by ICSI procedure. Primary end-points were fertilization rates calculated per warmed and per injected oocytes. Secondary end-points were zygote and embryo morphology.

RESULTS: A total of 244 oocytes were involved in this study. Of the 120 fresh sibling oocytes inseminated, 100 were fertilized (83.3%). Survival rate of sibling vitrified oocytes was 96.8% (120/124 oocytes). Fertilization rate after ICSI was 76.6% (95/124) per warmed oocyte and 79.2% (95/120) per survived/inseminated oocyte. No statistical difference in fertilization rates was observed between the two groups when calculated per sibling oocytes (absolute difference −6.73%; OR: 0.65; 95% CI = 0.33–1.29; $P = 0.20$) and per inseminated oocyte (absolute difference −4.17%; OR: 0.76; 95% CI = 0.37–1.53; $P = 0.50$). Embryo development was also similar in both treatment groups up till Day 2. The percentage of excellent quality embryos was 52.0% (52/100) in the fresh group and 51.6% (49/95) in the vitrification group (absolute difference −0.43%; OR: 0.98; 95% CI = 0.53–1.79; $P = 0.9$). The mean age of the 40 patients included in this study was 35.5 ± 4.8 years (range 26–42). Fifteen clinical pregnancies were obtained in the vitrification cycles of 39 embryo transfers performed (37.5% per cycle, 38.5% per embryo transfer), with an implantation rate of 20.2% (19/94). Three spontaneous miscarriages occurred (20%). Twelve pregnancies are ongoing (30.0% per cycle, 30.8% per embryo transfer) beyond 12 weeks of gestation.

CONCLUSIONS: Our results indicate that oocyte vitrification procedure followed by ICSI is not inferior to fresh insemination procedure, with regard to fertilization and embryo developmental rates. Moreover, ongoing clinical pregnancy is compatible with this procedure, even with a restricted number of oocytes available for insemination. The promising clinical results obtained, in a population of infertile patients, need to be confirmed on a larger scale.

Clinical Trials Registration number: iSRCTN60158641.

Key words: oocyte / cryopreservation / vitrification / ICSI / sibling oocyte study

Introduction

In Italy from March 2004 to May 2009, according to the Italian Law No. 40 that regulates assisted reproductive technology (ART), no more than three oocytes could be inseminated per cycle, all obtained embryos had to be transferred, and embryo cryopreservation and gamete donation were forbidden. Thus, no embryo selection, based on morphological and/or genetic evaluation, was possible (Benagiano

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and Gianaroli, 2004). As an alternative, in accordance with the legal restrictions imposed, oocyte selection (Chamayou *et al*., 2006; Rienzi *et al*., 2008) and cryopreservation (Borini *et al*., 2006a, b, 2007; La Sala *et al*., 2006; Levi Setti, 2006; Chamayou *et al*., 2006; De Santis *et al*., 2007; Parmegiani *et al*., 2008, 2009a,b; Magli *et al*., 2009) have been introduced as routine practice in Italian IVF centres. A recent edict of the Italian Constitutional Court (151/2009) declared that patient health is paramount, and thus embryo freezing is constitutional, if determined by the health of the patient. To this end, many changes have been introduced in the Italian IVF centres. In particular, to guarantee the best treatment for patients, the number of oocytes to be inseminated is now chosen by the physician (and not by the Parliament) according to the individual situation. It must however be underlined that the application of oocyte cryopreservation is not only limited to the Italian situation; this technique is also of paramount importance for fertility preservation (especially in the case of cancer patients), for oocyte donation programmes, and to help overcome ethical issues related to embryo cryopreservation.

