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ORIGINAL ARTICLE ***Infertility***

Cumulative ongoing pregnancy rate achieved with oocyte vitrification and cleavage stage transfer without embryo selection in a standard infertility program

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BACKGROUND: Recent advancement of minimum volume vitrification methods has resulted in a dramatic increase in the efficiency of the process. The aim of this study was to estimate the cumulative reproductive outcome of a cohort of infertile couples undergoing ICSI and oocyte vitrification in restrictive legal conditions, where only a limited number of oocytes could be inseminated per cycle and embryo selection and cryopreservation were forbidden.

METHODS: In this prospective longitudinal cohort study, the cumulative ongoing pregnancy rates obtained by the insemination of fresh and vitrified oocytes from the same cohort were calculated as primary outcome measures. Moreover, the effect of basal and cycle characteristics on clinical outcomes were assessed.

RESULTS: Between September 2008 and May 2009, 182 ICSI cycles were performed where oocyte vitrification was possible. A total of 104 first and 11 second oocyte warming cycles were then performed in non-pregnant patients of the same cohort. The overall ongoing pregnancy rates obtained in the fresh, and first and second warming cycles were 37.4, 25.0 and 27.3%, respectively. The overall cumulative ongoing clinical pregnancy rate observed per stimulation cycle was 53.3%. Maternal age was the only characteristic found to influence the reproductive outcome, with an inverse correlation between the age >40 and the ongoing pregnancy rates ($P = 0.04$, by Cox regression analysis).

CONCLUSIONS: High cumulative ongoing pregnancy rates can be obtained with transfers of embryos derived from fresh and cryopreserved oocytes in a typical infertile population. Female age significantly affects outcomes in this system.

Key words: oocyte vitrification / ICSI / cumulative pregnancy / female age

Introduction

Cryopreservation of embryos has become an indispensable part of assisted reproductive techniques. Frozen embryo transfer contributes 25% of all births achieved by assisted reproduction worldwide (ICMART, 2008), and with systematical application, up to 42% of implantations can be derived from frozen embryos (Edgar and Gook, 2007). In many clinics, birth rates after transfer of cryopreserved embryos are close or identical to those achieved with their fresh counterparts, increasing considerably the overall success rate of ART procedures measured by delivery per oocyte aspiration rates. However, legal issues and moral concerns may restrict the application of embryo cryopreservation. Additionally, due to the lack of a partner it cannot be applied in many cases of fertility preservation with medical or social indications, and may create controversial issues in case of divorce or separation of partners.

The most feasible solution for these problems is oocyte cryopreservation. Unfortunately, in spite of the relative early successes (Chen,

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1986; Van Uem et al., 1987), widespread application of oocyte cryopreservation was hampered for a long time by inconsistent efficiency of the available cryopreservation methods (Oktay et al., 2006). Stepwise adjustments of traditional slow freezing protocols as well as optimization of minimum volume vitrification methods (Arav, 1992; Kuwayama et al., 2005; Kuwayama et al., 2007) have resulted in breakthroughs in this field. Recent prospective randomized studies in oocyte donation programs (Cobo et al., 2008; Nagy et al., 2009) have found no significant differences between fresh and vitrified oocytes regarding the *in vitro* and *in vivo* developmental potential. According to a multicenter study, pregnancies and perinatal outcomes do not appear to be altered by oocyte vitrification (Chian et al., 2008). Our recent non-inferiority trial in a standard infertility program indicated that the oocyte vitrification procedure followed by ICSI is not inferior to the fresh insemination procedure, with regard to fertilization and embryo developmental rates (Rienzi et al., 2010).

This prospective cohort study aimed to evaluate the cumulative outcome of embryo transfers performed in infertile patients with the subsequent use of fresh and vitrified human oocytes between September 2008 and May 2009. To meet the Italian law No 40/2004 between March 2004 and May 2009 (Benagiano and Gianaroli, 2004; Rienzi et al., in press), three oocytes were subjected to ICSI in fresh and warming cycles and all obtained embryos were transferred without further selection.

