Successful ongoing pregnancies after vitrification of oocytes


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Objective: To demonstrate the efficiency of vitrifying mature human oocytes for different clinical indications.

Design: Descriptive case series.

Setting: Cryobiology laboratory, Centro Colombiano de Fertilidad y Esterilidad–CECOLFES LTDA. (Bogotá, Colombia).

Patient(s): Oocyte vitrification was offered as an alternative management for patients undergoing infertility treatment because of ovarian hyperstimulation syndrome, premature ovarian failure, natural ovarian failure, male factor, poor response, or oocyte donation. Mature oocytes were obtained from 33 donor women and 40 patients undergoing infertility treatment.

Intervention(s): Oocytes were retrieved by ultrasound-guided transvaginal aspiration and vitrified with the Cryotops method, with 30% ethylene glycol, 30% dimethyl sulfoxide, and 0.5 mol/L sucrose. Viability was assessed 3 hours after thawing. The surviving oocytes were inseminated by intracytoplasmic sperm injection. Fertilization was evaluated after 24 hours. The zygotes were further cultured in vitro for up to 72 hours until time of embryo transfer.

Main Outcome Measure(s): Recovery, viability, fertilization, and pregnancy rates.

Result(s): Oocyte vitrification with the Cryotop method resulted in high rates of recovery, viability, fertilization, cleavage, and ongoing pregnancy.

Conclusion(s): Vitrification with the Cryotop method is an efficient, fast, and economical method for oocyte cryopreservation that offers high rates of survival, fertilization, embryo development, and ongoing normal pregnancies, providing a new alternative for the management of female infertility. (Fertil Steril 2006;85:108–11. ©2006 by American Society for Reproductive Medicine.)

Key Words: Oocyte, vitrification, survival, pregnancy, ICSI, cryopreservation, cryobank

Cryopreservation of oocytes enables patients who are at risk of losing ovarian functions because of gonadotoxic treatment or surgery to store their oocytes for later use. Egg banking also provides women with the option to delay their childbearing plan and circumvents the ethical concerns associated with embryo preservation (1). Oocyte cryopreservation is being used by a limited number of assisted reproductive technology (ART) clinics in Italy, Germany, The United States, South America, and Asia (2–6). The objective of this study was to report the efficiency of vitrifying mature human oocytes for different clinical indications.

MATERIALS AND METHODS

Controlled Ovarian Stimulation

A subcutaneous dose (0.5 mg/d) of a GnRH agonist (leuprolide acetate; Lupron, Laboratorios Abbott, Bogotá, Colombia) was given on the first day of menstruation. Stimulation with recombinant FSH (150–250 IU/d; Puregon; Laboratorios Organon, The Netherlands) was administered starting on day 3 of the cycle until follicles reached 18–22 mm in diameter. Human chorionic gonadotropin (10,000 IU; Gonacor, Laboratorios Daxley) was injected, and 34–36 hours later transvaginal oocyte retrieval was performed under ultrasound guidance by traditional techniques (7).

Selection and Vitrification of Oocytes

The oocytes were denuded of cumulus cells with hyaluronidase (80 IU/mL) Sage In Vitro Fertilization, Trumbull, CT. Mature, metaphase II oocytes were vitrified with the Cryotop technique (8, 13) for a maximum period of 6 hours after retrieval. Vitrification was performed with minimum volume cooling (MVC) plates (Kitazato, Tokyo, Japan), with cryoprotectant solutions (Kitazato). The oocytes were equilibrated with equilibration solution (15% ethylene glycol (EG) plus dimethyl sulfoxide [DMSO]; Kitazato) for 10 minutes and then placed in vitrification solution (Kitazato) consisting of 15% EG plus 15% DMSO plus 0.5 mmol/L sucrose. The oocytes were loaded quickly onto an MVC plate containing approximately 2 μL of vitrification solution and immersed directly in liquid nitrogen, at a cooling rate of approximately −23,000°C/min.

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Recipients Preparation
Patients waiting for the transfer of embryos obtained from vitrified–thawed oocytes underwent endometrial preparation with estradiol valerate (Progynova; Shering Laboratories, Berlin, Germany) at increasing doses, beginning with 2 mg and increasing to 6 mg at day 14 of the cycle, maintaining the dose until the time of the pregnancy test. Three days before embryo transfer (ET) or starting on day 13, patients were also given natural injectable P (50 mg daily, IM; Arsal Laboratories, El Salvador).

