Original Article

Fertilization and Embryo Development of Fresh and Cryopreserved Sibling Oocytes

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Abstract

Background: Oocyte cryopreservation is potentially the best way to preserve female fertility for unmarried women or young girls at risk of losing ovarian function. The aim of this study was to compare fertilization and embryo development in frozen-thawed oocytes to their fresh siblings in women undergoing *in vitro* fertilization (IVF) and embryo transfer (ET).

Materials and Methods: Eleven infertile women undergoing infertility treatment, between the ages of 24 to 37 years (mean ± SD = 31.6 ± 3.5), were included in this study. Mature oocytes from each patient were randomized into cryopreserved and fresh groups prior to intracytoplasmic sperm injection (ICSI). One hundred and thirty nine oocytes were retrieved, of which 105 were at metaphase II (MII). Forty- five fresh MII oocytes were kept in culture whereas their sibling 60 MII oocytes were cryopreserved using a slow cooling protocol. The frozen oocytes remained in LN2 for 2 hours before thawing. ICSI was performed 1-2 hours after thawing for frozen oocytes and 4-5 hours after retrieval for fresh oocytes. Fertilization and embryo development were compared.

Results: Following thawing, 31 oocytes (51.6 %) survived and 22 fertilized (79%) while 32 fresh oocytes fertilized upon ICSI (71%). The mean ± SE scores for embryos developing from frozen-thawed oocytes were significantly lower at 48 and 72 hours post-ICSI than for embryos resulting from fresh oocytes ($p<0.05$).

Conclusion: Our data demonstrated that oocyte freezing resulted in acceptable survival rates following cryopreservation, and similar fertilization rates following ICSI as compared to the fresh sibling oocytes. However the number of blastomeres and the embryo quality on day three was superior in embryos from fresh oocytes when compared to the frozen oocytes.

Keywords: Cryopreservation, Fertilization, Embryo Quality, ICSI, Oocytes

Introduction

Human oocyte cryopreservation is a useful addition to the range of infertility treatments currently available. Unmarried women or young girls at risk of losing ovarian function prematurely as a result of surgery, or as a consequence of cancer therapy, women who work with toxins or teratogens, and women who are concerned about age-related infertility may now choose to preserve their fertility. In today's society, many women are focused on career development and are delaying marriage or having children until they are in their late 30s and early 40s. In such cases, oocyte cryopreservation may be an appropriate solution to protect fertility potential since oocyte quality declines with increasing age (1). In addition, cryopreserved oocytes of infertile couples who have completed their families could be an important source of oocyte donation to help other couples. Oocyte cryopreservation presents an attractive alternative to embryo freezing, which is often fraught with religious, ethical and legal complications. While oocyte cryopreservation success has increased over time, the oocyte thaw-survival and pregnancy rates remain relatively low. Morphological survival,including absence of cracks in the zona pellucida, disruption of the plasma membrane and extensive disorganization of the ooplasm, seems to depend on the quality of oocytes at freezing (2). Chen reported a survival rate of 76% for mature oocytes of very good quality (3), while a significantly lower survival rate (4%) was obtained in excess oocytes, mostly immature and of poor quality (4). Good fertilization and cleavage rates

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(4-8) as well as pregnancies (3, 9-12) have been reported using human oocytes that survived cryopreservation. Among the key concerns associated with oocyte cryopreservation is the disruption of cytosckeletal architecture associated with cooling, which is believed to lead to chromosomal anomalies as well as abnormal cytokinesis.
Insufficient information is available that directly compares the quality and development of embryos originating from oocytes, either fresh or cryopreserved from the same cycle (sibling oocytes). The objective of this study was to evaluate the oocyte freeze/thaw survival rate, fertilization rate, embryo quality and pregnancy rate from embryos originating from sibling fresh or frozen oocytes.

Materials and Methods

Patients

Eleven infertile females between 24 to37 years of age (mean ± SD = 31.6 ± 3.5) undergoing infertility treatment from April to September 2005 were included in this study. Retrieved oocytes were denuded and MII oocytes from each patient were randomized into cryopreserved and fresh groups prior to intracytoplasmic sperm injection (ICSI). The study protocol was approved by the Institutional Review Board of Mount Sinai Hospital, University of Toronto. All women participating in the study signed consent prior to treatment.

