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Pregnancies and births after oocyte cryopreservation

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Objective: To determine the potential of oocyte cryopreservation techniques.

Design: Retrospective data analysis.

Setting: A tertiary infertility center.

Patient(s): Sixty-eight patients (29 to 37 years of age) undergoing assisted reproduction procedures for infertility problems.

Intervention(s): Oocytes from women treated for infertility were cryopreserved with a slow cooling/rapid thawing protocol in which 1,2 propanediol and sucrose were used as cryoprotectants. Eighty-six thawing cycles were performed.

Main Outcome Measure(s): Rates of survival after thawing, fertilization after intracytoplasmic sperm injection, cleavage, implantation, and pregnancy.

Result(s): We treated 68 patients through 86 thawing cycles.. Seven hundred thirty-seven oocytes were thawed, and 59 transfer cycles were performed. The survival rate was 37%. The fertilization and cleavage rates were 45.4% and 86.3%, respectively. A total of 15 clinical pregnancies were achieved with pregnancy rates of 25.4% per transfer and 22% per patient. There were three miscarriages, resulting in an abortion rate of 20%. Seventeen of the 104 transferred embryos implanted, corresponding to an implantation rate of 16.4%. Thirteen babies were born, 8 females and 5 males.

Conclusion(s): Statistically significant results were obtained for fertilization, cleavage, and pregnancy rates. Our results show oocyte cryopreservation may represent an alternative to embryo storage in selected cases. (Fertil Steril® 2004;82:601–5. ©2004 by American Society for Reproductive Medicine.)

Key Words: Oocyte cryopreservation, freezing, cryoprotectants, oocyte survival, pregnancy

The importance of oocyte cryopreservation is widely recognized for its potential to circumvent several ethical and legal problems associated to embryo freezing. Egg storage would also represent a benefit for certain categories of patients, including [1] women at risk of losing ovarian function prematurely for surgical treatments or as a consequence of cancer therapy; [2] hyperstimulated patients; [3] women who, for various reasons, intend to preserve their fertility.

The first frozen egg pregnancy was achieved by Chen in 1986 (1). Few other births were reported in the following years (2, 3), and for a prolonged period the experiences conducted on oocyte freezing remained anecdotal. Gook et al. (4) introduced for the first time the use of intracytoplasmic sperm injection (ICSI) associated with oocyte freezing to avoid possible fertilization problems caused by premature cortical granule release and zona hardening, Since then, several other pregnancies have been obtained worldwide (5–8). The approach used by those investigators was based on a slow cooling/rapid thawing method using propanediol (PROH) and sucrose as cryoprotectants, in line with the conditions originally developed for embryo freezing (9). However, these protocols have shown to have limited efficiency in terms of survival rate in many cases. Recently, it has been suggested that protocol modifications may improve recovery after thawing (10). Some investigators have reported improved survival and fertilization rates as well as live births using vitrification as a freezing method (11).

Oocyte cryopreservation raises concerns in relation to sublethal damage that could cause

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detrimental effects at prenatal and/or postnatal stages, but this assumption has not been necessarily confirmed by studies conducted on the oocyte meiotic spindle (12) or on the chromosome complement of embryos developed from thawed oocytes (13). More comprehensive information is necessary to confirm those preliminary results. In the last few years, we have gained some experience in this field, and here report our data concerning 68 patient couples whose treatment required oocyte freezing in response to a variety of conditions.

MATERIALS AND METHODS

Human oocytes were obtained from 68 couples undergoing assisted reproduction technology (ART) treatment, from January 1997 to December 2000. To prevent generation of supranumerary embryos, in 51 patients only a minority (three) of the oocytes recovered were inseminated, and the remaining oocytes were cryopreserved. In another group of 17 patients, the whole cohort of oocytes was cryopreserved because no spermatozoa were available at the time of oocyte retrieval, after failed testicular sperm aspiration (TESA) or inability to produce a semen sample (seven cases). Approval for oocyte freezing treatment had been preliminarily obtained from the local institutional review board, and the patients had signed an informed consent form before the procedure.

