How does vitrification affect oocyte viability in oocyte donation cycles? A prospective study to compare outcomes achieved with fresh versus vitrified sibling oocytes

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STUDY QUESTION: How does vitrification affect oocyte viability?

SUMMARY ANSWER: Vitrification does not affect oocyte viability in oocyte donation cycles.

WHAT IS KNOWN ALREADY: Oocyte vitrification is performed routinely and successfully in IVF and oocyte donation programs.

STUDY DESIGN, SIZE, DURATION: This is a prospective study performed between June 2009 and February 2012 to compare ongoing pregnancy rates and other indices of viability between fresh and vitrified oocytes. A total of 99 donations with more than 16 oocytes (MII) in which oocytes were allocated both to a synchronous recipient (fresh oocytes) and to an asynchronous recipient (vitrified oocytes) were included.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The participants were consenting couples (donors and recipients) from the oocyte donation program. On the day of retrieval, the oocytes allocated to the synchronous recipient were inseminated and those allocated for banking were denuded of cumulus and vitrified. Vitrified oocytes were microinjected with spermatozoa 2 h after warming. Embryo transfer was performed on Day 2 of development in both groups, and the remaining embryos were cryopreserved on Day 3. Clinical pregnancy was defined by a positive fetal heartbeat at 6 weeks.

MAIN RESULTS AND ROLE OF CHANCE: A total of 989 oocytes were warmed and 85.6% survived. No significant differences were observed between fresh and vitrified oocytes: fertilization rate (80.7 versus 78.2%), ongoing embryo rate (71.0 versus 68.2%) or good-quality embryo rate (54.1 versus 49.8%). The mean number of embryos transferred was similar in both groups (1.82 ± 0.44 versus 1.90 ± 0.34). The implantation rate (33.3 versus 34.0%) and the multiple pregnancy rate (27.7 versus 20.8%) were also similar between both groups (P > 0.05). The live birth rate per cycle was 38.4% in the recipients of fresh oocytes and 43.4% in the recipients of vitrified oocytes (P > 0.05). Eightyfive frozen embryo transfers were also evaluated. Comparing embryos from fresh and vitrified oocytes there were no significant differences in the embryo survival rate (70.1 versus 65.8%), clinical pregnancy rate (40.8 versus 33.3%) or implantation rate (21.8 versus 26.8%).

LIMITATIONS, REASONS FOR CAUTION: The oocytes were donated by healthy, young women (≤35 years) and these results cannot be extrapolated to other populations.

WIDER IMPLICATIONS OF THE FINDINGS: Outcomes obtained with vitrified oocytes are as good as with fresh oocytes and the use of vitrification can be extended to new applications, e.g. accumulation of oocytes from successive stimulations for preimplantation genetic diagnosis, for patients at risk of ovarian hyperstimulation syndrome or in patients needing to preserve their fertility.
Introduction

Historically, slow freezing has been the most widely used method for the cryopreservation of embryos and oocytes since the first live births resulted from this technique in the mid-80s. The optimization of the vitrification method originally described by Rall and Fahy (1985) has brought about a complete revolution in the field of cryopreservation, successfully establishing vitrification as a routine procedure in many IVF laboratories.

During vitrification the cells are exposed to high cooling rates in media with high concentrations of cryoprotectant and so avoiding ice-crystal formation (intrinsic to slow freezing). This protection against ice-crystal formation has increased the survival rates of both oocytes and embryos as reported in two published meta-analyses (Oktay et al., 2006; Cobo and Diaz, 2011).

The first pregnancy with vitrified oocytes was published by Kuleshova et al. (1999), whose protocol included high concentrations of a cryoprotectant. Other subsequent isolated reports followed (Yoon et al., 2000; Wu et al., 2001) until Kuwayama’s protocol proposed in 2005 with the Cryotop® vitrification system rapidly expanded and proved to be the most efficient (Katayama et al., 2003; Kuwayama et al., 2005). Kuwayama’s method was used afterwards by other groups who reported no effects on the viability of oocytes in oocyte donation programs (Lucena et al., 2006; Cobo et al., 2008; Nagy et al., 2009).

