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Use of cryo-banked oocytes in an ovum donation programme: a prospective, randomized, controlled, clinical trial

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BACKGROUND: An efficient oocyte cryopreservation method is mandatory to establish a successful egg-banking programme. Although there are increasing reports showing good clinical outcomes after oocyte cryopreservation, there is still a lack of large controlled studies evaluating the effectiveness of oocyte cryo-banking. In this study, we aimed to compare the outcome of vitrified-banked oocytes with the gold standard procedure of employing fresh oocytes.

METHODS: A randomized, prospective, triple-blind, single-centre, parallel-group controlled-clinical trial (NCT00785993), including 600 recipients ($\alpha = 0.05$ and power of 80% for sample-size calculation) selected among 1032 eligible patients from November 2008 to September 2009, was designed to compare the outcome of vitrified-banked oocytes with the gold standard procedure of employing fresh oocytes. The study was designed to establish the superiority of the ongoing pregnancy rate (OPR) of fresh oocytes over that of vitrified oocytes, by performing a likelihood ratio test in a logistic regression analysis expressed as odds ratio (OR) with 95% confidence interval (CI). A limit of 0.66 for OR of vitrified versus fresh groups was defined to set up a possible conversion from superiority to non-inferiority. Randomization was performed 1:1 based on a computer randomization list in vitrification ($n = 300$) or fresh groups ($n = 300$). The primary end-point was the OPR per randomized patient i.e. intention-to-treat population (ITT). Secondary end-points were clinical pregnancy (CPR), implantation (IR) and fertilization rates, respectively. Additionally, embryo developmental characteristics were recorded.

RESULTS: There were no differences in donor ovarian stimulation parameters, demographic baseline characteristics for donors and recipients, ovum donation indications or male factor distribution between groups (NS). The OPR per ITT was 43.7 and 41.7% in the vitrification and fresh groups, respectively. The OR of OPR was 0.921 in favour of the vitrification group. Nevertheless, the 95% CI was 0.667–1.274, thus the superiority of fresh group with respect to OPR was not proven ($P = 0.744$). Non-inferiority of the vitrified group compared with the fresh group was shown with a margin of 0.667, which was above the pre-established non-inferiority limit of 0.66. CPR per cycle (50.2 versus 49.8%; $P = 0.933$) or per embryo-transfer (55.4 versus 55.6% ; $P = 0.974$), and IR (39.9 versus 40.9%; $P = 0.745$) were similar for patients receiving either vitrified or fresh oocytes. The proportion of top-quality embryos obtained either by inseminated oocyte (30.8 versus 30.8% for Day-2; and 36.1 versus 37.7% for Day-3, respectively) or by cleaved embryos (43.6 versus 43.8% for Day-2 and 58.4 versus 60.7% for Day-3, respectively) was similar between groups (NS).

CONCLUSIONS: This controlled-randomized, clinical trial confirmed the effectiveness of oocyte cryo-storage in an ovum donation programme, failing to demonstrate the superiority of using fresh oocytes with respect to the use of vitrified egg-banked ones in terms of OPR. Instead, the non-inferiority of vitrified oocytes was confirmed. These findings involve highly relevant issues that may open a new range of possibilities in ART.
Clinical Trials identifier: www.clinicaltrials.gov: NCT 00785993.

Key words: oocyte vitrification / oocyte donation / oocyte-bank / ongoing pregnancy rate/OPR / clinical trial

Introduction

Ovum donation is a well-established practice that is commonly applied as a response to many clinical situations, and leads to the highest pregnancy rates reported for any assisted reproductive method (Sauer and Kavic, 2006; Budak *et al.*, 2007). The successful clinical outcome of ovum donation programmes requires a receptive endometrium, usually prepared with exogenous steroids (Remohi *et al.*, 1995, 1997), and a well synchronized replacement of good-quality embryos. For synchronization, several strategies have been employed

with varying levels of success, but certainly the most crucial factor is the availability of banked oocytes. Moreover, current procedures of ovum donation involve drawbacks, such as long waiting lists subject to the availability of a suidonor. Both these reasons are often responsible for the poor efficiency of ovum donation programmes, which causes great stress and discomfort to patients.

Another relevant issue is safety. Current regulations demand that donors be tested to avoid the transmission of infectious diseases (Guidelines for gamete and embryo donation, 2008). However, it is not possible to impose an adequate quarantine period for ovums, as routinely occurs with donated semen, as cycles must be conducted with fresh oocytes. It is true that no major complication has been reported to date, but common sense suggests that a quarantine period is always advisable in cell and tissue transplantation. All these limitations could be solved with an efficient oocyte-banking programme.