Controlled ovarian stimulation and oocyte retrieval

Each woman underwent controlled ovarian stimulation using GnRH agonist (Suprefact; Aventis Canada) and follicle stimulating hormone (FSH) (Puregon; Organon, Canada, or Gonal-F; Serono, Canada). Follicular growth was monitored using vaginal ultrasonography and serum estradiol (E2) detection. When two leading follicles reached a mean diameter of 18 mm with proportional serum E2 levels, 10,000 IU of human chorionic gonadotropin (hCG) (Profasi; Serono) was given. Oocytes were retrieved 34 hours later under the guidance of vaginal ultrasound. The oocyte-cumulus complexes were cultured in human tubal fluid (HTF) medium supplemented with 10% Synthetic Serum Substitute (SSS; Irvine Scientific, USA) and incubated at 37°C in a humidified atmosphere of 5.5% CO_2 in air.

Preparation of oocytes for cryopreservation

After 1 h in culture, the oocytes were placed in hyaluronidase (80 IU/mL; Sigma, USA) for 30 sec and cumulus cells were removed by pipetting through a micropipette. Each oocyte was checked for the presence of the first polar body. The oocytes with an extruded first polar body (MII) were cryopreserved. Immature oocytes [metaphase I (MI), or germinal vesicle (GV)] were not frozen. Freezing was initiated within 2 hours post-retrieval.

Freezing and thawing

The cryopreservation solution consisted of sodium ion depleted medium containing 20% SSS, 1.5 mol/L propanediol and 0.2 mol/L sucrose (Sage Assisted Reproduction Products, ART-8017-A, USA). Denuded oocytes were transferred into 1 mL of cryopreservation solution in a central well culture dish and kept at room temperature (22-24°C) for 20 minutes. The oocytes shrank rapidly upon exposure to the freezing solution (Fig 1).
The dish was covered to minimize evaporation and a subsequent change in osmolality of the media. After 20 minutes equilibration, 1 to 3 oocytes were loaded into 0.25 mL straws (CryoBio System, I.M.V. Technologies, France) and placed into the freezing chamber. The freezing ramps were as follows: room temperature to -6.0°C at -2.0°C/minutes, held for 5 minutes, manually seeded then held an additional 10 minutes at -6.0°C, cooled at -0.3°C per minutes to -33°C, plunged into liquid nitrogen and stored for 1 to 2 hours.

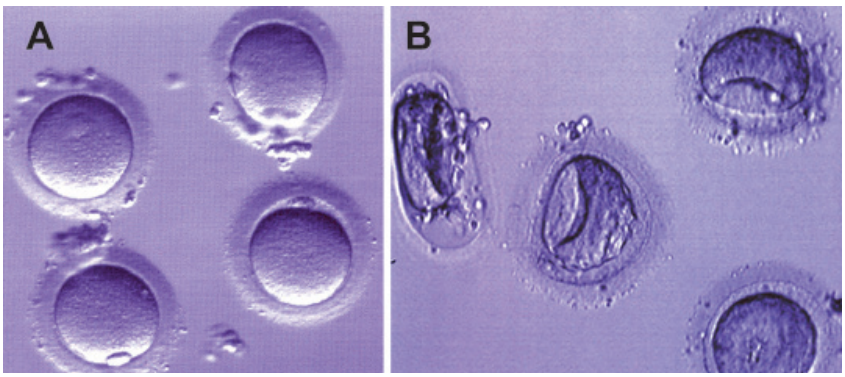

Fig 1: A. Mature (MII) denuded oocytes and B. Following exposure to freezing solution.

Thawing was performed in solutions obtained from Sage Assisted Reproduction Products, USA. The straw was held in air for 45 seconds and then immersed in a water bath at 35°C for 45 seconds. The straw was then wiped, and contents expelled into the lid of a 65 mm culture dish. The oocytes were identified and picked up in a minimal amount of medium and transferred into 1 mL of oocyte thawing solution containing 0.5M sucrose at room temperature for 10 minutes, followed by thawing solution containing 0.2M sucrose for 10 minutes. The oocytes were then washed through two dishes containing 1 mL of thawed oocyte wash medium and transferred to an overnight equilibrated P1 medium (Irvine Scientific, USA) supplemented with 10% SSS in humidified atmosphere of 5.5% CO_2 in air and 37°C.