The mean age ± standard deviation (SD) of the patients was 33 ± 4.3. The superovulation protocol included either subcutaneous administration of 0.1 mL/day of leuprolide (Enantone SC; Takeda, Catania, Italy) from day 21 of the previous cycle to hCG administration, or intramuscular administration of leuprolide depot (Enantone depot; Takeda) on day 21 of the previous cycle. Four ampules of FSH (Metrodin HP; Serono, Rome, Italy) were administered on day 2 of the cycle for 2 days, followed by two ampules per day. Ovulation was induced with 10,000 IU hCG (Profasi HP; Serono) when at least two follicles 22 mm in diameter were observed with a correspondent E_2 level. Transvaginal ultrasound–guided oocyte retrieval was performed 36 hours after the administration of hCG.

The oocyte-cumulus complexes were separated from their follicular fluid and transferred to human tubal fluid medium (HTF) supplemented with 10% human serum albumin (10 mg/mL) (Irvine Scientific, Santa Ana, CA) and cultured in 5% CO_2 at 37°C. Complete removal of the cumulus mass and corona cells was performed using hyaluronidase (80 IU/mL; Sigma Chemical, St. Louis, MO) and mechanical manipulation with fine-bore glass pipettes. Only oocytes showing an extruded polar body I and presumably at the metaphase II stage were frozen after culture for about 4 hours.

Freezing/Thawing Procedure

The oocytes were cryopreserved using a slow freezing method (12). All the gametes were washed in Dulbecco's phosphate-buffered solution (PBS) (GIBCO, Life Technologies Ltd., Paisley, United Kingdom) supplemented with 20% (10 mg/mL) plasma protein solution (PPS) (Baxter AG, Vienna, Austria). One or two oocytes were subsequently placed in an equilibration solution that contained 1.5 M 1,2 propanediol (PROH) (Fluka Chemika, Sigma Aldrich Srl, Milan, Italy) supplemented with 20% plasma protein solution for 10 minutes. Afterward, the oocytes were transferred to the loading solution containing 1.5 M PROH, 20% PPS, and 0.1 M sucrose at room temperature, loaded into plastic straws (Paillettes Cristal 133 mm; Cryo Bio System, France), and placed into an automated Kryo 10 series III biological freezer (Planer Kryo 10/1,7; Planer Plc., Sunbury, Middlesex, United Kingdom). The temperature was gradually lowered from 20°C to −8°C at a rate of −2°C/min. Manual seeding was induced during the 10-minute holding ramp at −8°C. The temperature was decreased then to −30°C at a rate of −0.3°C/min and, finally, rapidly to 150°C at a rate of −50°C/min. The straws were finally plunged into liquid nitrogen and stored for later use.

The thawing procedure was carried out at room temperature (12). The straws were air-warmed for 30 seconds and then plunged into a 30°C water bath for 40 seconds. The cryoprotectant was removed by using a PROH stepwise dilution in the thawing solution; oocytes were maintained 5 minutes in 1 M PROH + 0.2 M sucrose + 20% PPS; 5 minutes in 0.5 M PROH + 0.2 M sucrose + 20% PPS; and 10 min in PBS + 20% PPS. Variable amounts of stored material was used at each thawing cycle to obtain four morphologically normal oocytes suitable for ICSI, thereby avoiding the risk of generating spare embryos. Finally, the viable oocytes were cultured in HTF medium (Irvine Scientific) at 37°C in an atmosphere of 5% CO_2 in air for at least 2 to 3 hours before insemination.

Insemination and Embryo Culture

Insemination was routinely performed by ICSI as previously described elsewhere (14). Sixteen to 18 hours later, normal fertilization was checked by the presence of two pronuclei and two polar bodies. All the fertilized oocytes were transferred into P-1 Medium (Irvine Scientific). The embryos were graded on day 2 from 1 to 4 (best to worst) according to fragmentation rate, cytoplasm quality, and the presence of multinucleated blastomeres (MNB) (15).

Endometrial Preparation and Embryo Transfer

The women received a steadily increasing dosage of 100–300 μg, of micronized 17β-E_2 in patches (Esclima; Schering, Milan, Italy) administered over a period of time ranging from 10 to 18 days, depending on the patient (14). Progesterone (P) supplementation, either as 100-mg injections in oil (Prontogest; Amsa, Rome, Italy) or as 180-mg micronized doses in gel (Crinone 8; Serono) via vaginal route, was started on the day of oocyte thawing. Transfers were performed on the third day of P administration. Endo-

TABLE 1

Oocyte cryopreservation data.