Thereafter vitrification has become well established and is currently the most widely used technique for oocyte cryopreservation in daily practice as it achieves outcomes similar to those obtained with fresh oocytes (Almodin et al., 2010; Cobo et al., 2010; Rienzi et al., 2010; Parmegiani et al., 2011).

Oocyte donation is nowadays a widespread assisted reproductive technology (ART) which allows many women without or with defective oocytes to achieve a pregnancy. Up to now oocyte donation usually involved, the synchronization of the donor and the recipient. Oocyte cryopreservation avoids the scheduling step by creating oocyte banks. It allows the patients and the medical team to choose the best timing for both medical and the patient’s personal reasons.

The aim of the present study was to compare prospectively the outcomes with fresh and vitrified oocytes from the same donor but different recipients. The strategy of using sibling oocytes removes the contribution of between-donor variation and allows the possible effects of vitrification on the viability of the oocytes and the resulting embryos to be assessed more efficiently.

Materials and Methods

The study included all oocyte donation cycles performed between June 2009 and February 2012 in which some oocytes were used for a synchronous fresh donation to one recipient and others were vitrified, banked and subsequently allocated to a different recipient for an asynchronous donation. All recipients included in the study were informed and completed written consent forms.

The donors included in the study fulfilled the clinical and legal requirements requested by the Spanish regulations on ART (RD 1301/2006) described before by Clua et al. (2010). For the proper scheduling of the cycle all donors were treated with contraceptive pills during the month prior to stimulation. Five days after discontinuing the pills, stimulation with recombinant FSH (r-FSH) (Puregon Pen®, MSD, Madrid, Spain) was started at a dose of 150–200 IU/day according to the donor’s BMI. From Day 5 of stimulation donors were monitored by plasma estradiol measurements and transvaginal ultrasound scans every 2 days and doses were adjusted individually. Once a dominant follicle measuring >14 mm was observed a GnR antagonist (ganieplex acetate 0.25 mg; Orgalutran®, MSD, Madrid, Spain) was administered daily. Finally, when at least three follicles measuring ≥20 mm were observed recombinant hCG (r-hCG) or a bolus of GnRH agonist was administered (triptoreline 0.3 mg, Decapeptyl®, Ipsen Pharma, Barcelona, Spain) to trigger ovulation.

The recipients included in the study were women <50 years from the waiting list of our oocyte donation program. They were scheduled with the administration of an i.m. injection of leuprolrelin depot 3.75 mg (Ginecin Depot®, Abbott Laboratorones, S.A., Madrid, Spain) on Days 20–22 of the previous cycle if destined for synchronous donation this was timed when the donor was starting the last week of contraceptives. Endometrial preparation included estradiol valerate (EV) 6 mg/day (Progynova®, Bayer-Schering, Madrid, Spain) during 14–16 days followed by EV and vaginal micronized progesterone 600 mg/day (Utrogestan®, Seid, Barcelona, Spain) both for the fresh and vitrified cycles (Martinez et al., 2011).

A total of 2087 oocytes from 99 oocyte donation cycles in each of which a minimum of 16 metaphase II oocytes (MII) were obtained after follicular aspiration were included in the two groups of the study.

Oocyte vitrification was performed immediately after denudation, 38 h following the administration of r-hCG.

The vitrification method described by Kuwayama et al. (2005) was applied. Commercial kits (Vitrification and Warming KIT, Kitazato, Japan) were used throughout the study. The oocytes (up to 12 simultaneously) were equilibrated in the ‘equilibration solution’ for 8–15 min. After a 1-min wash in the ‘vitrification solution’, a maximum of four oocytes were loaded on the Cryotop strip. Once the excess medium was removed they were immediately plunged into liquid nitrogen.

