Cryopreserved oocytes of infertile couples undergoing assisted reproductive technology could be an important source of oocyte donation: a clinical report of successful pregnancies

Xiao-Hong Li1,3, Shee-Uan Chen2, Xiao Zhang1, Miao Tang1, Yuan-Rong Kui1, Xueqing Wu1, Song Wang1 and Ying-Lu Guo1

1Reproduction and Genetic Center, First Hospital of Peking University, Peking University, Beijing, China and 2Department of Obstetrics and Gynecology, National Taiwan University Hospital, Taipei, Taiwan

3To whom correspondence should be addressed at: Reproduction and Genetic Center, First Hospital of Peking University, Peking University, No. 8, Xishiku St., West District, Beijing 100034, China. E-mail: l_xiaoh@yahoo.com.cn

BACKGROUND: Surplus oocytes in assisted reproduction treatment cycles could be saved and donated to other couples. ICSI is usually performed for oocytes that have been stored frozen, considering possible exocytosis of cortical granules (CG). The unavoidability of ICSI merits further study. METHODS: We used a slow method to freeze excess oocytes from infertile couples. After thawing, oocytes were fertilized by either IVF or ICSI according to semen parameters. Some oocytes were examined for CG. RESULTS: Twenty-eight infertile couples cryopreserved a portion of their oocytes and 12 thawed their oocytes. Three couples used their own oocytes, whereas nine donated their oocytes to nine other couples for 12 cycles. The survival rate from thawing was 90% (73/81). The fertilization rate using IVF (83%) was similar to ICSI (82%). Seven pregnancies (47% per cycle) were achieved; one used her own oocytes and six received donated oocytes. Five women delivered six babies including one set of twins. Two pregnancies aborted. The frozen–thawed oocytes (15/15) revealed no exocytosis of CG. CONCLUSIONS: To freeze oocytes of infertile couples undergoing assisted reproduction treatment may help other couples. Our successful experience may facilitate oocyte banks to become a reality. Both IVF and ICSI are valuable for frozen oocytes.

Key words: cortical granules/cryopreservation/gamete donation

Introduction
The cryopreservation of sperm, oocyte and embryo has significant clinical value in reproductive medicine. Although sperm and embryo freezing have already been widely used for infertility treatment, oocyte freezing has not yet achieved an important role. However, oocyte cryopreservation can avoid ethical, religious and legal issues surrounding embryo preservation (Wennerholm, 2000). The freezing of oocytes may be an important method for preserving the fertility of women affected by radiotherapy, chemotherapy, surgical removal of the ovaries, or the physiological effects of age (Porcu et al., 2004; Chen et al., 2005). Otherwise, oocytes can be frozen in the event of failed sperm retrieval on the day of oocyte retrieval (Porcu et al., 1997, 1999).

In the past, oocyte cryopreservation was associated with poor survival, fertilization, and development. Using the same freezing and thawing procedures, oocytes have lower survival rates compared to embryos (Tucker et al., 1998). Exposure of metaphase II oocytes to cooling, cryoprotectants, and a freeze–thaw cycle causes disorganization of the meiotic spindles that deters normal fertilization and cleavage (Eroglu et al., 1998; Chen et al., 2003). Recently, several protocols for slow freezing and vitrification were proposed to improve the survival of frozen–thawed oocytes (Fabbri et al., 2001; Quintans et al., 2002; Yang et al., 2002; Yoon et al., 2003). After thawing, sufficient incubation in optimal conditions would allow the spindles to recover (Chen et al., 2001; Rienzi et al., 2004, Bianchi et al., 2005), and insemination at 3–4 h could improve normal fertilization and subsequent development (Bol dt et al., 2003; Yoon et al., 2003; Chen et al., 2005).

Fabbri et al. (2001) showed that slow freezing using 1.5 mol/l 1,2-propanediol (PROH) with an increase in the sucrose concentration to 0.3 mol/l yielded a high oocyte survival rate of 82%. Using Fabbri's regime, reproducible successes for survival (75–90%), fertilization (67–73%) and pregnancy (33–57%) of oocyte cryopreservation have been reported by Fosas et al. (2003) and Chen et al. (2005). Nonetheless, those case numbers were limited. Oocyte freezing is still not common in IVF centres, and further study is required for its authentication.
Chen (1986) achieved the first successful human pregnancy from frozen–thawed oocytes in the era of IVF. Porcu et al. (1997) reported the first live birth following oocyte cryopreservation and ICSI. Since then, ICSI has commonly been performed for frozen–thawed oocytes, considering the possible premature release of cortical granules (CG) resulting in zona hardening (Quintans et al., 2002; Boldt et al., 2003; Yoon et al., 2003; Borini et al., 2004; Chen et al., 2005). However, the effect of cryopreservation of mammalian oocytes on the premature release of CG has remained controversial (Vincent et al., 1990; George et al., 1992; Gook et al., 1993; Van Blerkom and Davis, 1994; Jones et al., 2004). The necessity for ICSI instead of IVF may merit further study.