ICSI and fertilization check

ICSI was performed 1-2 hours after thawing for frozen oocytes and 4-5 hours after retrieval for fresh oocytes by the same embryologist. Oocytes were examined 16-20 hours post-ICSI and the presence of two pronuclei (2PN) was taken as evidence of normal fertilization and oocytes that displayed one or three pronuclei were considered abnormal. Embryos were examined daily and scored at 48 and 72 hours post-ICSI (Fig 2).

Numerical embryo scoring, embryo development, and transfer

A numerical embryo scoring method was developed to objectively compare embryo development at 48 and 72 hours post-ICSI. Numerical embryo scoring was based on blastomere number and percentage of fragmentation. On day 2 (42-44 hours post-ICSI) an embryo having > 4 blastomeres was given a score of 10, an additional score of 10 was added if it had < 20% fragmentation and another score of 5 was awarded if there was no multinucleated blastomere. Similarly, on day 3 (66-68 hours post-ICSI) an embryo having > 8 blastomeres was given a score of 10, an additional score of 10 was added if it had < 20% fragmentation and another score of 5 was added if there was no multinucleated blastomere.

Embryo transfer (ET) was done on day 3 post-ICSI under ultrasound guidance using a Cook catheter (Sydney IVF ET set, Cook, USA). The number of ET was based on the number of available embryos, patient age and embryo quality. The embryos obtained from frozen/thawed oocytes were used for ET and embryos resulted from fresh oocytes were cryopreserved on day 3. In instances of no embryos from the frozen oocytes, embryos originating from fresh sibling oocytes were transferred. Natural progesterone was administered until menstruation or the first ten weeks following a positive β-hCG. Pregnancy test was carried out 14 days after ET. Clinical pregnancy was defined as the presence of the gestational sac in utero by ultrasound at 6 weeks of gestation. The subsequent development of a fetal heart beat was defined as an ongoing pregnancy.

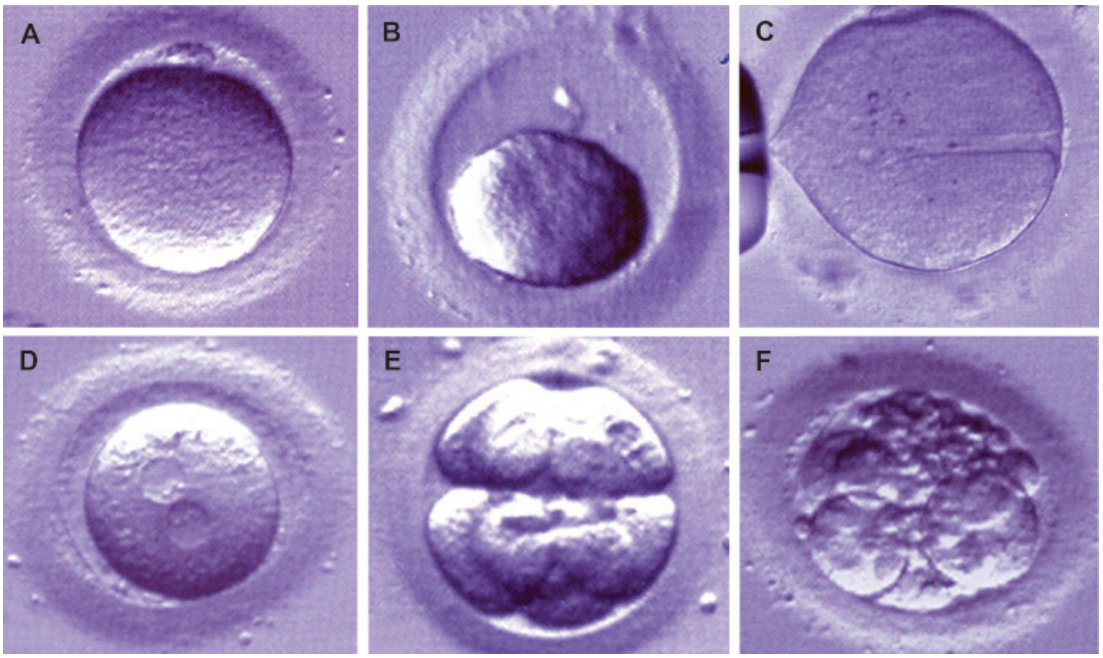

Fig 2: A. Normal mature oocyte, B. Following exposure to freezing solution, C. Thawed oocyte following ICSI, D. Fertilized oocyte at pronuclear stage, E. Day 3 embryo from fresh oocyte, F. Day 3 embryo from frozen-thawed oocyte.