Materials and Methods

Study design and outcome measures

The study was design as a prospective longitudinal cohort study. The baseline characteristics, embryological data, clinical pregnancy rate and ongoing clinical pregnancy rate were analyzed on a per cycle basis. The cumulative success rate was analyzed on a per patient basis.

Fertilization and Day 2 embryo development were evaluated under an inverted microscope at 16–18 and 44–46 h post-insemination, respectively. Fertilized oocytes were considered as morphologically normal when two equally sized, centrally located pronuclei were visible. Cleavage stage embryos were evaluated according to a cumulative embryo classification scheme (Rienzi et al., 2002). In accordance with this scheme, an embryo was considered as top quality when it scored 0–1.

Clinical pregnancy was defined as the presence of gestational sac in Week 7 after transfer. A miscarriage between Week 7 and Week 20 was defined as an abortion. The implantation rate was defined as number of gestational sacs per transferred embryo.

Ongoing pregnancy was defined when the pregnancy had completed $\geq$20 weeks of gestation. The ongoing implantation rate was defined as number of fetuses with heart activity beyond 20 weeks of gestation per transferred embryo.

Cumulative ongoing pregnancy rates obtained with fresh and vitrified oocytes from the same stimulation cycle were determined.

Female age, cause of infertility, basal FSH, stimulation protocol, sperm quality, number of oocytes retrieved and oocyte incubation time between retrieval and vitrification procedure were all assessed for their effects on clinical outcomes. The study and the informed consent procedure were approved by the Institutional Review Board of the Clinic.

Target population

All consecutives patients undergoing ICSI treatment in the Centre for Reproductive Medicine GENERA in Rome between 2 September 2008 and 15 May 2009 were considered for this study. Only patients with supernumerary oocytes available for cryopreservation were included. A single fresh attempt was included for each patient.

Ovarian stimulation, oocyte collection, denudation, evaluation and injection

Two protocols were used for controlled ovarian hyperstimulation: GnRH-agonist long protocol and GnRH-antagonist protocol as described previously (Rienzi et al., 2008).

Details of laboratory and embryo transfer procedures were described in a concomitant study (Rienzi et al., in press). Briefly, oocytes were collected at 35 h post-hCG administration. After 2–8 h incubation *in vitro*, cumulus-oocyte complexes (COCs) were exposed to 40 IU/ml hyaluronidase solution, and the corona radiata was removed mechanically by pipetting. Metaphase II (MII) oocytes were evaluated and selected under a stereomicroscope. Those with dark cytoplasm, a centrally located granular area, acuoles or a large polar body were excluded from insemination and vitrification.

Selected oocytes were allocated to fresh insemination or vitrification, respectively. Both procedures were performed immediately after denudation. Three oocytes were inseminated by ICSI using previously described techniques and instrumentations (Rienzi et al., 1998). Each inseminated oocyte was cultured separately in a 35 µl microdrop up to Day 2 in a humidified atmosphere containing 5% O_2 and 6% CO_2.

As the earlier referred Italian law did not allow any selection between viable embryos, all obtained viable embryos were transferred back to the patient 44–48 h after the microinjection procedure to avoid any potential negative effect of long-term *in vitro* embryo culture. In warmed oocyte cycles, embryos were transferred in the course of a natural cycle. The luteal phase was supported by vaginal micronized progesterone.

Oocyte vitrification and warming procedures

The vitrification and warming procedure has been described earlier (Rienzi et al., 2010). Briefly, oocytes were vitrified by using the Cryotop device and solutions (Kitazato BioPharma Co., Japan). The first equilibration was performed in 7.5% ethylene glycol (EG) and 7.5% dimethylsulfoxide (DMSO) at room temperature for 12–15 min. Subsequently, oocytes were transferred in 15% EG, 15% DMSO and 0.5 M sucrose for 1 min, then placed on the film strip of the Cryotop in a single small drop. The excess solution was removed to leave just a thin layer around each oocyte and the Cryotop was submerged into liquid nitrogen, the strip was covered with the cap and the sample was stored submerged in liquid nitrogen.