Oocyte cryopreservation, however, still represents a great challenge in ART (Gardner *et al*., 2007). The difficulties associated with oocyte cryopreservation are mostly related to the special structure and sensitivity of this cell. Differences observed in plasma membrane permeability to water and cryoprotectants, compared with later stages of development, makes oocyte survival rate very low with standard slow freezing protocols (Agca *et al*., 1998; Ford *et al*., 2000; Van den Abbeel *et al*., 2007). The extreme sensitivity of the meiotic spindle to temperature variation and to oocyte dehydration/rehydration (Chen *et al*., 2004; Rienzi *et al*., 2004; Bianchi *et al*., 2005; Coticchio *et al*., 2005; De Santis *et al*., 2007; Larman *et al*., 2007; Cobo *et al*., 2008a; Chen and Yang, 2009) may compromise subsequent chromosomal segregation. Precocious oocyte activation induced by cryoprotectants exposures may disturb future development (Larman *et al*., 2006; Gardner *et al*., 2007). Moreover, increased risk of oocyte ageing is reported with cryopreservation procedure (Parmegiani *et al*., 2008, 2009ab). Oocyte physiology seems also to be severely affected by cryopreservation (Gardner *et al*., 2007).

Novel approaches to slow freezing have been introduced (Fabbri *et al*., 2001; Borini *et al*., 2006a, b, 2007; Boldt *et al*., 2006) with improved oocyte survival rate and subsequent embryo development. Although these studies clearly describe a contribution of oocyte cryopreservation to the overall clinical success rate, they also show the limitations of the technique in terms of implantation rate per thawed oocyte. Very recently, reduced oocyte competence to development was reported in frozen cycles when compared with sibling fresh cycles (Magli *et al*., 2009), confirming the negative impact of slow-freezing procedure on oocyte potentiality. It is realistic to expect that cryopreservation in general (also of later stages of development) is less effective than fresh treatment, but the impact of the technique should not, in our opinion, exceed a certain range to justify its application in routine work.

As an alternative to slow freezing, vitrification procedure has been suggested for oocyte (and embryo) cryopreservation by different investigators (for review see Vajta and Nagy, 2006). The recent improvements in this approach, including increased cooling and warming rates using very small volumes and decreasing toxicity by reducing cryoprotectants concentrations, have allowed very high results in terms of oocyte survival, fertilization, embryo development rates and clinical outcomes (Kuleshova *et al*., 1999; Kuwayama *et al*., 2005; Lucena *et al*., 2006; Selman *et al*., 2006; Antinori *et al*., 2007; Kuwayama 2007; Yoon *et al*., 2007; Cobo *et al*. 2008b, c, Chian *et al*., 2008; Sher *et al*., 2008; Kim *et al*., 2009; Nagy *et al*., 2009). Moreover, when vitrified oocytes are compared simultaneously with fresh counterparts, in an oocyte donation programme, similar laboratory results are observed in terms of fertilization (76.3 and 82.2%, respectively), embryo development and blastocyst formation rates (48.7 and 47.5%, respectively) (Cobo *et al*., 2008b).

The difference in efficacy between slow freezing and vitrification procedures may be related to the fact that vitrification has a lower impact on oocyte physiology as compared with slow freezing (Gardner *et al*., 2007). A second aspect must be considered. Most of the published studies dealing with the oocyte vitrification procedure are performed in oocyte donation programmes or in patients with an extremely good prognosis (Kuwayama *et al*., 2005; Lucena *et al*., 2006; Yoon *et al*. 2007; Cobo *et al*., 2008b, c). Although oocyte competence with the vitrification procedure appears to have improved, further studies are needed (designed in a prospective way) to determine the efficacy of this approach in the population of infertile patients. The origin of the oocytes involved is, in fact, clearly a determining factor in the results, with an obvious advantage of young donors compared with older infertile women (Kim *et al*., 2009).

In order to validate the effectiveness of a vitrification approach for oocyte cryopreservation a prospective comparison was thus designed in our population of infertile patients. This study was set-up as a non-inferiority trial with a prospective target of 240 sibling metaphase II (MII) oocytes obtained from an estimated 40 ICSI patients (assuming that three oocytes per patient are inseminated in the fresh and in the vitrified groups). Oocyte fertilization rates after ICSI (per warmed oocyte and per injected oocyte) were evaluated as primary outcomes. Secondary outcomes were pronuclear morphology and embryo development.