Statistics

The survival, fertilization and development rate on days 2 and 3 were calculated for the fresh and frozen-thawed oocytes. Differences of scores at 48 and 72 hours were compared between the two groups using student's t test. Differences were considered significant at $p<0.05$.

Results

From 11 patients, 139 oocytes were retrieved of which 105 were at MII. Sixty MII oocytes were cryopreserved whereas their sibling 45 fresh MII oocytes underwent ICSI. Upon thawing, 31 oocytes (51.6 %) survived (Table 1).

Twenty-eight thawed oocytes were suitable for ICSI which resulted in fertilization of 22 oocytes (79%) compared to fertilization of 32 oocytes (71%) in fresh siblings. Mean ± SE scores of embryos originating from frozen-thawed oocytes at 48 and 72 hours were significantly lower than those in embryos from fresh oocytes ($p<0.05$). The mean number of embryos transferred did not differ between the two groups.

In one patient, none of her 9 frozen oocytes survived. Poor quality and slow embryos resulted from her 5 fresh MII oocytes and pregnancy was not achieved after transfer of fresh embryos. We suspected her failed oocyte cryopreservation was due to her poor oocyte quality. For a 35 year-old patient that became pregnant, 3 oocytes were cryopreserved, 3 survived, 3 fertilized, cleaved and were transferred. Two embryos implanted and she delivered twin girls. Three patients received embryos originating from fresh oocytes and one became pregnant and delivered a baby boy (Table 2).

Table 1: Comparison between some parameters of sibling embryos originating from either frozen-thawed or fresh oocytes

Variable	Frozen-thawed eggs (%)	Fresh eggs (%)
Number of patients	11	11
Number of frozen oocytes	60	-
Number of oocytes survived	31 (51.6)	-
Number of oocytes injected	28 (90)	45 (100)
Number of 2PN	22 (79)	32 (71)
Number of 3PN	2 (7)	4 (9)
Number of 1PN	1 (3.6)	0
Mean ± SD score 48 h	*10.9 ± 1.24/30	*15.8 ± 0.56/30
Mean ± SD score 72 h	*6.2 ± 1.28/30	*13.8 ± 1.47/30
Number of ET	8	3
Mean number of ET	2.6	2.7
Clinical pregnancy rate	1 / 8 (12.5)	1 / 3 (33.3)

Mean ± SD score obtained/total score possible

Table 2. Cycle outcome following thawing, ICSI and transfer of embryos resulting from frozen-thawed oocytes.

Patient No	MII	Frozen	Survived	Fertilized	*ET	Comments
1	8	4	4	2	2	**Negative β-hCG**
2	5	3	3	3	3	**Delivered twin girls (received embryos from frozen oocytes)**
3	12	6	2	2	2	**Negative β-hCG**
4	9	5	3	2	2	**Negative β-hCG**
5	8	5	3	2	2	**Negative β-hCG**
6	5	3	2	0	0	**Received 2 embryos from fresh oocytes, Negative β-hCG**
7	15	8	6	5	3	**Negative β-hCG**
8	8	5	2	1	0	**Delivered a boy (received 2 embryos from fresh oocytes)**
9	11	6	4	3	3	**Negative β-hCG**
10	14	9	0	0	0	**Received 2 embryos from fresh oocytes**
11	10	6	2	2	2	**Negative β-hCG**
Total	105	60	31	22	19	

Number of ET originating from frozen-thawed oocytes. The number "0" indicates that embryos from frozen-thawed oocytes were not available and the patient received embryos from fresh oocytes.