For the warming process, the Cryotop was removed from the liquid nitrogen and quickly immersed in the ‘warming solution’ at 37°C for 1 min. The oocytes were then placed in the ‘dilution solution’ for 3 min at room temperature (RT) before they were washed twice in the ‘washing solution’ at RT and finally transferred into 0.5 ml culture media (IVF, Vitrolife, Sweden). ICSI was done 2 to 3 h later.

Insemination of fresh oocytes was done either via conventional IVF or by ICSI 39–41 h after r-hCG administration, whereas oocytes from the bank were always inseminated by ICSI.

Fertilization was checked 16–20 h later, zygotes exhibiting two pronuclei were considered as fertilized. Embryos were cultured in microdrops (G1; Vitrolife, Sweden) under mineral oil (Ovoil; Vitrolife, Sweden) in GPS® dishes (LifeGlobal®, USA).

The number of blastomeres, their symmetry, cytoplasmic fragmentation and the occurrence of multinucleation were evaluated on Days 2 and 3. An embryo score ranging between 1 and 10 was used as previously described (Clua et al., 2012).
The third day of progesterone treatment (corresponding to Day 2 of embryo development), uterine embryo transfer of the highest quality embryos was carried out using an Edwards Wallace embryo replacement catheter (SIMS Portex Ltd, UK) coupled to an insulin syringe and guided by ultrasonography. No more than two embryos were transferred per recipient.

Clinical pregnancy was confirmed both by serum level of β-hCG (>75 IU/ml) measured 14–16 days after the transfer and ultrasound visualization of a gestational sac. Patients continued with estrogen and progesterone treatment until the results were known. If positive, hormonal treatment was maintained until the 10th week of gestation. Miscarriage was regarded as a spontaneous abortion described after a confirmed clinical pregnancy. Only clinical pregnancies were considered for data analysis.

 Supernumerary embryos that contained six or more than six cells were cryopreserved on Day 3 of development. Embryos with >35% fragmentation or multinucleated in Days 2 or 3 were discarded. The cryopreservation method used was slow freezing (Lassalle et al., 1985) with available commercial media (Freeze-Kit I and Thaw-Kit I; Vitrolife, Sweden) in 0.25 ml plastic straws (Cryo Bio System, France) in a programmable freezer.

Quantitative variables were compared with the Mann–Whitney test. The χ² test or Fisher’s exact test were used for the categorical variables. All tests were two-tailed with a significance level set to α = 0.05.

Statistical analyses were performed using the statistical package IBM SPSS Statistics 20.0.

Results

The results of this study are based on oocytes given by 99 donors, for each donor some oocytes were used fresh for synchronous recipients and some were vitrified and later allocated to a different assynchronous recipient. In each case, a minimum of eight mature oocytes were allocated per recipient. Table I summarizes the donors baseline characteristics and stimulation parameters (mean ± SD). The presence or absence of male factor of the patients included in the study was also assessed, the distribution being similar in both groups (NS).

The mean of oocytes received by the group of fresh oocytes was statistically higher (11.1 ± 2.53) than the mean of vitrified oocytes received from the bank (10.0 ± 1.68, P < 0.05). Although the survival rates reached 85.6%, the mean of inseminated oocytes after discarding lysed oocytes post-warming was significantly lower 8.6 ± 2.12 compared with the fresh oocytes inseminated (P < 0.01).

Both IVF (32.3%) and ICSI (67.7%) were used to inseminate fresh oocytes. The insemination technique did not affect the outcomes and a similar proportion of ongoing (66.9 versus 72.6%, NS) and good-quality embryos (56.2 versus 51.2%, NS) was obtained. The live birth rate per cycle (34.4 versus 40.3%, NS) was also similar.

No statistically significant differences were found between the two groups in all the embryonic variables analyzed: fertilization, ongoing embryo and good-quality embryo rates (Table II). However, the higher number of inseminated oocytes in the group with fresh oocytes resulted in highly significant differences between the two groups in the mean of fertilized oocytes (9.0 ± 2.77 versus 6.68 ± 2.33, P < 0.01), frozen embryos (4.5 ± 2.67 versus 2.65 ± 2.04, P < 0.01), good-quality embryos (4.8 ± 2.80 versus 3.3 ± 2.16, P < 0.01) and ongoing embryos (6.4 ± 2.59 versus 4.6 ± 2.01, P < 0.01).