Thawing of Oocytes and ET
Oocytes were thawed with fast warming rates (12,000°C/ min) by immersing the Cryotops in thawing solution (Kitazato) containing 1 mol/L sucrose for 1 minute at 37°C and then equilibrating the oocytes slowly in diluent solution (Kitazato) containing 0.5 mol/L sucrose for 3 minutes at room temperature and two consecutive flushes in washing medium (Kitazato). Oocytes were then immediately placed in fertilization media for incubation for 2–4 hours to evaluate their viability. Intracytoplasmic sperm injection (ICSI) was performed with the viable oocytes. Twenty-four hours later they were examined again for the presence of a pronucleus and then cultured in vitro for 72 hours until the day of intratransferine transfer. Vaginal P gel was applied to the recipients 4 hours before the procedure, which was performed under transabdominal ultrasound guidance with Soft Frydman Set Echo catheters (Laboratoire CCD, Paris, France).

RESULTS
In a 6-month period, 707 human oocytes were vitrified with the Cryotop method and stored in liquid nitrogen. The oocytes were cryopreserved for different clinical indications. Most oocytes were obtained from egg donors after they provided informed consent and after evaluation by the bioethics committee of CECOLFES. Patients who experienced ovarian hyperstimulation syndrome (OHSS) had the highest E2 peak scores and the greatest number of vitrified oocytes per woman. A second large group of patients were young women (average age, 29 years) with surplus oocytes after undergoing IVF, ICSI, or TTOMI (tubal transfer of oocytes micro-injected) treatments. Patients with the lowest E2 peak scores were those with advanced reproductive age and who were poor responders. A mean of 2.3 oocytes per patient per cycle were retrieved and cryopreserved. To optimize the chance of pregnancy in this group of patients, patients underwent multiple ovarian stimulation cycles and oocyte cryopreservation. Once an adequate number of oocytes was collected, ICSI and ET were performed.

Other indications, such as a plan to delay maternity and male factor–related problems, are shown in Table 1

A total of 23 patients, divided into two groups, underwent ET with embryos resulting from vitrified–thawed and fertilized oocytes. The first group of patients received their own embryos (n = 4), and the second group of patients received embryos derived from egg donors (n = 19).

Patients with a history of OHSS (n = 3) were the youngest in this study (mean age, 26.6 years) and had a mean of 4.6 embryos transferred, obtaining a pregnancy rate of 100% (Table 2). One patient with surplus oocytes stored from a previous cycles achieved pregnancy after her eggs were fertilized with her husband’s semen. The average time of storage for vitrified oocytes was 2 months before ET.

One of the main indications for transferring embryos derived from vitrified donor oocytes was functional ovarian failure (menopause). The mean age of these patients was 42.8 years. Among 14 patients, 8 achieved pregnancy. In this group, the average number of transferred embryos was 3.9.

The pregnancy rate was lower among patients with premature ovarian failure, who required donor eggs. Although these women were younger (36.5 years), only 1 of 4 patients

| TABLE 1 |
| Clinical indications for cryopreservation of oocytes, average age, E2 peak during controlled ovarian stimulation, and average number of vitrified oocytes per group of patients. |

<table>
<thead>
<tr>
<th>Indication</th>
<th>No. of patients</th>
<th>Mean age (y)</th>
<th>Mean peak E2 (pg/mL)</th>
<th>Total no. of vitrified oocytes</th>
<th>Mean no. of vitrified oocytes/woman</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocyte donors</td>
<td>33</td>
<td>25.6</td>
<td>2,966</td>
<td>337</td>
<td>10.2</td>
</tr>
<tr>
<td>Autologous oocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OHSS</td>
<td>7</td>
<td>30.8</td>
<td>6,161</td>
<td>99</td>
<td>14.1</td>
</tr>
<tr>
<td>Surplus oocytes</td>
<td>23</td>
<td>29.7</td>
<td>3,020</td>
<td>226</td>
<td>9.8</td>
</tr>
<tr>
<td>Poor response</td>
<td>6</td>
<td>38</td>
<td>1,103</td>
<td>14</td>
<td>2.3</td>
</tr>
<tr>
<td>Male factor</td>
<td>2</td>
<td>36</td>
<td>2,726</td>
<td>17</td>
<td>8.5</td>
</tr>
<tr>
<td>Postponed maternity</td>
<td>2</td>
<td>32.5</td>
<td>4,152</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>73</td>
<td>32.5</td>
<td>3,452</td>
<td>707</td>
<td>8.66</td>
</tr>
</tbody>
</table>

achieved pregnancy, with an average of 4.5 embryos transferred. Among this group, a patient with autoimmune disease (scleroderma) achieved pregnancy. The total pregnancy rate and the clinical indications are shown in Table 2.

**DISCUSSION**

In this study, we demonstrated that oocyte cryopreservation with the Cryotop vitrification technique for various clinical indications resulted in high recovery, postthaw survival, fertilization, embryo development, and pregnancy rates.