Semen and embryo cryopreservation are effective approaches that have been regular practices in IVF for some time now. However, in spite of the numerous studies conducted over the last 20 years, the reliability of oocyte cryopreservation has only recently been confirmed (Borini *et al.*, 2008; Cobo *et al.*, 2008c; Nagy *et al.*, 2008; Rienzi *et al.*, 2010). The subject of cyropreservation has been the focus of much research, as it has a potential use in several areas of infertility treatment apart from the abovementioned oocyte-banking for ovum donation. For example, it could be of interest in the contexts of cancer patients seeking to preserve their fertility before undergoing potentially sterilizing therapy (Cobo *et al.*, 2008b; Sanchez-Serrano *et al.*, 2009), women who wish to delay their motherhood due to a variety of reasons (Homburg *et al.*, 2008), IVF teams and couples subject to government restrictions (Boggio, 2005; Borini *et al.*, 2006), ethical objections to embryo cryopreservation, and the unavailability of a male gamete on the day of pick up (Vajta and Nagy, 2006; Cobo *et al.*, 2008a).

Despite this diverse range of potential applications, oocyte-banking is not a routine procedure: due to the poor track record of the methodology used to cryopreserve human oocytes, results have not always been reproducible. Recent reports point to an improvement, especially with respect to methods of vitrification (Kuwayama *et al.*, 2005; Cobo *et al.*, 2008c; Nagy *et al.*, 2008; Rienzi *et al.*, 2010). Nevertheless, no large, adequately designed, prospective studies have been conducted to evaluate the effectiveness of oocyte cryobanking. The aim of the current prospective, randomized, controlled-clinical trial was to assess whether there is a major difference between the ongoing pregnancy rates (OPR) obtained with fresh versus vitrified oocyte-banked oocytes in our ovum donation programme.

Materials and Methods

Participants and study design

This study was approved by the institutional review board on the use of human subjects in research at the Instituto Valenciano de Infertilidad and complies with the Spanish Law of Assisted Reproductive Technologies (14/2006, 26th May). This clinical trial was registered at the system ClinicalTrials.gov with the identification number NCT00785993 (accessed 11 November 2008).

Six hundred patients of an eligible population of 1032, who were treated between November 2008 and September 2009 and with indications for ovum donation, were randomized into two groups that received either vitrified oocytes from our oocyte-bank ($n = 300$) or fresh oocytes ($n = 300$).

The inclusion criteria for donors were (i) women of good physical and mental health, under 35 years old, with regular menstrual cycles of 21–35 days and no family history of hereditary or chromosomal diseases; (ii) normal karyotype; (iii) body mass index (BMI) of 18–29 kg/m^2; (iv) normal ovarian response to gonadotrophins (~8–15 follicles); (v) absence of polycystic ovaries, endometriosis, more than two previous miscarriages, or gynaecological or medical disorders and (vi) a negative result in a screening for sexually transmitted diseases. All donors signed a written informed consent form. Recipients were included in the study if they fulfilled the following criteria: (i) 18–49 years of age; (ii) BMI of 18–29 kg/m^2 at the time of randomization and (iii) less than three previous failed IVF attempts. All recipients also signed an informed consent form.

This study was designed following the revised recommendations of the CONSORT statement for improving the quality of reports of parallel-group randomized trials (Moher *et al.*, 2001). This was a randomized, triple-blind, single-centre, parallel-group study comparing the use of vitrified banked-oocytes with fresh oocytes in our ovum donation programme. Patients underwent one treatment cycle during the study. They were randomly assigned 1:1 to vitrification or fresh groups based on a computer-generated randomization list prepared by an independent statistician not involved in the rest of the study.

The randomization took place in a consultation prior to initiation of endometrial preparation on the same day recipients agreed to participate in the study. Recipients were prospectively assigned to one of the two groups by the study nurse. The patients were not aware of the origin of the oocytes received (fresh or vitrified).

In addition, the gynaecologist in charge of the patient was unaware of the treatment allocation; as a consequence, endometrial preparation was started in the same way in both groups, irrespective of the randomization assignment. The coordinating assistant of our ovum donation programme was informed about the treatment allocation in order to match the donors and recipients appropriately. In addition, on the day of ovum donation, the coordinating embryologist of our IVF laboratory was instructed by the donor-oocyte coordinating assistant about the assignment of vitrified or fresh oocytes. The laboratory personnel in charge of the warming procedure were also instructed. All the remaining embryologists and laboratory personnel in charge of evaluating fertilization, embryo development and embryo selection for transfers were blinded to the treatment allocation. Finally, the statistician responsible for the statistical analysis was also blinded to allocation of the study groups throughout the study. The primary end-point of the current study was the OPR per randomized recipients. Ongoing pregnancy was defined during transvaginal ultrasound as the presence of at least one viable fetus 10–11 weeks after embryo transfer. The secondary end-points were as follows; clinical pregnancy rate (CPR), confirmed by detection of one or more gestational sacs during transvaginal scan 3 weeks after embryo transfer; implantation rate (IR), defined as the number of gestational sacs observed during the aforementioned scan divided by the number of transferred embryos; fertilization rate (FR) and embryo quality. Miscarriage rate was defined as the percentage of pregnancies that terminated before the end of week 20 of gestation after detection of the embryo's heart beat during the ultrasound scan.