Materials and Methods

Study design and outcomes measures

A randomized sibling-oocyte trial was started to evaluate the effectiveness of oocyte vitrification when compared with fresh oocyte insemination in terms of fertilization rate per warmed and per injected oocyte. Randomization was performed at the moment of oocyte denudation by a different person from the one who performed the procedure. To each normal-appearing oocyte at MII stage a number was assigned by a computer-generated random list (www.random.org). Oocyte numbers 1, 2 and 3 were assigned to fresh ICSI procedure while the others to vitrification procedure. The warming procedure was performed by using the subsequent oocytes (starting from numbers 4, 5 and 6 and including other oocytes if necessary). Only the first warmed attempt was included in the study. By using a paired number of transfers from each patient, where the same number of sibling oocytes was used for insemination, differences in patient characteristics such as female age duration and origin of infertility, rank of trial were eliminated.

The primary efficacy end-points were non-inferiority in fertilization rates calculated per warmed and per injected oocyte. Secondary efficacy measures included pronuclear morphology and embryo development. In addition, patient's baseline characteristics and clinical outcomes were documented.

Target population

Between 2 September 2008 and 10 March 2009 consecutive patients not older than 42 years of age, presenting more than six normal-appearing MII oocytes and undergoing ICSI treatment with ejaculated sperm in the Centre for Reproductive Medicine GENERA in Rome, were considered for inclusion. To exclude potential negative paternal effect on embryo development, surgically extracted spermatozoa and very severe oligoastenoteratozoospermia (motile sperm count $<$500.000/ml after preparation) were not included. Patients enrolled in our polar body biopsy programme were also excluded.

The study and the informed consent were approved by the Institutional Review Board of the Clinic.

Ovarian stimulation, oocyte collection, denudation, evaluation and injection

Controlled ovarian hyperstimulation was performed using two different protocols: GnRH-agonist long protocol and GnRH-antagonist protocol as described previously (Rienzi *et al*., 2008).

Oocyte collection was performed at 35 h post-hCG administration. Denudation from the cumulus oophorus was performed by a brief exposure to 40 IU/ml hyaluronidase solution in fertilization media (Sage In-Vitro Fertilization, Inc., Trumbull, CT, USA), followed by mechanical removal of the corona radiata with the use of plastic pipettes of defined diameters (denuding pipette; COOK Ireland Ltd, Limerick, Ireland) in a controlled CO_2 and temperature environment (IncuChamber L-323, Ksystems, Birkerod, Denmark). This procedure was performed between 37 and 40 h post-hCG administration. MII oocytes were separated from the immature oocytes and evaluated at the stereomicroscope. Those with dark cytoplasm, centrally located granular area, giant, with vacuoles and/or with large polar bodies were considered of lower quality (according to Rienzi *et al*., 2008) and were thus excluded from randomization.

The selected oocytes were allocated to fresh insemination or vitrification, both performed immediately after denudation. According to the Italian law in force when this study was performed, a maximum of three oocytes were inseminated per patient in both groups. Oocytes were subjected to ICSI using previously described techniques and instrumentations (Rienzi *et al*., 1998). To be able to follow the developmental progression of individual oocyte, each inseminated oocyte was cultured separately in microdrops of 35 μl of cleavage medium under mineral oil (Sage) up to Day 2, in MINC incubators (COOK) (hypoxic atmosphere containing 6% CO_2). All obtained embryos were transferred back to the patient.

In oocyte warmed cycles, the obtained embryos were all transferred in the course of a natural cycle. When the mean diameter of the leading follicle was 17–18 mm and the endometrial thickness was $>$7 mm with a triple line pattern, the ovulation was triggered. Thirty-six to forty hours later, the oocytes were warmed and inseminated.

The luteal phase was supported by means of vaginal micronized progesterone, 400 mg/day (Progeffik 200 mg, Effik, Cinisello Balsamo, Milan, Italy) starting on the day of oocyte retrieval/warming and the embryo transfers were performed 44–48 h after the microinjection procedure.

Oocyte vitrification and warming procedures

The vitrification and warming procedures were performed according to Kuwayama *et al*. (2005, 2007). Commercial kits were used (Vitrification and Warming KIT, Kitazato BioPharma Co, Japan).

