Outcome of 518 salvage oocyte-cryopreservation cycles performed as a routine procedure in an in vitro fertilization program

Giovanni B. La Sala, M.D.,[a] *Alessia Nicoli, B.Sc.,*[a] *Maria T. Villani, M.D.,*[a] *Michela Pescarini, B.Sc.,*[a] *Andrea Gallinelli, M.D.,*[a] *and Isaac Blickstein, M.D.*[b,c]

[a] Department of Obstetrics and Gynecology, Arcispedale Santa Maria Nuova, Reggio Emilia, Italy; and [b] Department of Obstetrics and Gynecology, Kaplan Medical Center, Rehovot and the [c] Hadassah-Hebrew University School of Medicine, Jerusalem, Israel

Objective: To document outcomes of oocyte cryopreservation performed as a routine procedure in an IVF program.
Design: Describing the rate of oocyte survival, embryo transfer (ET), implantation, and live births of IVF–intracytoplasmic sperm injection performed on thawed oocytes.
Setting: Reproductive medicine center in Italy.
Patient(s): Women (n = 696) who failed to conceive after IVF–intracytoplasmic sperm injection with fresh oocytes.
Intervention(s): Surplus oocytes obtained during a failed cycle with fresh oocytes were frozen and then were thawed, micromanipulated, and transferred in a later cycle.
Main Outcome Measure(s): Rates of oocyte survival, ETs, implantation, and live births were calculated in the entire cohort and in patients aged ≤38 and >38 years.
Result(s): There were 29 pregnancies, for a total implantation rate of 6.3% (95% CI: 4.3, 9.0) per 456 ET cycles. The clinical-pregnancy rate was 19 (4.2%; 95% CI: 2.6, 6.4) of 456 ET cycles, with a take-home-baby rate of 7 (1.5%; 95% CI: 0.7, 3.0) of 456 ET cycles.
Conclusion(s): Cryopreservation performed as a routine procedure for so-called salvaging of surplus oocytes is associated with poor implantation rates and with a probability of 1 live birth in 65 ET cycles. (Fertil Steril® 2006; 86:1423–7. ©2006 by American Society for Reproductive Medicine.)

Key Words: IVF, ICSI, oocyte cryopreservation, assisted reproduction

Cryopreservation of sperm and embryos have been used for decades to preserve fertility with acceptable success rates. In contrast, the survival rate of oocytes after the traditional freezing–thawing methods has been unacceptably low for routine clinical use (1, 2). In fact, despite adequate fertilization and cleavage rates (1, 3, 4), the first live birth was not reported until 1997 (5). Recent advances in cryopreservation techniques partially overcome the vulnerability of metaphase II oocytes and increased the survival rates of frozen oocytes with a 57% fertilization rate after intracytoplasmic sperm injection (ICSI) of thawed oocytes, 91% cleavage rate, and satisfactory embryonic morphology (1).

Oocyte cryopreservation appears to be applicable in current assisted reproductive technology (ART) for several reasons. First, it may circumvent ethical or moral dilemmas for couples voicing negative feelings about embryo cryopreservation (6, 7). Second, it may preserve fertility in young women undergoing gamete-destructive cancer treatment (8, 9). Third, it may enable preservation of fertility beyond the normal reproductive age. Finally, oocyte cryopreservation may reduce the risks as well as the cost of oocyte retrieval from potential donors of oocytes (6).

Conceivably, all these indications are intended for women who presumably are fertile and for whom oocyte cryopreservation is an elective procedure. In contrast, a special situation exists in Italian ART centers, where, on one hand, insemination of no more than three oocytes is allowed under the new 2004 Italian legislation regulating ART (10) and where, on the other hand, freezing of surplus embryos is not permitted. Under such circumstances, oocyte cryopreservation becomes the only alternative for salvaging the excess oocytes obtained after IVF stimulation protocols, and thus, our report is, to the best of our knowledge, the first to

Received December 24, 2005; revised and accepted April 1, 2006.
Reprints will not be available. Correspondence requests: Isaac Blickstein, M.D., Department of Obstetrics and Gynecology, Kaplan Medical Center, 76100 Rehovot, Israel (FAX: 972-8-941-1944; E-mail: blick@netvision.net.il).

document outcomes of oocyte cryopreservation that is performed as a routine procedure in an IVF program.