Stimulation protocol for donors

The protocol for controlled ovarian stimulation (COH) in donors has been previously described (Soares *et al.*, 2005). In short, donors were down-regulated with daily doses of a GnRH-agonist (Decapeptyl®, 0.1 mg; Ipsen Pharma, Barcelona, Spain) and, after menses, COH was initiated

with 225 IU/day of recombinant FSH (Gonal-F®; Merck-Serono, Barcelona, Spain; or Puregon®; Shering-Plough, Madrid, Spain) or hMG (Menopur®, Ferring Pharmaceuticals, Madrid, Spain). The dose was adjusted to the ovarian response. Stimulation was carried out until leading follicles had a mean diameter of ≥18 mm. Recombinant human chorionic gonadotrophin (Ovitrelle®, Merck-Serono) was then administered and oocyte retrieval was performed 36 h later. Anonymous donors were matched with their recipients according to phenotype and blood groups. In all cases, all of the oocytes retrieved from one stimulated donor were donated to a single recipient.

Endometrial preparation for oocyte recipients

The protocol for endometrial preparation has also been described elsewhere (Soares *et al*., 2005). Women with ovarian function were first down-regulated in the luteal phase with a single-dose of GnRH-agonist depot (Decapeptyl®, 3.75 mg; Ipsen Pharm or Gonapeptyl® 3.75, Ferring). After menses, all subjects received oral estradiol valerate (EV) (Progynova®, Schering Spain, Madrid, Spain), starting with a daily dose of 2 mgs, that increased to 6 mgs. Approximately 10–15 days after initiation of EV, serum E_2 levels and endometrial thickness were determined. Administration of micronized progesterone (P) (800 mg/day, vaginally) (Progeffik, Effik Laboratories, Madrid, Spain) was initiated the day after oocyte donation.

Oocyte handling

The oocytes of donors intended for oocyte-banking were maintained in fertilization media (Sage Inc., A Cooper Surgical Company™; Bedminster, NJ, USA) for 2 h after ovum pick-up, and then enzymatically denuded (Sage Inc., A Cooper Surgical Company™). Vitrification was carried out immediately after assessing nuclear maturity. Only Metaphase II (MII) oocytes were vitrified. Insemination was performed 2 h after warming by means of ICSI.

Fresh oocytes were maintained in the media for 4 h following oocyte retrieval. They were then denuded and inseminated by ICSI. Zygotes and embryos derived either from vitrified or fresh oocytes were cultured to beyond Day 3 in cleavage media (Sage Inc., A Cooper Surgical Company™). Embryo culture was performed following the routine protocol in our IVF laboratory under oil in 20 μl drops of culture media.

Embryo quality was assessed on Day-2 or Day-3. Top quality Day-2 embryos were defined as those with 2–4 symmetric cells and ≤15% Types I–II fragmentation and absence of multinucleation. Top quality Day-3 embryos were defined as 6–8 cells and ≤20% Types I–II fragmentation, symmetry Grades 1–2 and less than 20% of multinucleation. Fragmentation of Type I was minimal in volume and typically associated with only one blastomere; fragments of Type II were localized predominantly and occupied the periviteline space (Alikani *et al*., 1999).

In all cases, embryo transfer was performed on Day 3 and surplus embryos suitable for additional cryopreservation were vitrified (Kuwayama *et al*., 2005). In the case of patients receiving cryo-banked oocytes, this additional cryopreservation is referred to as 're-vitrification' because it is related to the vitrification of embryos developed from vitrified oocytes.

Oocyte vitrification/warming

The cryotop method employed for oocyte vitrification was that described by Kuwayama *et al.* (2005), with minimal modifications.

Oocytes were equilibrated at room temperature for 15 min in 7.5% (v/v) ethylene glycol (EG) + 7.5% dimethylsulfoxide (DMSO) in TCM199 medium +20% synthetic serum substitute (SSS), referred to as 'equilibrium solution' (ES). As in most cases more than eight oocytes were equilibrated at the same time, they were checked for recovery of their initial shape at 12 min; if possible, they were subjected to vitrification step at this point. They were then placed in 'vitrification solution-VS' that was the same as ES except that the concentrations were 15% EG + 15% DMSO + 0.5 M sucrose. After 1 min in this solution, oocytes were placed on the cryotop strip (Kuwayama *et al*., 2005) and immediately submerged in liquid nitrogen (LN). No more than four oocytes per cryotop were loaded. For warming, the cryotop was removed from the LN and instantly placed in 1.0 M sucrose in TCM199 + 20% (SSS) at 37°C. After 1 min, oocytes were placed in 0.5 M sucrose in M199 + 20% SSS at room temperature for 3 min. Finally, one 5-min wash followed by one 1-min wash was performed with TCM199 + 20% SSS at room temperature prior to incubating the oocytes in fertilization media for 2 h before ICSI. All materials required for vitrification were obtained from Kitazato (Tokyo, Japan). Vitrified cryo-banked oocytes were quarantined for a minimum of 6 months and were assigned after verifying the sero-negativity of the donor.