The vitrification procedure was performed at room temperature (RT). Oocytes were first equilibrated in 7.5% ethylene glycol (EG) and 7.5% dimethylsulfoxide (DMSO). The equilibration was performed gradually. After 12–15 min incubation, the oocytes (one to three, contemporaneously) were then transferred in 1 ml of vitrification solution (VS) containing 15% EG, 15% DMSO and 0.5 M sucrose for 1 min. The oocytes were then placed on the Cryotop strip in a single small drop of VS. Much care was taken to re-aspirate the excess of VS in such a way to leave just a thin layer around each oocyte. The Cryotop was then immediately submerged into liquid nitrogen. Finally, the plastic cap was pulled over the Cryotop inside the liquid nitrogen and the sample was stored submerged in liquid nitrogen.

The first step of warming procedure was performed at 37°C. The cap was removed in liquid nitrogen and the cryotop was immediately submerged in 1 ml of warming solution containing 1.0 M sucrose. After 1 min, oocytes were placed in 1 ml solution containing 0.5 M sucrose, and incubated at RT for 3 min. Finally, the oocytes were washed at RT for 6 min in two different dishes containing 1 ml basic medium, then transferred into 1 ml culture media (Cleavage media, Sage). Degenerated oocytes were removed from the cohort.

The surviving oocytes were cultured at 37° (6% CO_2 and 5% O_2) in mini Sanyo incubators (48L, MCO-5M Sanyo, Japan) for exactly 2 h before ICSI.

Pronuclear and Day 2 embryo assessment

Fertilization was assessed at 16–18 h after ICSI. Fertilized oocytes were considered morphologically normal when two equally sized centrally located pronuclei were visible. Asymmetry, eccentric position and/or distance between the two pronuclei were considered as abnormal patterns.

Cleaving embryos were evaluated on Day 2 after ICSI (44–46 h post-insemination) with the use of a cumulative embryo classification scheme taking into account cleavage speed, blastomere symmetry, extent of fragmentation and the presence or absence of multinucleated blastomeres (Rienzi *et al*., 2002). In accordance with this scheme an embryo was considered excellent when it scored 0–1, good when it scored 2–3, fair when it scored 4–5 and poor quality when it scored $>$5.

Clinical outcome

Pregnancy was confirmed by serial rise in serum HCG concentrations on two consecutive occasions 13 days after embryo transfer. According to Farquharson *et al*. (2005) the absence of an identifiable pregnancy on ultrasound examination was named 'Biochemical pregnancy loss'. Clinical pregnancy was determined by ultrasound demonstration of gestational sac at 7 weeks. Miscarriage was classified as 'early' (before 12 weeks) or 'late' (after 12 weeks) according to Farquharson *et al*. (2005).

Implantation rate and ongoing implantation rate were defined as number of gestational sacs per transferred embryo, and number of fetuses with heart activity beyond 12 weeks of gestation per transferred embryo, respectively.

Sample specifications and statistical analysis

The non-inferiority margin of this study was set at 17% because this threshold was considered to indicate clinically important differences, in particular on the number of embryos available for transfer in our setting. Considering a mean fertilization rate of 84%, based on our experience with ICSI procedure with selected fresh MII oocytes, the lower confidence limit would be not inferior to 67% for the insemination performed with vitrified oocytes. By using three oocytes for ICSI, the mean number of fertilized oocytes in the vitrified group would not fall below 2, which is the expected number of embryos needed for transfer. It must be underlined that in this setting the embryos are not selected.

To demonstrate the non-inferiority of vitrification procedure on fertilization rate based on a maximum absolute difference of 17% with a power of 80% and a confidence of 95% (non-inferiority design with one-sided tests performed) a minimum of 111 oocytes were required per group (222 total). It was decided to include 40 patients (considering

three oocytes inseminated per patient, per group) in order to reach the calculated number of oocytes.

Baseline characteristics (continuous data: female age, rank of trial, baseline FSH, total dose of gonadotrophin, days of stimulation, number of cumulus corona cell oocyte complexes retrieved, number of MII obtained, number of embryos transferred, number of oocytes vitrified, number of oocytes warmed) are presented as absolute, mean with standard deviation (SD) and range. Categorical variables (oocyte survival, oocyte fertilization, normal and abnormal pronuclear (PN) morphology, 1PN and 3PN occurrence, oocyte degeneration, embryo quality, clinical pregnancy, biochemical pregnancy loss, early miscarriage, ongoing pregnancy, implantation rates) are presented as absolute and percentage frequency.