Table III summarizes the clinical outcomes. A similar number of embryos was replaced per recipient in both groups, never more than two. As shown in this table no statistically significant differences were observed in clinical pregnancy, ongoing pregnancy, implantation or live birth rates per cycle.

The frozen embryo transfer (FET) from the oocyte donations were also evaluated (Table IV). A total of 54 frozen/thawed embryo transfers have been performed so far in the fresh oocyte group and 30 in the vitrified oocyte group.

The mean of thawed and replaced embryos was similar. No statistically significant differences were observed in the embryo survival rate or in the implantation and clinical pregnancy rates per transfer between the two groups (NS). Four hundred and twenty-eight embryos still remain cryopreserved for future FET.

Discussion

The design of the present study enabled us to analyze the impact of vitrification on the functionality of the oocyte and its capacity to produce an ongoing embryo, the subsequent pregnancy and a healthy live birth in an oocyte donation program. Comparing the results obtained with fresh

Table I  Donor baseline characteristics and stimulation parameters (mean ± SD).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fresh oocytes</th>
<th>Vitrified oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of donors</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>Age (years)</td>
<td>26.1 ± 4.33</td>
<td>24.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.7 ± 2.88</td>
<td>23.2 ± 3.98</td>
</tr>
<tr>
<td>Days of stimulation</td>
<td>8.5 ± 2.59</td>
<td>10.0 ± 1.68</td>
</tr>
<tr>
<td>Total dose of gonadotrophin IU</td>
<td>1515.0 ± 714.92</td>
<td>85.6</td>
</tr>
<tr>
<td>Number of oocytes retrieved</td>
<td>24.9 ± 5.73</td>
<td>91.9</td>
</tr>
</tbody>
</table>

*p < 0.05.
**p < 0.01.
ongoing embryo and good-quality embryo rates were compared. The effects of vitrification with different parameters. Fertilization, implantation rate, and ongoing pregnancy rate were also considered for the analysis without observing differences among recipients since in the work of Ubaldi et al. (2010) the ongoing pregnancy and implantation rates were significantly higher in the fresh oocyte group. These results differ from ours. This may be due to the high mean age of our recipients since in the work of Ubaldi et al. (2010) these differences were not noticed among patients <35 years, which is the matching group by age of the donors in our study. In this regard, it could be speculated that oocytes from young women may be more resistant to vitrification than those from women of a more advanced age.

We compared ongoing pregnancy, implantation and live birth rates between the two groups to confirm the suitability of the vitrification technique, and found no statistically significant differences.

Our results correlate with the comparative studies performed in recipients of oocytes from different cohorts in which no differences were found in terms of clinical pregnancy or implantation rates when comparing vitrified and fresh oocytes (Cobo et al., 2010; Garcia et al., 2011).

Furthermore, the two studies with an experimental design similar to the present work show pregnancy and implantation rates comparable and vitrified oocytes from the same cohort allowed us to assess the effect of vitrification avoiding the extrinsic variables related to oocyte quality.

The presence of male factor and the insemination technique in the recipients was considered for the analysis without observing differences between the two groups. Other aspects that could have an impact on the clinical results of the donation cycles such as the age of the recipients or their BMI were also taken into account, not finding differences between both groups.

The comparison of the results with sibling oocytes allowed the assessment of the effects of vitrification with different parameters. Fertilization, ongoing embryo and good-quality embryo rates were compared between both groups. No statistically significant differences were found in any of the parameters studied between the two groups.