Statistical analysis

Calculation of sample size was based on the comparison of two binomial proportions, assuming OPRs of 40–52% with the donation of vitrified and fresh oocytes, respectively. This analysis, based on a two-sided significance level, α of 0.05 and a power of 80%, revealed that at least 287 cycles would be necessary for each group.

This study was designed to establish the superiority of fresh cycles versus vitrified ones in terms of OPR. For this purpose, a likelihood ratio-based test in a logistic regression analysis expressed as odd ratios (ORs) with 95% confidence intervals (CIs) was employed.

A possible conversion from superiority to non-inferiority was set up between vitrified and fresh oocytes with respect to the OPR, based on a pre-defined non-inferiority limit of 0.66 for the OR of vitrified versus fresh oocytes (corresponding to limits of −9 to −10% in the difference in proportion scale with an overall OPR of 40–50%). The non-inferiority criteria were based on our clinical data and those differences which were considered clinically relevant.

The primary end-point was analysed with respect to the intention-to-treat (ITT) population (all randomized patients) and treated patients, according to the number of oocytes allocated to the recipients. Additionally, clinical and OPRs were calculated per-transfer population to confirm the robustness of findings. Secondary end-points were analysed by means of the χ^2 test and Student's *t*-test for categorical comparisons or Fisher's exact test, as appropriate. A *P* value of < 0.05 was considered significant.

A logistic regression analysis was conducted to verify the influence of male factor (MF) as a potential confounding variable possibly influencing the FR. The statistical analysis was performed using the Statistical Package for Social Sciences for Windows version 15.0 (SPSS, Chicago, IL, USA) and MedCalc software (Belgium).

Results

A total of 1032 potential recipients were screened for eligibility, of whom 600 were randomized to one of two groups. Five recipients allocated to the vitrification group discontinued the treatment due to personal reasons, and 11 from the group of patients receiving fresh oocytes were cancelled due to endometrial bleeding while awaiting the availability of a suitable donor. As a result, 295 (vitrified cryo-banked oocytes) and 289 (fresh oocytes) recipients were finally treated (Fig. 1).

Indications for oocyte donation in the group of women receiving cryo-banked oocytes were: advanced maternal age ($n = 161$); low