Differences in frequencies of fertilization (primary outcome), pronuclear morphology and embryo grade (secondary outcomes) were evaluated with Pearson's chi-squared test with Yates' continuity correction and Fisher's exact test. Data are presented as odds ratio (OR), 95% confidence interval (95% CI), and *P*-value. The differences between arms are summarized as absolute difference with 95% CI.

To verify the influence of potential confounding variables (female age, rank of trial, baseline FSH, stimulation protocol, the total dose of gonadotrophin, days of stimulation, number of cumulus corona cell oocyte complexes retrieved, number of MII obtained) on primary outcomes, logistic regression analysis was conduced. Moreover, McNemar's test was used to test whether the proportion of cycles (on a per woman basis) where at least one of the three oocytes successfully fertilized, was equal for both groups. Embryo scores were aggregated to the level of each patient for each arm and a paired *t*-test was performed. $P < 0.05$ was considered statistically significant.

All statistical analyses were performed using R version 2.8.0 (The R Foundation for Statistical Computing). This trial has been registered and the following number has been assigned ISRCTN60158641.

Results

Baseline characteristics and clinical outcomes

During the study period 366 patients underwent 391 ICSI cycles with ovarian stimulation in our centre. Twenty-four cycles were performed in females aged >42 years old, in 182 cycles less than six normal-appearing oocytes at MII stage were obtained, in 17 cycles testicular sperm was used and in 11 cycles less than 500 000 motile sperm were obtained after preparation. Polar body biopsy was performed in two cycles, and 31 patients did not agree to sign the informed consent. All these cases did not meet with the inclusion criteria and were excluded from the study.

One hundred and twenty four cycles, from 124 patients (33.8% of the treated couples) meet with the inclusion criteria. Fifty four patients (43.2%) obtained a clinical pregnancy in the fresh cycle and were therefore not, involved in the warming cycle during the study period. Of these 6 had an early miscarriage (11.1%) and 48 are ongoing (beyond 12 weeks of gestation) (38.8%). Sixty-nine sacs were observed by ultrasound examination with an implantation rate of 21.7% (69/318) and an ongoing implantation rate of 19.2% (61/318).

Of the remaining 70 patients, 40 performed the first warming cycle during the study period and represent our patient population (per-protocol analysis). The mean age of these included patients was

Table I **Patient's baseline characteristics and fresh cycle parameters**

	Patients included (*N* = 40)
Female age (mean years ± SD)	35.5 ± 4.8
Baseline FSH (mean mU/ml ± SD)	6.44 ± 3.1
Previous IVF attempts (mean ± SD)	0.58 ± 1.0
GnRH-agonist long protocol (%)	31/40 (77.5)
Antagonist protocol (%)	9/40 (22.5)
Days of stimulation (mean ± SD)	10.8 ± 1.95
Total gonadotrophin amount IU (mean ± SD)	2201.65 ± 765.7
Number of CCOCs retrieved (mean ± SD)	13.3 ± 4.5
Number of MII oocytes (mean ± SD)	10.7 ± 3.6
Number of MII oocytes vitrified (mean ± SD)	6.3 ± 2.8

CCOC, cumulus corona oocyte complex; MII, metaphase II.

35.5 ± 4.8 years (range 26–42). In Table I patient's baseline characteristics and cycle parameters are described. Three oocytes were inseminated per patient in the fresh cycle ($N = 120$ oocytes) and the remaining good quality oocytes were vitrified ($N = 251$; mean 6.3 ± 2.8; range 3–14).

The fertilization rate obtained with fresh oocytes was 83.3% (100/120). All obtained viable embryos ($N = 100$), independently from their morphological appearance, were transferred. The mean number of embryos transferred per patient was 2.5 ± 0.5 (range 1–3). In one case no embryo was available for transfer.