The oocyte survival rate obtained, 85.6%, was slightly lower than reported in other studies that used the Cryotop® system for donor oocytes, these range from 89.0 to 92.5% (Nagy et al., 2009; Cobo et al., 2010; Garcia et al., 2011; Trokoudes et al., 2011). In a recent study, to assess the real biological efficiency, live babies born per oocyte were reported in a retrospective analysis. Patient’s cycles and oocyte recipients were included obtaining increased birth rate per oocyte in cycles where <15 oocytes were obtained (Patrizio and Sakkas, 2009). There is still a debate about whether obtaining large oocyte cohorts (e.g. high ovarian response to stimulation) affects oocyte quality or not.

Our results show a fertilization rate per inseminated oocyte of 78.2% and a good-quality embryo rate per fertilized oocyte of 49.8% in the group receiving vitrified oocytes.

These results concur with those of two other groups reporting a fertilization rate after vitrification/warming of donor oocytes of 85.4% and a good-quality embryo rate of 55.9% (Cobo et al., 2011). In the other study in which the transferred embryos were at the blastocyst stage the fertilization rate was 84.4% and the blastocyst rate 41.3% (Garcia et al., 2011).

Three other studies comparing vitrified and fresh oocytes from the same cohort of donor oocytes have been published. These studies also reported comparable fertilization and embryo viability rates between the two groups. The first study, which included only data on embryo quality to assess the Cryotop method, was published in 2008 by Cobo et al. (2008) reporting a fertilization rate of 76.3% in the group receiving vitrified oocytes and 82.2% in the group receiving fresh oocytes, with a good-quality embryo rate of 59 and 67%, respectively. The second study (Nagy et al., 2009) analyzed a very small sample and the fertilization rate was significantly higher in the group of vitrified oocytes (87 versus 67%). The third study included 36 recipients of vitrified oocytes and 41 recipients of fresh oocytes from the same cohort (Trokoudes et al., 2011). The fertilization rate reported was 84.4% for vitrified oocytes versus 86.6% for fresh oocytes. This group did not find differences in the embryo quality either with a good-quality embryo rate of 58% in recipients of vitrified oocytes and 62% in the group receiving fresh oocytes.

Studies comparing outcomes between fresh and vitrified oocytes in IVF cycles have also been published. In these studies part of the oocytes were allocated to fresh insemination and some were vitrified for subsequent attempts. In the first one (Almodin et al., 2010) the results are comparable between both groups except that statistically significant differences were observed in the number of blastomeres per embryo obtained. In the second study (Ubaldi et al., 2010) the ongoing pregnancy and implantation rates were significantly higher in the fresh oocyte group. These results differ from ours. This may be due to the high mean age of our recipients since in the work of Ubaldi et al. (2010) these differences were not noticed among patients <35 years, which is the matching group by age of the donors in our study. In this regard, it could be speculated that oocytes from young women may be more resistant to vitrification than those from women of a more advanced age.

We compared ongoing pregnancy, implantation and live birth rates between the two groups to confirm the suitability of the vitrification technique, and found no statistically significant differences.

Our results correlate with the comparative studies performed in recipients of oocytes from different cohorts in which no differences were found in terms of clinical pregnancy or implantation rates when comparing vitrified and fresh oocytes (Cobo et al., 2010; Garcia et al., 2011).

Furthermore, the two studies with an experimental design similar to the present work show pregnancy and implantation rates comparable

### Table III: Clinical outcomes of cycles performed with fresh and vitrified sibling donor oocytes.

<table>
<thead>
<tr>
<th></th>
<th>Fresh oocytes (n = 99)</th>
<th>Vitrified oocytes (n = 99)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transferred embryos</td>
<td>1.82 ± 0.44</td>
<td>1.90 ± 0.34</td>
</tr>
<tr>
<td>Clinical pregnancy rate</td>
<td>47 (47.5)</td>
<td>53 (53.5)</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>33.3</td>
<td>34.0</td>
</tr>
<tr>
<td>Ongoing pregnancy rate</td>
<td>39 (39.4)</td>
<td>44 (44.4)</td>
</tr>
<tr>
<td>Miscarriage rate (%)</td>
<td>9 (19.1)</td>
<td>11 (20.8)</td>
</tr>
<tr>
<td>Live birth rate/transfer</td>
<td>38 (38.4)</td>
<td>42 (43.4)</td>
</tr>
<tr>
<td>Multiple pregnancy rate</td>
<td>27.7</td>
<td>20.8</td>
</tr>
</tbody>
</table>

n.s., non-significant.