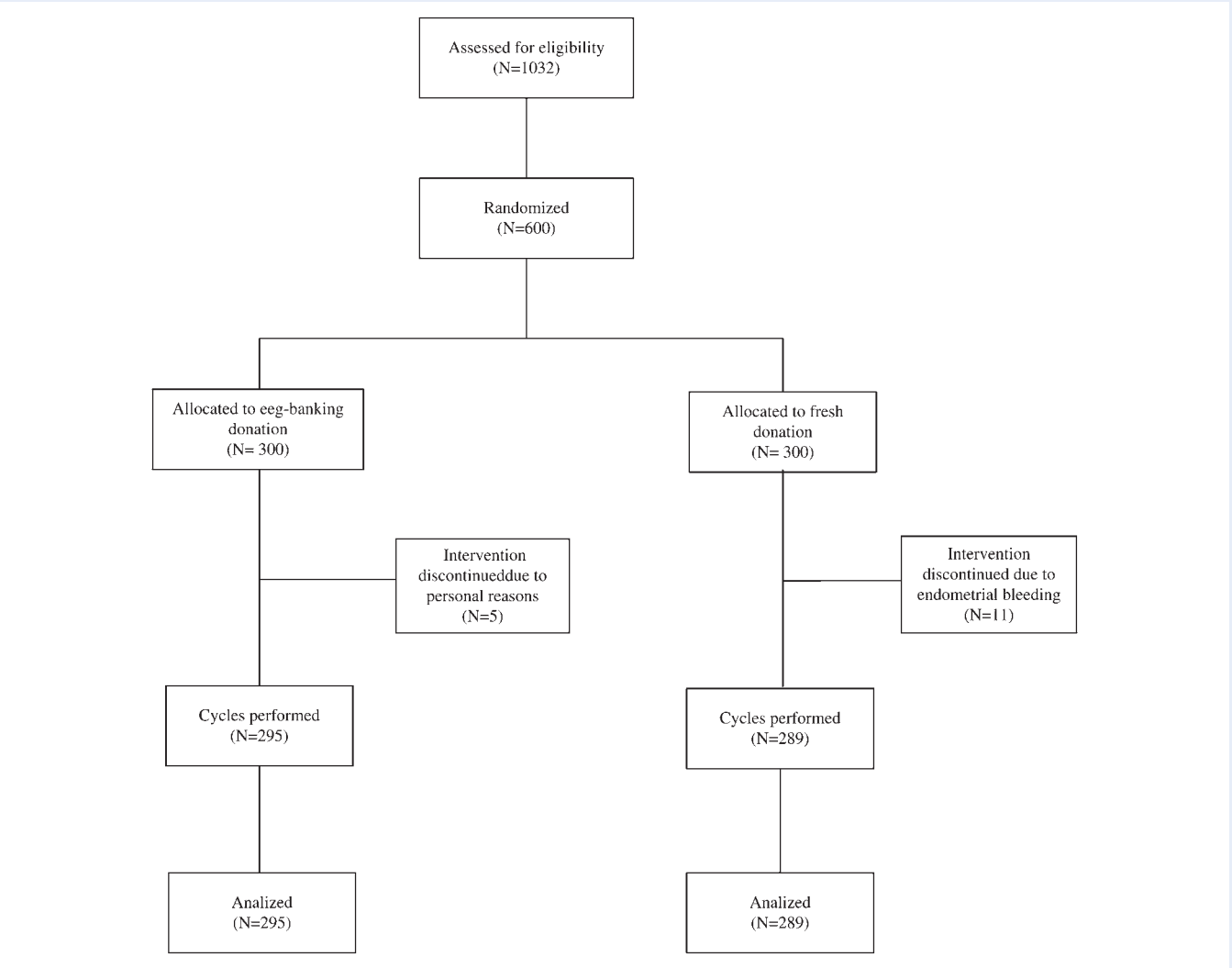

Figure 1 Study flow chart.

response to gonadotrophins ($n = 50$); premature ovarian failure ($n = 26$); endometriosis ($n = 20$); menopause ($n = 14$); recurrent implantation failure in IVF ($n = 10$); genetic conditions ($n = 8$); poor oocyte quality ($n = 7$) and recurrent miscarriage ($n = 4$). Women receiving fresh oocytes underwent ovum donation due to advanced maternal age ($n = 165$); low response to gonadotrophins ($n = 52$); premature ovarian failure ($n = 28$); endometriosis ($n = 13$); menopause ($n = 14$); repeated implantation failure ($n = 13$); genetic conditions ($n = 4$); poor oocyte quality ($n = 7$) and recurrent miscarriage ($n = 4$) ($P = 0.823$).

The MF distribution in the group of patients receiving vitrified cyro-banked oocytes was as follows: no severe MF ($n = 241$); severe MF ($n = 28$); sperm bank semen ($n = 25$); obstructive MF ($n = 5$) and secretory MF ($n = 1$). The distribution of patients receiving fresh oocytes was: no severe MF ($n = 247$); severe MF ($n = 35$); sperm bank ($n = 14$) obstructive MF ($n = 3$) and secretory MF ($n = 1$). There were no statistical differences between the distributions of the groups ($P = 0.365$). In addition, logistic regression analysis revealed no correlation between MF and FRs.

Demographic baseline characteristics of donors and ovarian stimulation parameters are shown in Table I. No difference was found between groups with respect to age or BMI ($P > 0.05$). Regarding ovarian stimulation parameters, there was no difference in the duration of COH, number of days of administration of GnRHa, rFSH dose or serum progesterone and E_2 levels on day of hCG administration between donors whose oocytes were banked and those who donated fresh oocytes. In addition, the number of MII oocytes retrieved was similar in both groups (Table I). As shown in the same table, a total of 3039–3826 oocytes survived vitrification (92.5%).

Table II summarizes recipients' baseline characteristics and embryo development parameters according to the type of oocytes received. No difference in age, BMI, endometrial thickness or mature oocytes received was found between the two groups. The duration of the endometrial preparation among patients receiving vitrified versus fresh oocytes was statistically different (15.5 ± 4.6 and 22.4 ± 5.4 days, respectively; $P < 0.0001$). There was no difference between patients receiving vitrified cryo-banked oocytes versus those receiving

Table I Demographics, baseline characteristics and ovarian stimulation of donors.