One hundred twenty four oocytes were warmed in the 40 cycles included (mean 3.1 ± 0.3, range 3–4), of which 120 survived the procedure (96.7%). In all cases three oocytes were available for the ICSI procedure ($N = 120$) and 95 fertilized (79.2%). All available embryos were transferred ($N = 93$). Also in this group, one case was cancelled because no embryo was available. Seventeen pregnancies were obtained (43.6% per cycle, 45.9% per embryo transfer), two of which had a biochemical pregnancy loss. Of the 15 clinical pregnancies recorded (37.5% per cycle, 38.5% per embryo transfer), 3 had an early miscarriage (20%) and 12 are ongoing (beyond 12 weeks of gestation) (30.0% per cycle, 30.8% per embryo transfer). Nineteen gestational sacs were observed by ultrasound examination with an implantation rate of 20.4% (19/93) and an ongoing implantation rate of 17.2% (16/93) (Table II).

Primary and secondary outcome measures: fertilization rates, pronuclear morphology and embryo development

Table III shows fertilization rates obtained per warmed oocyte and per injected oocyte, pronuclear morphology, embryo development and quality in the fresh ICSI group and in the vitrified/warmed ICSI group. No statistical differences were found between the two groups for all the parameters analysed. The lower limit of the

confidence intervals (CIs) of the differences between groups for primary outcomes excluded the predefined non-inferiority margin of 17%.

No correlations were found by logistic regression analysis between potential confounding variables (female age, rank of trial, baseline FSH, stimulation protocol, total dose of gonadotrophin, days of stimulation, number of cumulus corona cell oocyte complexes retrieved, number of MII obtained) and primary outcome measures. Moreover, the probability of obtaining at least one fertilized oocyte per cycle was not significantly different for fresh and vitrified oocytes ($P = 0.48$ by McNemar's test).

The mean embryo score obtained in the fresh ICSI group and in the vitrified/warmed ICSI group was also similar (1.39 ± 1.40 and 1.48 ± 1.47, respectively) ($P = 0.68$).

Table II **Clinical outcomes of cycles performed with vitrified/warmed oocytes**

	Patients included (*N* = 40)
Number of warmed oocytes (mean ± SD)	3.1 ± 0.30
Number of embryos transferred (mean ± SD)	2.3 ± 0.88
Number of embryo transfer performed (%)	39/40 (97.5)
Clinical pregnancy rate per cycle (%)	15/40 (37.5)
Clinical pregnancy rate per transfer (%)	15/39 (38.5)
Ongoing pregnancy rate per cycle (%)	12/40 (30.0)
Ongoing pregnancy rate per transfer (%)	12/39 (30.8)
Implantation rate (%)	19/93 (20.4)
Ongoing implantation rate (%)	16/93 (17.2)

Discussion

The aim of this study was to compare the *in vitro* performance of fresh and vitrified oocytes post-ICSI procedure. A randomized trial on sibling oocytes was set up. Oocyte fertilization was not significantly different when calculated per warmed and per injected oocytes. Moreover, embryo development was similar in the two groups.

Vitrification procedure was recently introduced in our laboratory as an alternative to slow freezing for oocyte cryopreservation. This choice was based on recent evidences that the vitrification procedure is less invasive, better preserves oocyte physiology as compared with slow freezing (Gardner *et al*., 2007) and can obtain excellent clinical outcomes (Kuwayama *et al*., 2005; Selman *et al*., 2006; Lucena *et al*., 2006; Antinori *et al*., 2007; Kuwayama 2007; Yoon *et al*., 2007; Cobo *et al*. 2008b, c, Chian *et al*., 2008; Sher *et al*., 2008; Kim *et al*., 2009; Nagy *et al*., 2009).

Different approaches in the vitrification procedure have been described for the human oocyte, embryo and blastocyst stages (see review by Vajta and Nagy, 2006). Although these approaches work on the same principle, they differ by type and concentration of cryoprotectants and/or for device used. In this study, the protocol firstly described by Kuwayama *et al*. (2005, 2007) was applied. Accordingly, the combination of EG and DMSO at a final total concentration of 30% and 0.5 M sucrose were used as cryoprotectants. Oocytes were loaded, vitrified and stored in Cryotops (Kitazato). To achieve vitrification, oocytes were covered by a thin film of cryoprotectant mixture and exposed to direct contact with liquid nitrogen. Although this is the most efficient approach to maximize cooling rates and minimize biological damages to the oocytes (Vajta *et al*., 2009, in press), it potentially exposes the cells to risk for contamination (Bielanski *et al*., 2000). The results reported in this study are however strictly related to the vitrification method used, other vitrification approaches in our hands were significantly less effective (data not shown). We are now evaluating the possibility to eliminate the cross-infection potential risk without modifying the principles of the protocol. This is possible with the use of highly