At warming, the cap was removed under liquid nitrogen and the film strip of Cryotop was quickly submerged in 1 ml of 37°C warming solution containing 1.0 M sucrose for 1 min, then oocytes were transferred to a room temperature solution containing 0.5 M sucrose, and incubated for 3 min. After two subsequent washing procedures in basic medium at room temperature for 6 min in total, oocytes were transferred into 1ml culture medium (Cleavage medium, Sage). Degenerated oocytes were removed from the cohort. Surviving oocytes were cultured at 37°C (6% CO_2 and 5% O_2) in mini Sanyo incubators (48L, MCO-5M Sanyo, Japan) for 2 h before ICSI. When possible, oocytes with dark cytoplasm, vacuoles or signs of degeneration were excluded from insemination.

Sample specifications and statistical analysis

Baseline characteristics (continuous data: female age, baseline FSH, number of cumulus corona cell oocyte complexes retrieved, number of MII obtained, number of oocytes fertilized, number of embryo transferred, number of oocytes vitrified, number of oocytes warmed) are presented as

absolute mean with standard deviation (SD) and range. Categorical variables are presented as absolute and percentage frequency. Differences in clinical and ongoing pregnancy and abortion rates between fresh and vitrified cycles were evaluated with Pearson's χ^2 test with Yates' continuity correction and Fisher's exact test.

The cumulative ongoing pregnancy rates were calculated by life table analysis and are expressed as percentage probabilities with 95% confidence intervals (CI). Differences between groups were calculated by Kaplan–Meier procedure.

The effect of covariates (female group of age, infertility factors, basal FSH, stimulation protocol, number of COCs and MII oocytes retrieved, sperm quality and oocyte incubation time between retrieval and vitrification procedure) on clinical outcome were assessed using Cox regression analysis.

All statistical analyses were performed using R version 2.8.0 (The R Foundation for Statistical Computing).

Results

There were 408 patients who underwent 456 ICSI cycles with ovarian stimulation in our centre during the study period. Oocyte vitrification was performed for 182 patients in 182 cycles (44.6% of patients, 39.9% of cycles) which represent our patient's population. Vitrification was performed for the following reasons: in 173 cycles supernumerary oocytes were available; in five cycles hyperstimulation syndrome needed to be prevented; in one cycle no sperm was available on the day of ovum pick-up; and in three cycles for genetic analysis was performed.

The mean age of the included patients was 35.81 ± 4.19 years (range 21–43). Infertility factors were endometriosis in 8 cases (4.4%), male in 76 cases (41.8%), tubal in 39 cases (21.4%), idiopathic in 43 cases (23.6%), ovulatory in 4 cases (2.2%) and combined in 12 cases (6.6%). Testicular sperm was used in 10 cycles, and in additional 15 cycles less than 500 000 motile spermatozoa were obtained after preparation. These cycles where grouped as 'bad sperm quality' to analyze the potential negative paternal effect on outcomes.

The mean numbers of recovered COCs and obtained MII oocytes were 12.8 ± 4.7 (range 6–31) and 10.1 ± 3.50 (range 5–24), respectively. There were 511 fresh MII oocytes inseminated in 173 cycles (2.95 ± 0.40; range 2–3). The fertilization rate with fresh oocytes was 87.1% (445/511). All obtained embryos, independent of their morphological appearance, were transferred. The mean number of embryos transferred per patient was 2.52 ± 0.59 (range 1–3) in 172 cycles. In nine cycles, fresh ICSI procedure was not performed, for reasons stated above, and all available mature oocytes were vitrified, and in one cycle no embryo was available for transfer after fresh ICSI. A total of 1132 oocytes were vitrified (mean 6.22 ± 3.08).