	Egg-bank	Fresh
Number of subjects	295	289
Age (years)	26.7 ± 3.9	26.6 ± 3.8
BMI (Kg/m^2)	22.6 ± 3.2	22.5 ± 3.0
Days of stimulation	11.1 ± 2.9	11.8 ± 21.8
GnRHa (days)	18.1 ± 3.2	18.3 ± 3.4
rFSH dose (IU)	1814 ± 635	1774 ± 621
E_2 on day of hCG (pg/ml)	2879 ± 1172	2892 ± 1201
P_4 (ng/ml)	1.0 ± 0.7	0.9 ± 0.7
MII oocytes retrieved (mean ± SD)	3286 (11.1 ± 3.2)	3185 (11.0 ± 2.8)
Survival rate	3039 (92.5)	–

Unless otherwise indicated values are mean ± SD or *n* (%). BMI, body mass index; rFSH, recombinant FSH; GnRHa,GnRH agonist.

Table II Recipients' base line characteristics and embryo development according to the type of oocytes received.

	Egg-bank	Fresh
Number of subjects	295	289
Age (years)	41.10 ± 4.3	41.35 ± 4.5
BMI (Kg/m^2)	24.2 ± 4.2	23.4 ± 4.2
Previous ovum donation attempts	0.33 ± 0.8	0.34 ± 0.7
Days of endometrial preparation	15.5 ± 4.6	22.4 ± 5.4*
Oocytes received (mean ± SD)	3039 (10.3 ± 2.9)	3185 (11.2 ± 3.4)
Endometrial thickness (mm)	8.2 ± 0.4	7.9 ± 1.1
Fertilization rate (2PN)	2256 (74.2)	2334 (73.3)
Embryo cleavage Day-2	2151 (95.3)	2240 (96.0)
Embryo cleavage Day-3	1877 (87.3)	1976 (88.2)
Top quality Day-2 embryos/ inseminated oocyte	938 (30.8)	983 (30.8)
Top quality Day-2 embryos/ cleaved embryo	938 (43.6)	983 (43.8)
Top quality Day-3 embryos/ inseminated oocyte	1098 (36.1)	1201 (37.7)
Top quality Day-3 embryos/ cleaved embryo	1098 (58.4)	1201 (60.7)

Unless otherwise indicated values are mean ± SD or *n* (%). BMI, body mass index; GnRHa, GnRH agonist.
* < 0.0001.

fresh oocytes with respect to fertilization (74.2 versus 73.3%; $P = 0.393$) or embryo cleavage on Day-2 (95.3 versus 96.0%; $P = 0.297$) or on Day-3 (87.3 versus 88.2%; $P = 0.335$), respectively. Moreover, the proportion of top-quality embryos obtained by inseminated oocyte (30.8 versus 30.8% for Day-2 and 36.1 versus 37.7% for Day-3, respectively) or by cleaved embryos (43.6 versus 43.8% for Day-2 and 58.4 versus 60.7% for Day-3, respectively) was similar in both groups ($P > 0.05$).

Table III Clinical outcome according to the type of oocytes received

	Egg-bank	Fresh
Number of embryos transferred	267 (90.5)	259 (89.6)
Mean number of embryos replaced	513 (1.74 ± 0.7)	498 (1.72 ± 0.7)
Number of cycles with embryo 're-vitrification'/cryopreservation	196 (66.7)	216 (74.7)*
Mean number of re-vitrified or cryopreserved embryos	592 (2.0 ± 2.1)	743 (2.5 ± 2.3)*
Implantation rate	205 (39.9)	204 (40.9)
Positive hCG test/cycle	165 (55.9)	159 (55.0)
Clinical pregnancy rate/cycle	148 (50.2)	144 (49.8)
Positive hCG test/transfer	165 (61.8)	159 (61.4)
Clinical pregnancy rate/transfer	148 (55.4)	144 (55.6)
Twin pregnancy rate	48 (32.4)	54 (37.5)

Unless otherwise indicated values are mean ± SD or *n* (%).
*$P < 0.05$.

Table IV Primary outcome, OPR, according to the type of oocytes received.

	Egg-bank	Fresh
Ongoing pregnancy rate/ITT	131 (43.7)	125 (41.7)
Ongoing pregnancy rate/cycle	131 (44.4)	125 (43.3)
Ongoing pregnancy rate/transfer	131 (49.1)	125 (48.3)

Unless otherwise indicated values are mean ± SD or *n* (%).
ITT, intention to treat.

IVF outcomes were assessed in all cases and are shown in Table III. A total of 526 patients underwent embryo transfer, corresponding to 90.5% ($n = 267$) and 89.6% ($n = 259$) of patients receiving vitrified or fresh oocytes, respectively ($P = 0.783$). The main reason for cancellation of embryo transfer was a lack of transferable embryos on Day 3 due to the poor quality of the embryos available. The mean number of embryos transferred was similar in the two groups (1.74 ± 0.7 versus 1.72 ± 0.7; $P = 0.772$). Both the number of cycles with surplus embryo cryopreservation and the number of embryos vitrified or 're-vitrified' were higher for the group of patients receiving fresh oocytes ($P < 0.05$).