Table III **Primary and secondary outcomes measures: fertilization, pronuclear morphology, embryo development and embryo morphology of fresh and vitrified sibling oocytes**

	Fresh ICSI	Vitrified/Warmed ICSI (%)	Absolute difference (%) (95% CI)	OR (95% CI)	*P*
Fertilization (2PN) per sibling oocyte	100/120 (83.3)[b]	95/124 (76.6)[a]	−6.73 (−16.6 to 3.39)	0.65 (0.33 to 1.29)	0.20
Fertilization (2PN) per injected oocyte	100/120 (83.3)[b]	95/120 (79.2)[b]	−4.17 (−14.0 to 5.7)	0.76 (0.37 to 1.53)	0.50
Normal 2PN morphology	96/100 (96.0)[c]	86/95 (90.5)[c]	−5.47 (−13.4 to 1.84)	0.39 (0.08 to 1.49)	0.16
1PN oocytes	3/120 (2.5)[b]	6/120 (5.0)[b]	2.5 (−2.82 to 8.22)	2.05 (0.42 to 12.9)	0.50
3PN	1/120 (0.83)[b]	2/120 (1.66)[b]	0.83 (−3.09 to 5.1)	2.01 (0.10 to 119.9)	1
Degenerated oocytes post-ICSI	1/120 (0.83)[b]	4/120 (3.34)[b]	2.51 (−1.75 to 7.47)	4.08 (0.39 to 203.5)	0.37
Day 2 embryo development	100/100 (100)[c]	93/95 (97.9)[c]	−2,11 (−7.3 to 1.9)	0.0 (0.00 to 0.23)	0.24
Excellent quality embryos	52/100 (52.0)[d]	49/95 (51.6)[d]	−0.43 (−14.2 to 13.3)	0.98 (0.53 to 1.79)	0.90
Good quality embryos	38/100 (38.0)[d]	41/95 (43.2)[d]	5.16 (−8.49 to 18.6)	1.24 (0.67 to 2.28)	0.47
Fair/poor quality embryos	10/100 (10.0)[d]	3/95 (3.16)[d]	−6.84 (−14.6 to 0.42)	0.29 (0.05 to 1.19)	0.10

[a]Percentages, expressed per warmed oocyte.
[b]Percentages, expressed per inseminated oocyte.
[c]Percentages, expressed per 2PN fertilized oocyte.
[d]Percentages, expressed per cleaved oocyte.

purified liquid nitrogen for vitrification (Vajta *et al*., 1998) or sterilized nitrogen by ultraviolet irradiation (Parmegiani *et al*., 2009a, b) allowing retention of the direct contact, and hermetical devices for storage (pre-cooled straws heat-sealed and stored in liquid nitrogen containers) (Vajta *et al*., 1998, 2009, in press). Alternatively, for the latter, dry systems have been proposed (Cobo *et al*., 2008c).

Some other technical aspects of this study must be highlighted. The vitrification procedure was performed immediately after denudation and always between 37 and 40 h post-hCG administration. It was shown that timing is a crucial aspect when dealing with oocytes prior to insemination (Parmegiani *et al*., 2008, 2009a, b). Conversely to pronuclear, embryo and blastocyst stage, oocytes are developmentally arrested (at metaphase II stage) and thus much more sensitive to *in vitro* culture (Yanagida *et al*., 1998; Dozortsev *et al*., 2004). Long incubation periods, prior to cryopreservation, presumably affects oocyte competence. Moreover, after denudation, oocytes are particularly vulnerable to suboptimal *in vitro* conditions (Edwards *et al*., 1998). These drawbacks, not strictly related to cryopreservation itself but often associated with it, may explain part of the low success rate generally reported with oocyte freezing. To minimize oocyte stress, the denudation process was performed in a chamber with controlled temperature and gas atmosphere, in our laboratory (L-323, Ksystems). In this way, the randomization process was also performed without exposing the oocytes to suboptimal conditions. It was decided not to observe the oocytes under the inverted microscope for fine morphological assessment in this study. This extra manipulation was considered potentially harmful. According to our experience (Rienzi *et al*., 2008) only oocytes with determined abnormal morphological features, assessable under the stereomicroscope at 40× magnification, were considered severely compromised and were not included in the study.