Thawing cycle	86
Oocytes	
Frozen	772
Thawed	737
Survived (%)	273 (37.0)
Microinjected (%)	273 (100)
Fertilized	
2PN (%)	124 (45.4)
1PN (%)	11 (4.0)
3PN (%)	15 (5.5)
Lysed after ICSI (%)	40 (14.6)
Embryos	
Cleaved (%)	107 (86.3)
Transferred (%)	104 (97.2)
Transferred mean ± SD	1.1 ± 1.0

Borini. Oocyte cryopreservation. Fertil Steril 2004.

metrium thickness was checked before starting P administration, and the cycle was suspended if the lining was thinner than 8 mm or thicker than 12 mm. In case of pregnancy, hormone therapy (HT) was continued 60 days after transfer. In our clinic since February 1992, no more than three embryos have been transferred with the aim of decreasing the incidence of triplets. Clinical pregnancy was defined as the presence of a gestational sac at ultrasound examination.

RESULTS

Eighty-six thawing cycles were performed in our center. Survival, fertilization, and cleavage rates were 37.0%, 45.4%, and 86.3% respectively (Table 1). Oocyte degeneration occurred in most cases (85%) during or immediately after thawing, but the loss of viability in the following 2 to 3 hours was a relatively rarer event. In our ICSI program using fresh oocytes, fertilization and cleavage rates are around 65.0% and 93.9%. respectively.

Tables 2 and 3 describe the clinical and pregnancy outcomes, respectively. Of the 13 babies born, 8 were female and 5 male. The weight ± SD at birth was 3.19 ± 0.62 kg. All were healthy, with a normal karyotype and no malformations. The follow-up of one ongoing pregnancy was not available.

TABLE 2

Clinical data.

Patients	68
Age ± SD	33 ± 4.3
Transfers	59
Pregnancies	15
Gestational sacs	17
Pregnancy rate/patients	22
Pregnancy rate/thawing cycle	17.4
Pregnancy rate/transfer	25.4
Implantation rate	16.4
Abortions (%)	3 (20)

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TABLE 3

Pregnancy outcome.

Children	13
Weight at birth (kg ± SD)	3.19 ± 0.62
Singleton pregnancies	9
Twins	2
Follow-up not available	1
Males born	5
Females born	8

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DISCUSSION

Because of its size, unicellular nature, and unique characteristics, the oocyte poses special problems in relation to its cryopreservation. In particular, in most studies applying conventional slow freezing protocols, survival rates have been reported to be low. Our study confirmed that inadequate survival (37%) could be obtained when mature oocytes were frozen with a slow freezing protocol that ensured excellent recovery when used for the storage of early cleavage embryos.

To increase dehydration and reduce the risk of intracellular ice formation during slow freezing, Fabbri et al. (10) used higher sucrose concentrations (0.2 M and 0.3 M) in the freezing solution, achieving proportionally improved survival rates (58% and 83%, respectively). The beneficial effect of 0.3 M sucrose on the survival rate has been confirmed by Fosas et al. (16). Other investigators (17) have also described a high survival rate (74%) using a modified slow freezing protocol, but from their data it is not possible to discriminate whether improved results are attributable to the removal of sodium from the freezing solution, higher seeding temperature (−6°C), or increased sucrose concentration (0.5 M) in the thawing solution. Oocytes stored with these improved protocols have shown the ability to support development to term. However, these results need to be confirmed by larger studies. Vitrification protocols (11, 18) have been suggested to produce high survival (65% to 70%), but, again, data need to be expanded before drawing unambiguous conclusions.