Implantation (39.9 versus 40.9%; $P = 0.745$) and CPR per cycle (50.2 versus 49.8% ; $P = 0.933$) and per embryo-transfer (55.4 versus 55.6%; $P = 0.974$) were similar for patients receiving vitrified or fresh oocytes. No differences were observed between the two groups regarding twin-pregnancy rate. No multifetal pregnancy occurred (Table III).

The OPR per ITT was 43.7% in the vitrification group and 41.7% in the fresh group (Table IV). The OR of ongoing pregnancy was 0.921 in favour of vitrification group. Nevertheless, the 95% CI was 0.667–1.274, and consequently, the superiority of the OPR of the fresh

group was not confirmed ($P = 0.744$). The non-inferiority of the vitrified group in relation to the fresh group was demonstrated with a margin of 0.667, which is above the pre-established non-inferiority limit of 0.66. These findings were supported by the analysis of the primary end-point on a per-treated patient basis: OPR was 44.4% and 43.3% for patients receiving vitrified or fresh oocytes, respectively. The OR of ongoing pregnancy was 0.954 in favour of the vitrification group. Nevertheless, the superiority of the fresh group was not proven (95% CI = 0.688–1.323; $P = 0.779$). The non-inferiority of vitrified cryo-banked oocytes versus fresh oocytes was reconfirmed, as the inferior limit of 0.688 was well above the pre-established non-inferiority limit of 0.66.

Discussion

This study of a large number of patients aimed to confirm the effectiveness of oocyte cryo-storage in an ovum donation programme. No differences were confirmed with respect to the primary end-point (i.e. OPR) when calculated on a per-ITT or per-treated patient basis. Moreover, the results failed to demonstrate the superiority of fresh oocytes over that of vitrified oocyte-banked oocytes. These findings raise highly relevant issues that may open a new range of possibilities in ART.

The non-inferiority of vitrified oocytes in relation to OPR was also assessed on the basis of a predefined non-inferiority limit. However, it is necessary to underline that the non-inferiority test used in the current study, initially designed as a superiority trial, could be interpreted as border-line and subjected to relatively arbitrary limits. A strictly non-inferiority design would be better to arrive at this conclusion. Nonetheless, the clinical efficiency of vitrified oocytes has been clearly demonstrated herein.

One of the greatest advantages of oocyte cryo-banking is the elimination of waiting lists, as these oocytes can be donated as soon as the recipient's endometrial preparation is completed. Our protocol of endometrial preparation has been previously described as a 'prolonged follicular phase protocol' (Remohi *et al.*, 1995, 1997; Soares *et al.*, 2005; Budak *et al.*, 2007). Patients with ovarian function are desensitized through administration of GnRHa, which is followed by estrogen replacement via application of E_2 valerate. Although recipients are ready to receive embryos within ~2 weeks, the unopposed administration of E_2 valerate can be maintained for a maximum of 50 days until a suitable donation becomes available. If vaginal spotting or bleeding occurs in the meantime, recipients must initiate a new cycle of endometrial preparation. In the current study, after applying the same schedule of endometrial preparation to both groups, the mean number of days of E_2 replacement was statistically higher in the group of patients receiving fresh oocytes. This may be explained by the fact that, in the case of cycles using cryo-banked oocytes, the patient and donor could be matched as soon as the former were ready for donation. These data clearly demonstrate the positive impact of oocyte-banking on the management of an oocyte donation programme.

An additional aspect concerning oocyte-banking for oocyte donation is that this strategy makes the procedure safer by permitting a more accurate screening of infectious diseases among donors, as occurs with cryopreserved semen. In the current study, cryo-stored oocytes were considered for donation after a minimum of 6 months quarantine, after which a donor's serology was confirmed. To the best of our knowledge, the present study, together with another by our group (Cobo *et al.*, 2010), is the first to report 6-month banking of oocytes (with the aim of screening donors twice prior to donation) and to provide evidence that oocyte banking is technically feasible and yields OPRs similar to those obtained with fresh embryos. We believe that these data may represent a breakthrough in the current practice of oocyte donation, as they highlight the feasibility of a safer approach to oocyte donation. We did not analyse the health of the infants born within this study, as our primary end-point was pregnancy rates. This could be possible study bias, due to the knowledge of the safety aspects, and all possible implications of any new clinical application is of a great value. Obviously, the comparison of obstetric and perinatal outcomes of babies born after the use of fresh versus frozen oocytes is completely advisable. However, our own experience (unpublished data on 250 babies born after oocyte vitrification) and previous reports have found no difference in malformations between children born after vitrification of oocytes and those born through IVF (Chian *et al.*, 2008; Noyes *et al.*, 2009).