There were 104 first and 11 second oocyte warming cycles performed in non-pregnant patients of the same cohort. The mean numbers of warmed, survived and inseminated MII phase oocytes were 4.23 ± 1.23; 3.80 ± 0.89 and 2.97 ± 0.16, respectively. The overall survival rate was 89.7% (437/487). After morphological selection, 342 oocytes (maximum three per patient) were inseminated. The fertilization rate with vitrified oocytes was 85.4% (292/342). As in the fresh cycles, all obtained embryos, independent of their morphological appearance, were transferred. The mean number of embryos transferred per patient was 2.40 ± 0.80 (range 1–3). In four cases, (3.48%) no embryo was available for transfer.

Baseline patient's characteristics, as well as laboratory outcomes of fresh and warmed cycles, are shown in Table I. No differences in fertilization rates (87.1 and 85.4%, respectively) and top quality Day 2 embryo rates (58.7 and 57.9%, respectively) were observed between fresh and vitrified cycles.

Clinical outcomes according to the female's age are shown in Table II. The overall ongoing pregnancy rates obtained in the fresh, I and II warming cycles were 37.4% (68/182), 25.0% (26/104) and 27.3% (3/11), respectively ($P = 0.05$). Regarding the different age groups, ongoing pregnancy rates of: 40.3% (29/72), 45.7% (16/35) and 0% (0/2) were observed in women aged <34 years (NS); 41.7% (20/48), 11.1% (3/27) and 33.3% (1/3) in women aged 35–37 years ($P = 0.02$); 36.6% (15/41), 20.0% (5/25) and 20.0% (1/5) in women aged 38–40 years (NS); and 19.0% (4/21), 11.8% (2/17) and 1/1 (100%) in women aged 41–43 years (NS).

The highest achieved cumulative ongoing pregnancy rate (62.5%) was obtained in the ≤34 years group. The ongoing clinical pregnancy rate showed a declining tendency with the increasing maternal age (Table III). A significant difference was found between age group <34 years and age group 41–43 years ($P = 0.006$).

According to the Cox regression analysis, infertility factors, basal FSH levels, stimulation protocols, number of retrieved COCs and MII, sperm quality and oocyte incubation time between retrieval and vitrification all did not influence the ongoing pregnancy rates. An inverse correlation was found between the maternal age >40 and the ongoing pregnancy rates (Table IV).

Table I Baseline patient's characteristics, fresh and warming cycles laboratory outcomes.

Baseline patient's characteristics	
No. patients	182
Mean age (mean ± SD)	35.81 ± 4.19
Mean basal FSH (mean ± SD)	6.5 ± 2.23
Agonist protocol (%)	143/182 (78.6%)
Antagonist protocol (%)	39/182 (21.4%)
Fresh cycle: laboratory outcomes	
COC (mean ± SD)	12.8 ± 4.7
MII (mean ± SD)	10.1 ± 3.5
Inseminated MII (mean ± SD)	2.95 ± 0.40
2 PN (mean ± SD)	2.57 ± 0.61
Top quality embryos (mean ± SD)	1.51 ± 0.96
Embryo transferred (mean ± SD)	2.52 ± 0.59
Oocyte vitrified (mean ± SD)	6.22 ± 3.08
Warmed cycles: laboratory outcomes	
Warmed MII (mean ± SD)	4.23 ± 1.23
Survived MII (mean ± SD)	3.80 ± 0.89
Inseminated MII (mean ± SD)	2.97 ± 0.16
2 PN (mean ± SD)	2.54 ± 0.65
Top quality embryos (mean ± SD)	1.47 ± 0.91
Embryo transfer (mean ± SD)	2.40 ± 0.80

COC, cumulus-oocyte complex; MII, metaphase II; PN, pronucleus.

Table II Clinical outcomes of fresh, and warming cycles according to female age.