Discussion

In this study we compared fertilization and embryo development of sibling frozen-thawed and fresh oocytes. This comparison will highlight the impact of cryo-injury during slow cooling-rapid thawing on oocyte ultrastructure and developmental potential. In our study, we achieved a live birth rate of 3.3% per oocyte thawed, 7.1% per oocyte injected and 12.5% per ET. Our results are comparable with the meta-analysis of 26 published studies in 2006 where the live birth rate per oocyte thawed was 1.9%, per injected oocyte was 3.4% and 21.6% per ET. Pregnancy rates with vitrification appear to be improved and superior to slow freezing and further studies will determine the efficiency of this technique (13). The survival rate in this study was 51.6 % which is similar to 60% reported by Fabbri et al. (14). Until recently, a poor survival rate has been the major hurdle to the application of oocyte cryopreservation. Methodological improvements have made egg freezing more efficient with an increase in survival, fertilization and pregnancy rates. One approach involves a slow cooling/rapid thawing method using propanediol (PrOH) and sucrose as cryoprotectants, similar to the methodology used for embryo cryopreservation. On the other hand, some investigators have reported improved survival and fertilization rates as well as live birth rates using vitrification which is less expensive and less time consuming (12, 15). Despite interest in the potential benefits of vitrification as an alternative laboratory approach to long-term oocyte preservation, there is little agreement on how safe and applicable this procedure is. High concentrations of cryoprotectants used in vitrification and their toxic effects on oocytes and resulting embryos have been questioned. Nevertheless, the recent improvements in this approach, including increased cooling and warming rates using very small volumes and decreasing toxicity by reducing cryoprotectant concentrations, have allowed for very high results of vitrification in terms of oocyte survival, fertilization rate, embryo development rates and clinical outcomes (13). Rienzi et al. have found that oocyte vitrification procedure followed by ICSI is not inferior to the fresh insemination procedure, with regard to fertilization and embryo developmental rates. Although they found some, non-significant differences in pronuclear morphology; embryo quality was similar in the two groups. The percentage of top quality embryos per fertilized oocyte was about 52% in the fresh and vitrified group of oocytes (15). Moreover, when vitrified oocytes are compared simultaneously with fresh counterparts, in an oocyte donation program, similar laboratory results have been observed in terms of fertilization (76.3 and 82.2%, respectively), embryo development and blastocyst formation rates (48.7 and 47.5%, respectively) (16).

Using the slow cooling method based on PrOH, Fabbri et al. have reported a very high survival rate as a consequence of the increase in sucrose concentration to 0.3 mol/L in the cryopreservation mixture (17). A different approach has been applied by Stachecki et al. who replaced sodium with the less toxic organic ion choline (18). This is believed to reduce the solution effect and made it possible to attain higher rates of post-thaw survival, fertilization and pre-implantation development of mouse oocytes. The use of sodium-depleted freezing solutions has also been adopted for human embryos by some researchers (19) and was the method used in our study. However, similar to our findings, Magli et al. have shown reduced fertilization and cleavage rates in oocytes following a slow cooling protocol when compared with sibling fresh oocytes and suggested that, even if surviving thawing, the process of slow freezing has a negative impact on the potential of further growth (20) Studies using MII oocytes demonstrate that the oocyte survival rate after cryopreservation could be affected by morphological and biophysical factors (21). Among the morphological factors, particularly important are oocyte characteristics such as maturity, quality and oocyte size. Frozen-thawed oocytes can be fertilized by conventional insemination, although ICSI has become the accepted means of insemination of oocytes post-thaw (15). This is to avoid any reduction in sperm penetration of the zona pellucida due to premature cortical granule release or general hardening of the zona following freezing and thawing. The abnormal fertilization rate of cryopreserved oocytes, as seen in this study, is low and similar to that of fresh oocytes. The application of ICSI for cryopreserved oocytes did not seem to increase the degeneration rate after injection. Similarly embryo development, cleavage rate and morphological quality did not seem to be severely compromised by cryopreservation because a reasonable percentage of embryos were of good or fairly good quality.

Conclusion

The comparison of *in vitro* outcome of sibling fresh and frozen oocytes is important for setting up an oocyte banking program for patients at risk of losing their ovaries. Cryopreservation of oocytes, especially in very young female patients, remains a controversial technique particularly when the numbers of oocytes to be frozen are

few. An increased number of oocytes retrieved or several oocyte-freezing cycles will provide more peace of mind and may guarantee a larger number of oocytes that survive and fertilize after thaw. Banking oocytes to extend reproductive age is not a guaranteed procedure as the survival rate of frozen oocytes is variable and several egg retrievals to obtain a large number of mature eggs may be required. Given the current pregnancy rate for oocyte cryopreservation, the success rate is similar to the pregnancy rate for women in their late 30s and early 40s, the important question is whether oocyte freezing is efficient enough to be used electively to delay child bearing? Recent guidelines published by the Practice Committee of the American Society for Reproductive Medicine (ASRM) and the Practice Committee of the Society for Assisted Reproductive Technology (SART) consider oocyte freezing an experimental procedure and only to be performed as an investigational protocol under the auspices of an IRB (22). It is likely that further studies of oocyte vitrification as one possible means to improve outcome are required to address this question.

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