The current study reveals statistical differences between groups in the number of patients who vitrified surplus embryos and the number of cryopreserved embryos. It is difficult to explain the causes of such differences, but it may be relevant that the number of vitrified oocytes eventually inseminated was slightly lower than, but not statistically different from, the number of fresh oocytes subjected to ICSI. A larger sample would probably be required to clarify the possible role of embryo quality in the lower performance of cryo-banked oocytes related to the availability of surplus embryos for additional vitrification in comparison with that of fresh oocytes. Nonetheless, re-vitrified embryos were achieved in 66.7% of patients, and we consider that this percentage, in conjunction with the OPR of 49.1%, reflects an excellent yield of the cryo-banking strategy. Other advantages of this technique are discussed below.

The cryopreservation technique employed in this study, namely, the cryotop vitrification method, was introduced in our laboratory in late 2006. This approach circumvents the chilling injury caused by low temperatures by employing extremely high cooling rates (Kuwayama, 2007). Furthermore, the low volume of VS used for loading the oocytes and embryos helps to minimize the probability of ice nucleation (Arav, 1992). An additional advantage of this procedure is related to the lower cryoprotectant concentration required, which reduces the cytotoxic effect of the substances used in vitrification (Vajta and Kuwayama, 2006). Together, these aspects contribute to the high performance of this methodology, which has been reported by several authors, including our own group (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.*, 2008a, b, c; Chang *et al.*, 2008; Keskintepe *et al.*, 2008; Nagy *et al.*, 2008; Rienzi *et al.*, 2010).

Oocyte donation using cryotop-vitrified/warmed oocytes has also been evaluated previously by others. Nagy *et al.* (2008) reported high FRs and similar embryo development when comparing the use of vitrified and fresh oocytes in their ovum donation programme, with both methods leading to high pregnancy and IRs. These conclusions have been confirmed very recently in a prospective, randomized sibling study conducted with autologous oocytes (Rienzi *et al.*, 2010). Fertilization and embryo developmental rates after oocyte vitrification using the cryotop method were shown not to be inferior to those obtained after insemination of fresh oocytes.

It is worth mentioning that this very efficient approach needs the direct contact with LN during the vitrification step in order to achieve the extremely high cooling rates required (Vajta and Kuwayama, 2006). This condition has been considered as a major inconvenience due to safety issues related to the potential role of LN as a source of cross contamination. Increasing concerns have been generated since the report of cross contamination with hepatitis B virus within a bone marrow transplantation programme (Tedder *et al.*, 1995). However, there is no other evidence of such contamination after the cryo-storage of biological samples and their subsequent use in humans, including within the ART clinical practice. The contamination of LN can occur during transportation between the manufacturer and IVF laboratory, as a result of exposure to environmental agents, while re-filling the tanks once in the IVF facilities, or by introducing infected samples. Theoretically once the LN is contaminated, samples subsequently stored inside can also be affected. From a practical point of view, there are two hypothetical steps in which samples vitrified using open devices may become contaminated: firstly during de vitrification itself and secondly during the storage period. Consequently, as suggested by Vajta *et al.* (2009), it sounds reasonable to separate cooling from storage. Several strategies have been suggested to prevent the potential risk of transmission of pathogen agents at both points. With respect to the first point, some authors have recommended the use of double packing or wrapping of the samples (Kuleshova and Shaw, 2000). However, such measures may thermally isolate the sample, therefore altering its viability (Vajta *et al.*, 2009). A more appropriate approach to safe vitrification would be to isolate the open device once the samples have been vitrified for storage purposes. This alternative can be carried out as proposed by this author by using sterile pre-cooled straws that could be heat sealed after vitrification (Vajta *et al.*, 1998). Needless to say, it is advisable to carry out the vitrification step in a completely sterile environment. The use of commercially available filters that can guarantee a 0.2 μm filtration or the UV irradiation of LN have been recommended (Parmegiani *et al.*, 2009). On the other hand, the authors are involved in the current development of a filter device suitable to be used at IVF facilities, and capable of offering a solution for a safer vitrification process. Regarding the storage, the use of vapour freezers has been also recommended. Theoretically, contamination of vials stored in the vapour phase of nitrogen should be significantly lower than that of those submerged in LN since the density of environmental airborne contaminants is lower than in the liquid phase. In a very recent report, we have proposed the use of vapour storage freezers in order to avoid the direct contact with LN during the storage time (Cobo *et al.*, 2010). This approach, in combination with the use of purified LN, would allow us to work within a security margin that may guarantee the elimination of the risk of cross contamination while maintaining the effectiveness of the cryopreservation method.

The OPR obtained in the current study after oocyte vitrification/storage demonstrates that cryo-banking can provide successful clinical outcome in oocyte donation programmes. The validation of this strategy will no doubt be of great importance to ovum donation procedures, as it allows traditional drawbacks associated with the use of fresh oocytes to be overcome.

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