After warming, the oocytes were cultured for 2 h before insemination. This period was considered necessary to allow the oocyte cytoskeletal apparatus to fully restore after vitrification, and in particular the meiotic spindle. We previously showed that oocyte meiotic spindle needs 3 h to fully reform after slow freezing procedure (Rienzi *et al*., 2004). This observation was confirmed by other studies using different oocyte freezing and/or vitrification procedures (Bianchi *et al*., 2005; Coticchio *et al*., 2007; Cobo *et al*., 2008a). Spindle recovery seems however to be faster after vitrification than slow freezing (Larman *et al*., 2007; Chen and Yang, 2009). To minimize oocyte ageing post-warming it was decided to reduce the *in vitro* culture period prior to insemination from 3 to 2 h. Recent evidences suggest that this period could further diminish to 1 h where the higher rate of normal spindle configuration is observed (Bromfield *et al*., 2009, in press).

The general idea of the study was thus to minimize extra stress on oocytes often related with cryopreservation procedures, namely: (i) long exposure to Hepes-buffered media, with uncertain temperature control, for oocyte denudation and selection under the inverted microscope, (ii) prolonged oocyte *in vitro* culture without the protection of cumulus and corona cells, (iii) oocyte ageing. In this way, by using randomized sibling oocytes, the only difference between the fresh and the vitrified group was the vitrification procedure itself followed by 2 h of *in vitro* culture.

According to our results, embryo development up to Day 2 is not affected by vitrification procedure. Oocyte survival rate was higher than 95%. The number of oocytes degenerated during warming procedure was negligible and did not affect the overall fertilization rate. Although some, non-significant, differences in pronuclear morphology were observed, embryo quality was similar in the two groups. The percentage of top quality embryos per fertilized oocyte was about 52% in fresh and vitrified group of oocytes. These observations are further confirmed by the clinical outcomes obtained. Although, the evaluation of pregnancy and implantation rates were beyond the purpose of this study, it is interesting to report the promising clinical results recorded in the analysed warming cycles. An ongoing clinical pregnancy of 30% and an ongoing implantation rate of 17% were obtained in our population of infertile couples subjected to severe legal restrictions (where only three vitrified oocytes could be inseminated per cycle). Thus, the ongoing implantation rate per warmed oocytes was 12.9% (16/124). Moreover four ongoing pregnancies (30.7%) were obtained in women aged >38 years. It cannot be excluded that endometrium receptivity may also be involved in successful implantation of embryos derived from vitrified oocytes. By transferring embryos in a natural unstimulated cycle, synchronization of embryo and endometrial development can be, in fact, probably better obtained (Ubaldi *et al*., 1997).

We believe that these results will help the spread of vitrification for human oocytes cryopreservation. This possibility is particularly important to overcome legal and ethical issues related to embryo storage. Moreover, this study demonstrates that the same number of oocytes is needed in fresh and vitrified cycles to obtain similar results in terms of embryo development. The number of oocytes to be warmed per cycle should thus be chosen accordingly. We believe that this finding is particularly important in oocyte donation programmes where a limited number of oocytes are available for recipients. Moreover, in order to avoid the production of supernumerary embryos and thus the risk of double vitrification, a limited number of oocytes should be warmed per cycle in patients who have cryopreserved oocytes because they are at risk of premature ovarian failure.

To our knowledge, this is the first clinical trial, designed in a prospective randomized way, which demonstrates the efficacy of the vitrification procedure to maintain oocyte competence to develop *in vitro*, in a population of infertile patients. We believe that this study strongly supports the laboratory efficacy of the technique. Further studies, performed on a larger scale, are however needed to confirm clinical outcomes of vitrified oocytes and the safety of the technique.

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