Based on the assumption that, following thawing, premature release of cortical granules may compromise the oocyte fertilization ability by causing zona hardening, frozen-thawed human oocytes are routinely inseminated via ICSI. Protocols that have shown to generate improved survival rates (8, 16, 18) also appear to ensure high fertilization rates (70% to 80%). In association with reduced survival, we have

observed a suboptimal fertilization rate (45%). These findings may suggest that the incidence of survival somehow reflects also the oocyte fertilization ability, that suboptimal protocols can cause sublethal damage associated with limited fertilization ability. Despite reduced survival and fertilization rates, in our study we observed a cleavage rate of 86%, approaching the value normally achieved with embryos from fresh oocytes (92% to 94%). Other reports have confirmed this finding, describing similar cleavage rates (7, 17, 19). Also, oocyte freezing did not appear to compromise the morphology of day-2 embryos because over 97% of them were considered suitable for transfer.

Several live births derived from cryopreserved oocytes have been reported. Attempts have been generally sporadic, as embryo freezing is a well-established method for the preservation of the reproductive potential. Very few studies have described the treatment of more substantial numbers of patients (8, 17, 18, 20, 21). In most cases, pregnancies have been achieved with the application of slow freezing protocols. However, more recently, vitrification methods have also shown the potential to give rise to viable pregnancies (11, 18). In our case, pregnancy (per cycle) and implantation rates were 22% and 16%, respectively. Similar pregnancy rates were described by Porcu et al. (21) and Yoon et al. (18) (18% and 21%, respectively), although in these studies implantation rates were not reported or were lower (6.4%), respectively. Other investigators have described better results. In particular, Yang et al. (8) were able to achieve pregnancies in 45% of their patients, with a 25% implantation rate. However, it should be noticed that in the work of Yang et al. the frozen oocytes were presumably of superior quality because they were obtained from donors, whereas our data are derived from patients of diverse ages and treatment indications. More generally, exhaustive evaluation of the clinical efficiency of diverse protocols remains unfeasible, as substantial differences are found among the patient populations of the various reports. Ultimately, efficiency assessment among the different protocols would require comparison of implantation rates derived from the treatment of homogeneous groups of patients and estimations on the basis of the number of frozen-thawed oocytes. Also, for oocyte freezing to become an established option, developing methods to obtain success rates similar to embryo freezing is essential. In our center, over the period in which the present study was conducted, frozen-thawed embryos were able to implant with a rate of 3.9%, considering the number of oocytes initially inseminated. This rate is markedly higher compared with the efficiency estimated on the basis of our oocyte freezing data (2.2%).

The safety of oocyte freezing has always been a reason of major concern, especially in relation to the susceptibility of the meiotic spindle to low temperatures and the consequent increased risk of aneuploidy incidence. Three (20%) of our pregnancies failed to develop to term. We have no information on whether these abortions were caused by either chromosomal anomalies or other factors. Data on abortion rates from other studies are generally lacking or are insufficient in terms of sample size (22), leaving the question still open. But an increase in aneuploidy frequency may not be necessarily associated with oocyte freezing (23). It has been found that, after freezing, the proportion of MII oocytes with a morphologically normal spindle is only moderately reduced (61% and 81% of normal oocytes in test and control groups, respectively) (12). Moreover, it appears that cryopreservation before in vitro maturation does not affect spindle organization; over 8l% of oocytes were found to be morphologically normal in frozen groups, as in controls (24). Cytogenetic analysis also suggests that slow freezing does not affect the rate of aneuploidy in human oocytes (25). By performing an in situ hybridization fluorescence (FISH) analysis and employing specific probes for chromosomes 13, 18, 21, X, and Y, Cobo et al. (13) found comparable aneuploidy frequencies in embryos obtained from fresh or frozen oocytes (28% and 26%, respectively). Nevertheless, further information on potential adverse effects of cryopreservation on oocyte viability is needed. Investigations should not be restricted to the cytoskeletal apparatus and associated chromosomes because other cellular attributes, such as mitochondria, may be affected by freezing-thawing (26).

Our results have shown limited efficacy of conventional slow freezing methods in maintaining oocyte viability. However, it appears that oocytes that are able to survive freezing and can be fertilized, allow preimplantation development and implantation with acceptable rates. Without a doubt, survival rate must be improved, perhaps by increasing the sucrose concentration (10), and the potential for sublethal cell damage from increased osmotic stress must be elucidated (27). Studies on the status of the meiotic spindle and mitochondrial function after thawing are ongoing in our clinic to establish with objective criteria the efficiency of different cryopreservation protocols.

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