### Table IV: Clinical outcomes following the transfer of cryopreserved surplus embryos derived from fresh or vitrified oocytes.

<table>
<thead>
<tr>
<th></th>
<th>Fresh oocytes</th>
<th>Vitrified oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cryotransfer cycles</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>Total thawed embryos</td>
<td>187</td>
<td>85</td>
</tr>
<tr>
<td>Thawed embryos/recipient (mean ± SD)</td>
<td>3.46 ± 1.73</td>
<td>2.83 ± 1.64</td>
</tr>
<tr>
<td>Survival rate (%)</td>
<td>70.1</td>
<td>65.8</td>
</tr>
<tr>
<td>Cycles without embryo transfer</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Cycles with embryo transfer</td>
<td>49</td>
<td>24</td>
</tr>
<tr>
<td>Transferred embryos (mean ± SD)</td>
<td>1.98 ± 0.63</td>
<td>1.64 ± 0.81</td>
</tr>
<tr>
<td>Clinical pregnancy/transfer (%)</td>
<td>40.8</td>
<td>33.3</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>21.8</td>
<td>26.8</td>
</tr>
</tbody>
</table>

n.s., non-significant.
between recipients of fresh and vitrified oocytes (Nagy et al., 2009; Trokoudes et al., 2011). Trokoudes et al. describe pregnancy rates of 55.6% in recipients of vitrified oocytes and 48.8% in recipients of fresh oocytes, with implantation rates of 24.7 and 25.6%, respectively.

As for the miscarriage rate, no significant differences were seen between groups similar to what has been previously reported in other studies with donor oocytes (Trokoudes et al., 2011).

When analyzing the outcomes of FET with surplus embryos, cryopreserved by slow freezing on Day 3, the first remarkable observation is that the embryo survival rate is similar in embryos obtained from fresh or from vitrified oocytes. It seems that the double cryopreservation (oocyte-embryo) did not affect any of the parameters analyzed: embryo survival, clinical pregnancy, ongoing pregnancy and implantation rates, suggesting that the slow freezing method can be successfully used on embryos arising from vitrified oocytes.

In our study the number of oocytes allocated to the group with fresh oocytes was higher than to the the group with vitrified oocytes. Also 14.4% of the vitrified oocytes did not survive the warming process. Consequently, the number of produced, ongoing and frozen embryos in the group of vitrified oocytes was lower than in the group of fresh oocytes. Under these circumstances and considering that one of the value indicators for success is the cumulative pregnancy rate, a possible decrease in this rate could be expected in the group that received vitrified oocytes. In this regard, the loss of oocytes associated with the vitrification/warming process will always have an impact on the cumulative pregnancy rate, although a follow-up is necessary to reach decisive data.

In conclusion, our data demonstrate that vitrification affects neither the functionality of the oocytes nor their capacity to produce and give rise to ongoing embryos, pregnancies and live birth.

These results encourage the use of this technique for other applications, such as fertility preservation, the accumulation of oocytes from successive stimulations for a preimplantation genetic diagnosis or low responders and in patients at risk of ovarian hyperstimulation syndrome. Oocyte vitrification can also be performed due to patient’s choice during IVF therapy as a means to an elective limited insemination to avoid the cryopreservation of surplus embryos (Tucker et al., 2004).

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Authors’ roles

M.S. collected the data. M.S., A.V. and J.S. designed the study, interpreted the data and wrote the manuscript. I.R. performed the statistical analysis of the data. M.B., E.C., F.M., B.C. and P.N.B. reviewed the manuscript.

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Conflict of interest

None declared

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