MATERIALS AND METHODS

This study was approved by our institutional review board. We analyzed the data set of IVF-ICSI cycles that were performed during the period March 22, 2004 to November 30, 2005 in our public clinic at Arcispedale Santa Maria Nuova, in Reggio Emilia, Italy. After an IVF-ICSI cycle with insemination of up to three oocytes, the excess oocytes were submitted to cryopreservation. We used the same freezing–thawing protocol that was described in detail by Fabbri et al. (1)

Cryoprotectants

We used Dulbecco's phosphate-buffered solution (PBS; Medicult, Mollehaven, Denmark), 1,2-propanediol (PROH; Medicult), and a serum protein supplement (SPS; Medicult) as cryoprotectant solutions. For the freezing process, we used an equilibration solution containing 1.5 mol/L PROH + 30% SPS (15 mg/mL of plasma proteins) in PBS, and a loading solution containing 1.5 mol/L PROH + sucrose 0.3 M + 30% SPS in PBS (Medicult). Oocytes dehydrated in 0.3 M sucrose are thawed in solution containing 0.3 mol/L sucrose.

Cryopreservation Protocol

We used a slow-freezing–rapid-thawing protocol. After incubation of about 1 hour, the cumulus cells surrounding each oocyte were removed by brief exposure (about 30 seconds) in hyaluronidase solution (80 IU/mL; Medicult), using a Pasteur pipette and a denuding Flexi-pet pipette (140-μm diameter; Cook, Eight Mile Plains, Queensland, Australia). We transferred all the oocytes to Petri dishes containing PBS supplemented with 30% SPS at room temperature. One to three oocytes are placed in 0.5 mL of the equilibration solution and maintained for 10 minutes at room temperature before transfer to 0.5 mL of the loading solution.

Oocytes are loaded in plastic straws (Technologies Paillettes Cristal, 133 mm; Cryo Bio System, Paris, France) and frozen with an automated Planer Kryo 360-1.7 biological freezer (Sapio Life, Como, Italy). The freezing process started at a chamber temperature of 20°C, which is reduced to –7°C at a rate of –2°C/min. Manual seeding is induced at –7°C. After a hold time of 10 minutes at –7°C, the straws are cooled slowly to –30°C at a rate of –0.3°C/min and then rapidly to –150°C at a rate of –50°C/min. After 10–12 minutes of stabilization temperature, the straws were transferred into liquid nitrogen (−196°C) and stored until thawing.

Thawing was achieved by air-warming the straws for 30 seconds, followed by immersion in a 30°C water bath for 40 seconds until deicing occurred. A two-step dilution of PROH (1.0 M and 0.5 M) containing 0.3 M sucrose (Medicult) is carried out at room temperature. After thawing, the contents of the straws are put in 1.0 mol/L PROH + sucrose 0.3 mol/L solution + 30% SPS, and the oocytes were equilibrated for 5 minutes. Oocytes then are transferred to 0.5 mol/L PROH + sucrose 0.3 mol/L + 30% SPS for an additional 5 minutes and then in a sucrose or 0.3 mol/L solution + 30% SPS for 10 minutes before a final dilution in PBS solution + 30% SPS for 20 minutes (10 min at room temperature and 10 min at 37°C). At the end of the process, the oocytes were cultured in Universal IVF Medium (Medicult) at 37°C in an atmosphere of 5.5% CO_2 in air, for an extended period of 4 hours.

Survival of the oocytes was established by observing an intact zona pellucida and plasma membrane, a clear perivitelline space of normal size, and no evidence of cytoplasmic leakage or oocyte shrinkage. We further classified oocytes as high quality if they had a regular form, a homogenous cytoplasm, and no vacuoles or granulations. It should be mentioned that during ICSI of fresh oocytes, an intact polar body (fragmented or not) always is seen, whereas in thawed oocytes, only a so-called ghost polar body (i.e., a membrane without cytoplasm) is sometimes observed. Surviving oocytes were submitted to insemination by ICSI. Although we usually freeze oocytes in metaphase II, we nevertheless continued with the freezing protocol when some or all oocytes were in metaphase I. After thawing, however, we verified whether metaphase I oocytes had matured in vitro at 4 hours after thawing.

Endometrial Preparation, Time of Thawing, and ET

We began the endometrial preparation on the 2nd day of the menstrual cycle, with E_2 valerate (2 mg twice per day, by mouth, Progynova; Shering, Milan, Italy). The first ultrasonographic assessment was performed, typically, on day 12 of the menstrual cycle. When the endometrial thickness ranged between 8 to 12 mm, we added intravaginal micronized P (200 mg, twice per day, Prometrium; Rottapharm, Milan, Italy). Oocyte thawing was performed on the day that ultrasonography demonstrated adequate thickness of 8 to 12 mm.