	Overall	≤34 years	35–37 years	38–40 years	41–43 years
Fresh cycles: clinical outcomes					
No. of cycles	182	72	48	41	21
No. of ET	172/182 (94.5)	66/72 (91.6)	46/48 (95.8)	40/41 (97.6)	20/21 (95.2)
Clinical pregnancy rate per cycle	77/182 (42.3)[a]	32/72 (44.4)	22/48 (45.8)	18/41 (43.9)	5/21 (23.8)
Clinical pregnancy rate per ET	77/172 (44.8)[b]	32/66 (48.5)	22/46 (47.8)	18/40 (45.0)	5/20 (25.0)
Implantation rate	101/435 (23.2)[c]	46/153 (30.0)	29/116 (25.0)[f]	21/112 (18.7)	5/54 (9.2)
Abortion rate	9/77 (11.7)	3/32 (9.4)	2/22 (9.0)	3/18 (16.7)	1/5 (20.0)
Ongoing pregnancy rate per fresh cycle	68/182 (37.4)[d]	29/72 (40.3)	20/48 (41.7)[g]	15/41 (36.6)	4/21 (19.0)
Ongoing implantation rate	90/435 (20.7)[e]	42/153 (27.4)	26/116 (22.4)[h]	18/112 (16.1)	4/54 (7.4)
Warmed cycles: clinical outcomes					
No. of cycles	115	37	30	30	18
No. of ET	111 (96.5)	35/37 (94.6)	29/30 (96.7)	30/30 (100)	17/18 (94.4)
Clinical pregnancy rate per cycle	35/115 (30.4)[a]	17/37 (45.9)	7/30 (23.3)	7/30 (23.3)	4/18 (22.2)
Clinical pregnancy rate per ET	35/111 (31.5)[b]	17/35 (48.6)	7/29 (24.1)	7/30 (23.3)	4/18 (22.2)
Implantation rate	43/266 (16.1)[c]	21/77 (27.3)	8/73 (10.9)[f]	9/75 (12.0)	5/41 (12.2)
Abortion rate	6/35 (17.1)	1/17 (5.9)	3/7 (42.8)	1/7 (14.3)	1/4 (25.0)
Ongoing pregnancy rate per warmed cycle	29/115 (25.2)[d]	16/37 (43.2)	4/30 (13.3)[g]	6/30 (20.0)	3/18 (16.6)
Ongoing implantation rate	35/266 (13.2)[e]	19/77 (24.7)	5/73 (6.8)[h]	8/75 (10.7)	3/41 (7.3)

Data are expressed as absolute and percentage frequency. ET, embryo transfer.
[a,b,c,d,e,f,g,h] $P < 0.05$.

Table III Cumulative ongoing pregnancy rate after the fresh cycle, first warming cycle and second warming cycle according to female age.

	Overall	≤34 years	35–37 years	38–40 years	41–43 years
Fresh cycle	68/182 (37.4%)	29/72 (40.3%)	20/48 (41.7%)	15/41 (36.6%)	4/21 (19.0%)
(95% CI)	(31.2–45.1)	(29.7–51.9)	(28.8–55.8)	(23.6–52.0)	(7.8–40.3)
I warming cycle	94/182 (51.6%)	45/72 (62.5%)	23/48 (47.9%)	20/41 (48.8%)	6/21(28.6%)
(95% CI)	(44.4–58.8)	(50.9–72.8)	(34.4–61.7)	(34.2–63.6)	(13.9–50.2)
II warming cycle	97/182 (53.3%)	45/72 (62.5%)[a]	24/48 (50.0%)	21/41 (51.2%)	7/21 (33.3%)[a]
(95% CI)	(40.0–60.0)	(50.9–72.8)	(36.3–63.6)	(36.4–65.8)	(17.2–54.9)

Data are expressed as absolute, percentage frequency and 95% CI.
[a] $P = 0.006$.

At the date of submission of this manuscript, 10 and 18 patients failed to complete the first and second warming cycle, respectively. The average numbers of available oocytes per patients are 5.22 ± 2.41 ($n = 47$) and 4.94 ± 2.26 ($n = 89$) for the first and second warming cycle, respectively. Moreover, 509 vitrified oocytes are still available in the pregnant patient group.