A mature human oocyte, measuring approximately 120 μm in diameter, is one of the largest cells in mammals. Other distinguishing features of a mature oocyte include high surface-volume ratio, low permeability to water, and a short life span if it is not fertilized. For fertilization and embryo development to occur, the oocyte must maintain its structural integrity, which is vulnerable to subphysiologic changes.

It has been well documented that oocytes suffer from reversible and irreversible damage during cryopreservation. This damage includes hardening of the zona pellucida, premature cortical granules release, depolymerization of the microtubules, and misalignment of the chromosomes.

Studies with mice demonstrated that cryopreservation induced hardening of the zona pellucida and premature release of the cortical granule content (1, 10), inhibiting sperm penetration and fertilization. During the freezing process, fracture of the zona pellucida and ooplasmic membrane disruption could also occur. In the field of clinical ART, zona hardening can be overcome by ICSI.

The meiotic spindles are highly sensitive to temperature changes, especially to cooling, leading to disruption of the meiotic spindles, dispersion of chromosomes, and possibly to increases in the risk of aneuploidy. Previous studies demonstrated the beneficial effect of DMSO in spindle polymerization. Therefore, the use of DMSO for oocyte cryopreservation might have a protective effect (2, 11–13).

Since 1986, when Chen (14) achieved the first pregnancy from frozen–thawed oocytes, the slow freezing method has been the procedure of choice for oocyte and embryo freezing, using low cooling rates (1°C/min) and cryoprotectant concentrations of approximately 1.5 mol/L. However, the results of oocyte cryopreservation by the slow freezing method were highly variable. Gook et al. (10) reported oocyte survival, embryo cleavage, and clinical pregnancy rates of 76%, 60%, and 28.5%, respectively. The first reported birth out of a frozen oocyte and ICSI was in 1997, with a survival rate of 33.3%, 50% fertilization rate, and 50% cleavage rate, where only one embryo was transferred, leading to pregnancy (3). In another study, Polak de Fried et al (9) obtained a survival rate of 30%, a 66.6% fertilization rate, and one pregnancy out of 10 donor frozen eggs.

The main factors affecting survival and subsequent embryo development relate to cryoprotectant and intracellular

<table>
<thead>
<tr>
<th>Table 2: Rates of recovery, viability, fertilization, cleavage, and pregnancy after thawing oocytes under different indications.</th>
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<tbody>
<tr>
<td><strong>Indication</strong></td>
</tr>
<tr>
<td>Autologous oocytes</td>
</tr>
<tr>
<td>Surplus oocytes</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Menopause</td>
</tr>
<tr>
<td>Premature ovarian failure</td>
</tr>
<tr>
<td>Other diseases</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

Note: Values in parentheses are percentages.
crystal formation. This is why oocyte cryopreservation is not being routinely offered by ART programs to patients with reproductive failure (3).

The Cryotop vitrification technique did not seem to affect the meiotic spindles and chromosome alignment, thus avoiding genetic abnormalities related to the slow freezing technique (15). Vitrification is a cryopreservation method with which solutions become solid when cooled at an extremely high speed (>2,000°C/min), forming a glass-like state, thus avoiding crystallization (16). The vitrification technique is simpler, more convenient, and more effective than the slow cooling method (8).

In 2001, Kuwayama et al. (15) modified the vitrification technique by increasing the high rates of cooling and warming to reduce the concentration of cryoprotectants to 15% DMSO, 15% EG, and 0.5 mol/L sucrose in an MVC plate (2, 9, 13). This technique minimizes the damage associated with slow freezing, such as intracellular and extracellular ice crystal formation, chilling injuries, and osmotic shock. To reduce the cryoprotector toxicity we used MVC, which has been proven to result in high survival and pregnancy rates (8, 13).

This is the technique that we applied in our ART program. Within a 4-month period, 707 eggs were retrieved from 73 patients (with informed consent) and vitrified. To date, 159 eggs have been thawed and transferred to 23 patients, achieving 13 ongoing pregnancies.

According to these results, it is feasible to offer oocyte vitrification as an alternative to embryo cryopreservation for patients with OHSS, those with surplus oocytes, poor responders to ovarian stimulation, and women who wish to delay their maternity. Oocyte cryopreservation is also indicated in cases of male factor infertility or problems associated with difficulty for sperm collection, inadequate seminal samples, or nonviable spermatozoa at the time of oocyte retrieval. Finally, egg donor programs can become more simplified and efficient by using oocyte cryopreservation. The oocytes can be quarantined, enabling the donors to be screened for transmissible disease. Furthermore, the donors and recipients are not required to synchronize their menstrual cycles, thus simplifying the egg donor program and reducing the cost for the patients because the use of donor eggs is optimized.

As the number of patients undergoing transfer of embryos derived from vitrified–thawed oocytes increases, the success rates of the Cryotop vitrification technique will improve. Oocyte vitrification represents a feasible option for women who wish to delay their maternity.

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REFERENCES