The number of thawed oocytes for each patient depended on the number of frozen oocytes available for that patient and on results of the thawing process of the first straw. If the first three oocytes degenerated during the thawing process, another set of frozen oocytes was submitted for thawing, and so on.

Fertilization is established at 18–20 hours after the ICSI, using the same criteria as with fresh oocytes, and embryo transfer (ET) is carried out 2 days later (day 3 embryos). Treatment with E_2 valerate and micronized P is continued until the day of β-hCG testing (typically about 12–14 d after ET), but the dose of micronized P was increased (to 200 mg, 3 times per day), starting from the ET day. In case of a positive β-hCG test and an ongoing pregnancy, we continued the hormonal support for 8 more weeks.

TABLE 1

Outcome of oocyte cryopreservation.

Characteristic	Total	Age ≤38 y	Age >38 y
Patients	414	343 (82.9)	71 (17.1)
Age (y)	35.3 ± 3.9	34.1 ± 3.3	40.6 ± 1.1
Thawed cycles	518	425 (82.0)	93 (18.0)
Frozen–thawed oocytes	1,647	1,358 (82.4)	289 (17.6)
Surviving oocytes	1,200/1,647 (72.8)	988/1,358 (72.8)	212/289 (73.4)
Cycles available for ICSI	502/518 (96.9)	413/425 (97.2)	89/93 (95.7)
Oocytes undergoing ICSI	1,132/1,200 (94.3)	932/988 (94.3)	200/212 (94.3)
Per cycle	2.3 ± 0.7	2.3 ± 0.7	2.3 ± 0.7
High-quality oocytes undergoing ICSI	880/1,200 (73.3)	730/988 (73.9)	150/212 (70.7)
Per cycle	1.8 ± 0.9	1.8 ± 0.8	1.7 ± 0.9
ET cycles	456/502 (90.8)	375/413 (90.8)	81/89 (91.0)
Total embryos	846/1,132 (74.7)	703/932 (75.4)	143/200 (71.5)
High-quality embryos	704/846 (83.2)	582/703 (82.8)	122/143 (85.3)
Implantations per ET cycles	29/456 (6.3)	25/375 (6.7)	4/81(4.9)

Note: Data are presented as n/N (%) or as mean ± SD. Differences between patients aged ≤38 or >38 years were not statistically significant.

La Sala. Routine "salvage" oocyte cryopreservation. Fertil Steril 2006.

In this analysis, we included all patients who had failed a previous IVF cycle with fresh oocytes. These patients included mainly (estimated to be >95%) women of Italian origin, who were all, with but few exceptions, nulliparous. Pregnancies were defined as chemical (positive β-hCG with subsequent decline and without a gestational sac seen on ultrasound), empty sac (gestational sac without an embryo), and clinical (when an embryo with a heartbeat was visualized by sonography). Pregnancy outcomes (live births and take-home-baby rates) were calculated as per the date of submission of this article.

Statistical Analysis

We compared the outcomes of oocyte cryopreservation by patient age (≤38 or >38 years). We performed Fisher's exact test by using True Epistat Software (Math Archives, Round Rock, TX) to derive odds ratios and Corenfield's 95% confidence intervals (CIs). We also calculated the strength of the relationship between the cleavage rates and patient's age by using Pearson's coefficient (R^2 value) and compared the slope of the least-square regression line with a zero-slope line (i.e., no change with increasing age).

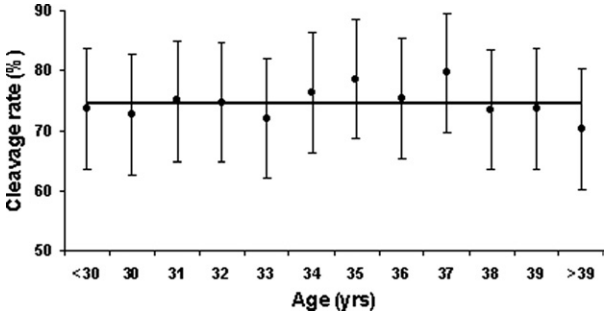
RESULTS

During the study period, there were 696 patients who underwent 1,022 freezing cycles, in which a total of 4,548 oocytes were cryopreserved. These patients come from the 81.9% of patients who failed IVF with fresh oocytes. The study group comprised 414 patients who underwent 518 thawing cycles of 1,647 oocytes. We performed ICSI in 502 cycles only because 16 cycles were canceled (15 because all the oocytes degenerate and 1 because all the oocytes remained in metaphase I). The mean age of this patient cohort was 35.3 ± 3.9 years. The cohort included 71 (17.1%) patients aged >38 years who underwent 93 (17.9%) cycles.