Discussion

Until recently, cryopreservation of human oocytes was regarded as an inconsistent and generally inefficient procedure; accordingly its application was restricted to very special situations where alternative solutions were not available (Porcu et al., 2000; Borini et al., 2004, 2008).

In spite of the difficulties related to the oocyte structure and its cryosensitivity, considerable advancement has been achieved in traditional slow freezing during the past decade (Boldt et al., 2006; Borini et al.,2006a, b, 2008), however, results achieved with frozen occytes compared with sibling fresh oocytes are still different (Magli et al., 2009). In parallel, and in spite of concerns and aversions, certain techniques based on the vitrification (i.e. ice-free cryopreservation) approach have gained an increasing acceptance (Kuwayama et al., 2005, 2007; Selman et al., 2006; Lucena et al., 2006; Antinori et al., 2007; Yoon et al., 2007; Cobo et al., 2008; Chian et al., 2008; Sher et al., 2008; Nagy et al., 2009; Kim et al., 2009; Rienzi et al., in press). The minimum volume vitrification methods including the Cryotop technique (Kuwayama et al., 2005) used in our study to

Table IV Effect of patients and cycle characteristics on cumulative ongoing pregnancy based on Cox regression analysis (per patient basis).

Covariate	*P*-value	OR	(95% CI)
Female age groups			
≤ 34 years (reference)	–	–	–
35–37 years	0.36	0.78	0.47 to 1.31
38–40 years	0.36	0.77	0.45 to 1.33
41–43 years	0.04	0.44	0.18 to 0.64
Infertility factors			
Male (reference)	–	–	–
Idiopathic	0.75	0.90	0.48–1.67
Endometriosis	0.19	0.38	0.09–1.60
Ovulatory	0.54	0.53	0.07–3.91
Tubal	0.81	0.93	0.52–1.65
Combined	0.25	1.56	0.73–3.32
Basal FSH	0.60	0.82	0.82 – 1.23
Number of COC	0.85	1.01	0.92–1.09
Number of MII oocytes	0.57	1.03	0.92–1.14
Incubation time prior to ICSI/ vitrification	0.77	1.02	0.88–1.17
Stimulation protocol	0.41	0.79	0.45–1.37
Sperm quality	0.84	1.07	0.56–2.03

OR, odds ratio; 95% CI, 95% confidence interval.

increase the cooling and warming rates, consequently decrease the chilling injury (Yavin and Arav, 2007), the chance of ice nucleation (Fuller and Paynter, 2004) and allow the application of a relatively low cryoprotectant concentration (Vajta and Nagy, 2006; Gardner *et al*., 2007).

According to the recent review of Tulandi *et al*. (2008), pregnancy rates after oocyte vitrification are approximately twice as high as after traditional freezing (10–20 versus 21–45%, respectively). In prospective randomized studies performed in oocyte donation programs, the results obtained with vitified oocytes were similar to those achieved with fresh counterparts (Cobo *et al*., 2008; Nagy *et al*., 2009). The estimated number of babies born after oocyte cryopreservation is ~1000–1200 worldwide (approximate half of them derived from vitrification), and the number of reported cases and the contribution of vitrification are increasing exponentially.

Unfortunately, even this impressing development seems to be insufficient. Situations where oocyte cryopreservation offers a realistic solution also increase exponentially, and the need is at least three orders of magnitude higher than the actual achievement. Indications of oocyte vitrification may be distributed in three distinct groups including (i) fertility preservation for medical or social reasons (Tulandi *et al*., 2008; Homburg *et al*., 2009); (ii) establishment of oocyte banks for donation programs (Cobo *et al*., 2008); and (iii) infertility treatment, to replace embryo cryopreservation.

The latter solution is imperative in countries or situations where legal or ethical/religious reasons hamper cryopreservation of embryos but can also be a viable general option to avoid potential entanglement in case of divorce and separation of couples (Benagiano and Gianaroli, 2004; Heng, 2007). In a more general sense, cryopreservation of supernumerary oocytes instead of embryos may also be regarded as a step towards decreasing differences between genders regarding choice in reproduction (Homburg *et al*., 2009).