Table 1 shows the outcome of oocyte cryopreservation in this cohort, as well as in the subgroups of patient age ≤38 and >38 years. The comparison (not shown) did not reveal statistical differences (all CI included 1) between these age groups. The relationship between cleavage rates and patient age showed no statistical correlation (R^2 = 0.0001; Fig. 1), and the regression slope was not different from a

FIGURE 1

Mean and SD cleavage rates by maternal age. The least-square regression trendline suggests that cleavage rates are independent of the patient's age (R^2 = 0.0001).



La Sala. Routine "salvage" oocyte cryopreservation. Fertil Steril 2006.

zero slope. The data in Table 1 and Figure 1 indicate that outcomes of oocyte cryopreservation were independent of patient age.

There were 29 pregnancies, for an implantation rate of 5.8% (95% CI: 3.9, 8.2) per 502 cycles with available oocytes for ICSI and for an implantation rate of 6.4% (95% CI: 4.3, 9.0) per 456 ET cycles. Seven (24.1%) of the 29 pregnancies were chemical, 3 (10.3%) resulted in an empty sac, and 19 (65.5%) resulted in a clinical pregnancy involving an embryo with a positive heartbeat. Thus, the clinical-pregnancy rates were 3.8% (19/502 cycles with available oocytes for ICSI; 95% CI: 2.3, 5.8) and 4.2% (19 of 456 ET cycles; 95% CI: 2.6, 6.4). Of the 19 clinical pregnancies, 9 (47.4%) ended with a first-trimester abortion, 7 (36.8%) ended with live birth, and 3 (15.8%) are ongoing pregnancies, with a minimum gestational age of 6 weeks. It follows that the take-home-baby rates were 1.4% (7/502 cycles with available oocytes for ICSI; 95% CI: 0.6, 2.8) and 1.5% (7/456 ET cycles; 95% CI: 0.7, 3.0).

The seven babies (three male and four female) were delivered (four, spontaneous vaginal birth; three, cesareans for various obstetrical indications) to mothers whose median age was 35 years (range, 29–39 y; 1 mother was aged >38 y), at a median gestational age of 39 weeks (range, 37–42 wk) and with a median birth weight of 3,400 g (range, 2,610–4,325 g). These apparently normal babies are thriving.

DISCUSSION

Cryopreservation of human sperm and embryos currently is commonplace in fertility centers worldwide (6). Clearly, cryopreservation of female gametes circumvents many religious, ethical, and legal problems involved in embryo freezing. Moreover, using frozen oocytes may increase the safety of fertility treatment by avoiding ovarian hyperstimulation and repeated ovum-pickup procedures. The greatest advance, however, appears to be for gynecological-cancer patients, who may use cryopreserved oocytes for ICSI treatment at a later stage. However, what is considered now for cancer patients may eventually be the safeguard against infertility that is caused by advanced age because of socio-demographic rather than medical reasons (6). One also can envision storage of surplus oocytes that are cryopreserved for anonymous oocyte donation.

In contrast to cryopreservation of human sperm and embryos, the cryobiology of oocytes still is more a potential solution than a practical remedy. Our study is the first to report on cryopreservation performed as a routine procedure for so-called salvaging of surplus oocytes that cannot be fertilized under the recent Italian legislation regulating ART (10). As opposed to the elective use of oocyte cryopreservation to maintain or preserve fertility described in the previous paragraph, oocyte cryopreservation in our center is used in infertile patients who failed IVF-ICSI with fresh oocytes. Thus, the results of our series enable one to appreciate the role of this method for treating existing rather than potential infertility.

The data indicate that existing freezing–thawing methods result in a reasonable survival rate (>70%) of oocytes and, moreover, that >70% of those surviving cryopreservation appear to be high-quality oocytes by the currently used criteria. These oocyte-survival rates translate to the observation that 75% of the oocytes submitted to micromanipulation undergo cleavage and that >80% of the resulting embryos appear to be of high quality by the currently used criteria. The data shown in Table 1 and Figure 1 may suggest further that outcomes of oocyte cryopreservation appear to be independent of the patient's age. Regardless, and in contrast to these apparently favorable method-related outcomes, the overall implantation per ET rate (6.3%) was poor, and moreover, one third of the pregnancies did not result in embryonic development, and of those who reached this clinical stage, almost half were aborted. The net result was an extremely low take-home-baby rate (<1.5%).

The discussion of our results relates to the two obvious discrepancies between poor pregnancy outcome and [1] the apparently favorable oocyte and embryonic characteristics and [2] the formidable investment in the process. The discrepancy between the encouraging oocyte-survival rates as well as the cleavage rates, on one hand, and the poor pregnancy rates on the other hand, may suggest that serious functional damage occurs to the oocytes en route to their becoming fertilized after being frozen and thawed. This discrepancy implies that the criteria used to characterize the process after ICSI of fresh oocytes may be unsuitable, or even deceptive, in characterizing the reproductive potential of cryopreserved oocytes undergoing the same ICSI technique.

Because the available criteria are morphological in nature, they conceivably miss submicroscopic functional damage to the oocytes or developing embryos after cryopreservation; however, whether the damage is structural, biochemical, or genetic; or represents an antigenic shift that increases the rejection risk of products of conception; or is due to a combination of these factors is at present unknown. In addition, the presence of uterine factors influencing fertility in these patients cannot be excluded. We did not perform endometrial evaluation or genetic analysis after abortions in our patients. Thus, because the damage to the oocytes is indefinable, the stage at which it occurs also is elusive, and therefore the damage cannot be contained at present.

The second aspect of our results refers to the discrepancy between the poor outcomes and the laborious and painstaking efforts involved in oocyte cryopreservation. Our data suggest (Table 1) that the probability of live birth is 1:65 (7/456) ET cycles, a rate that is far below the live birth rate after ICSI of fresh oocytes. Given these results, one may question the wisdom of spending so much effort for such little yield, unless one is obliged to do so under legislative restrictions.

Having voiced this pessimistic acknowledgement, we should at the same time admit that classical IVF at its inception had worse results than tubal surgery, and cryopreservation of sperm and embryos had much worse initial results than that achieved with improved cryobiological techniques. Because oocyte cryopreservation is only at its preliminary stages, one may expect that future advances and collaboration will improve pregnancy rates to levels that are acceptable for routine infertility treatment.

Acknowledgments: The authors thank Giuseppe Nucera, M.D., and Silvia Resta, B.Sc. (Department of Obstetrics and Gynecology, Arcispedale Santa Maria Nuova, Reggio Emilia, Italy), for their invaluable assistance in data management.

REFERENCES

1. Fabbri R, Porcu E, Marsella T, Rocchetta G, Venturoli S, Flamigni C. Human oocyte cryopreservation: new perspectives regarding oocyte survival. Hum Reprod 2001;16:411–6.
2. Borini A, Bonu MA, Coticchio G, Bianchi V, Cattoli M, Flamigni C. Pregnancies and births after oocyte cryopreservation. Fertil Steril 2004; 82:601–5.
3. Van der Elst J. Oocyte freezing: here to stay? Hum Reprod Update 2003;9:463–70.
4. Borini A, Sciajno R, Bianchi V, Sereni E, Flamigni C, Coticchio G. Clinical outcome of oocyte cryopreservation after slow cooling with a protocol utilizing a high sucrose concentration. Hum Reprod 2006;21: 512–7.
5. Porcu E, Fabbri R, Seracchioli R, Ciotti PM, Magrini O, Flamigni C. Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. Fertil Steril 1997;82:993–8.
6. Bredkjaer HE, Grudzinskas JG. Cryobiology in human assisted reproductive technology. Would Hippocrates approve? Early Pregnancy 2001;5:211–3.
7. ESHRE Task Force on Ethics and Law. Taskforce 7: ethical considerations for the cryopreservation of gametes and reproductive tissues for self use. Hum Reprod 2004;19:460–2.
8. Porcu E, Fabbri R, Damiano G, Fratto R, Giunchi S, Venturoli S. Oocyte cryopreservation in oncological patients. Eur J Obstet Gynecol Reprod Biol 2004;113:S14–6.
9. Practice Committee of the American Society for Reproductive Medicine. Ovarian tissue and oocyte cryopreservation. Fertil Steril 2004;82: 993–8.
10. Ragni G, Allegra A, Anserini P, Causio F, Ferraretti AP, Greco E, et al. The 2004 Italian legislation regulating assisted reproduction technology: a multicentre survey on the results of IVF cycles. Hum Reprod 2005;20:2224–8.