The perspective to cryopreserve oocytes instead of embryos in infertility programs has been outlined earlier (Tucker *et al*., 2004), and the recent increase of efficiency reported by various groups has made it a realistic possibility. However, data obtained from a selected group of young healthy women in oocyte donation programs cannot be directly applied to patients seeking treatment at an infertility clinic.

In this article, the cumulative ongoing pregnancy rate after transfers performed with embryos from fresh and vitrified oocytes among patients in a standard infertility program was analyzed. Principles of the Cryotop procedure applied for oocyte vitrification have been described earlier (Kuwayama *et al*., 2005; Kuwayama, 2007). Some technical aspects applied were discussed in detail in our earlier publication (Rienzi *et al*., 2010). Efforts were made to minimize oocyte damage by performing rapid denudation in bicarbonate buffered media and an appropriate gas atmosphere followed by morphological selection under a stereomicroscope by using the same media and environment. The time of *in vitro* culture of oocytes without the protection of cumulus-corona radiata cells was decreased to minimal, and the *in vitro* culture period after vitrification was restricted to 2 h.

Our previous work has investigated the *in vitro* performance of fresh and vitrified oocytes after insemination with ICSI. The vitrification procedure had no detectable effect on the *in vitro* developmental competence of oocytes. After ICSI and culture for 2 days, 52% of oocytes had developed to top quality embryos both from the fresh and vitrified group. This observation is confirmed by the present study where no statistical differences were found in the laboratory outcomes between fresh and vitrified oocytes. Although a negative trend was observed in the overall clinical outcomes when embryos derived from vitrified oocytes were transferred, the fresh and vitrified transfer groups cannot be compared in this study since the former group included all patients although the latter group included only those who did not become pregnant after the fresh transfer cycle. Furthermore, the fresh transfer cycle involved ovarian stimulation although the vitrified transfer was performed in a normal hormonal cycle. Previous reports (Cobo *et al*., 2008; Nagy *et al*., 2009), have found no difference between pregnancy rates achieved with embryos from fresh versus vitrified oocytes. These studies were performed in oocyte donation programs, although our study investigated the application of oocyte vitrification in a standard infertility program. Further investigations and a different experimental design would be required to determine whether or not ongoing pregnancy rates after vitrification are compromised.

Nevertheless, the cumulative ongoing pregnancy rate obtained in our study was high and comparable with transfer of fresh and cryopreserved embryos (Ubaldi *et al*., 2004). With Cox regression analysis, we did not find any association between the etiology of infertility, stimulation protocol or number of retrieved oocytes and the ongoing pregnancy rate. The decline of the ongoing pregnancy rate with augmenting maternal age is a commonly acknowledged phenomenon; it has also been confirmed in this study dealing with cumulative pregnancy rates after oocyte vitrification in a standard infertility program. The fact that the patients in general were good responders

to hormonal stimulation may explain the lack of correlation between the basal FSH levels, number of oocytes retrieved and ongoing pregnancy rates. In contrast to an earlier observation (Parmegiani *et al.*, 2008), in our study the incubation time between oocyte retrieval and cryopreservation (up to 8 h) had no influence on the ongoing pregnancy rates. These contradicting results may be explained by the different cryopreservation procedure (slow rate freezing versus vitrification). Another possible explanation is that Parmeggiani *et al*. had denuded the oocytes up to 1 h before cryopreservation (personal communication), although in our work the COCs were preserved during the *in vitro* incubation until the beginning of the vitrification procedure. The lack of relationship with sperm quality may be related to the exclusive use of ICSI for insemination of both fresh and vitrified oocytes.

In conclusion, high cumulative ongoing pregnancy rates were achieved in a standard infertility program with transfers of embryos derived from fresh and subsequently vitrified oocytes. Among various infertility factors, only female age influenced significantly the outcome. The overall efficiency justifies the application of this strategy in routine infertility work.

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