In China, donation of embryos to infertile couples is not allowed according to the relevant laws. Redundant cryopreserved embryos of women who have completed their families using assisted reproductive technology are normally discarded. With significant improvements in oocyte freezing techniques in recent years, surplus oocytes in assisted reproduction treatment cycles could potentially be saved and donated to other infertile couples. In this study, we used Fabbri’s method to freeze excess oocytes from infertile couples for their own use or possibly for donation, a new strategy for an oocyte-sharing programme. We chose ICSI or IVF for insemination of frozen–thawed oocytes based on the semen parameters. In addition, we examined CG of oocytes after the freezing and thawing procedures.

Materials and methods

Patients

Candidates for oocyte cryopreservation were recruited from women with tubal factor or severe male factor infertility undergoing assisted reproduction treatments who had >16 oocytes available. Female subjects were limited to those aged 25–33 years. The infertile couples were counselled on the advantages and disadvantages of oocyte cryopreservation, in comparison with embryo freezing. The information included ethical and religious aspects and the possibility of donating the oocytes to other infertile couples. The current success rate of oocyte cryopreservation was explained to the couples according to recent articles (Porcu et al., 2000; Yang et al., 2002; Boldt et al., 2003; Fosas et al., 2003). Patients with infectious or hereditary diseases were excluded from this study. Those infertile couples with premature ovarian failure, physiological menopause, or extremely poor ovarian reserve who asked for oocyte donation were enrolled as candidates for recipients. This research was approved by the Ethics Committee of First Hospital of Peking University. All women participating in the study signed an approval consent form prior to treatment.

Controlled ovarian stimulation (COS) and oocyte retrieval

Each woman underwent COS with a long protocol of GnRH agonist. FSH (Gonal-F; Serono, Switzerland) was used for ovarian stimulation. Follicular growth was monitored using vaginal ultrasonography and detection of serum estradiol (E2) levels. When the leading follicles reached a mean diameter of 18 mm with proportional serum E2 levels, 10 000 IU of HCG (Profasi, Serono) was given. Oocytes were retrieved 34 h later under the guidance of vaginal ultrasound. The oocyte–cumulus complexes were cultured in human tubal fluid (HTF) medium with 20% synthetic serum substitute (SSS, Irvine Scientific, USA) and incubated at 37°C in an atmosphere of 5% CO2 in air.

Cryopreservation of a proportion of oocytes

Six to eight oocytes were generally used for IVF or ICSI. The additional mature oocytes were cryopreserved. The cumulus cells were dissected using 23-G needles followed by brief exposure (30 s) to hyaluronidase (80 IU/ml; Sigma, USA). The corona cells were completely removed by pipetting through micropipettes. The oocytes were checked for the presence of the first polar body in the perivitelline space (metaphase II). Immature (metaphase I or germinal vesicle stage) oocytes were not frozen. Freezing was routinely initiated within 1–2 h after retrieval.

Preparation of the pretreatment, freezing, and dilution solutions

The solutions for cryopreservation and dilution were prepared using phosphate-buffered saline (PBS; Irvine Scientific) plus 20% SSS. The pretreatment solution consisted of 1.5 mol/l PROH (Sigma). The freezing solution contained 1.5 mol/l PROH plus 0.3 mol/l sucrose. For the thawing procedures, oocytes were thawed in stepwise dilution solutions of 1.0 mol/l PROH plus 0.3 mol/l sucrose, 0.5 mol/l PROH plus 0.3 mol/l sucrose, and 0.3 mol/l sucrose.

Equilibration and slow freezing of oocytes (Fabbri et al., 2001)

The oocytes were transferred into 0.8 ml of the pretreatment solution and maintained at room temperature (22–24°C) for 10 min. They were then transferred to the freezing medium. After 15 min of equilibration, oocytes were loaded into a 0.25 ml plastic straw (IMV, France) and transferred into an automated biological vertical freezer (Kryo-360, Planer Product Ltd, UK). The temperature was slowly reduced to −7°C at a rate of −2°C/min. Ice nucleation was induced manually at −7°C. After a holding time of 10 min at −7°C, the straws were slowly cooled to −30°C at a rate of −0.3°C/min and then rapidly to −150°C at a rate of −50°C/min. The straws were then transferred into liquid nitrogen tanks and stored until thawing.

Treatment cycles with frozen–thawed oocytes

For the thawing cycles, the endometrial preparation involved a natural ovulatory cycle or a hormonal replacement cycle. For the protocol of the hormonal replacement cycle, the female received oral estradiol valerate (2 mg) with incremental doses of 4 mg from day 3 to day 8; 8 mg from day 9 to day 11; and then 12 mg from day 12. On day 14, serum E2, LH and progesterone were determined and the endometrial thickness was measured using vaginal ultrasonography. If the endometrial thickness was ≥28 mm, with no evidence of ovulation, progesterone in oil (40 mg daily) was administered intramuscularly. The cryopreserved oocytes were thawed, and IVF or ICSI was performed on the day in which progesterone administration was begun. If pregnancy was achieved, hormonal replacement was carried out until 12 weeks of gestation.

To thaw the straws, they were air-warmed for 30 s and then immersed in a 30°C water bath for 40 s until all traces of ice had disappeared (Fabbri et al., 2001). The contents of the straws were expelled into 1.0 mol/l PROH plus 0.3 mol/l sucrose solution, and the oocytes were equilibrated for 5 min. They were then transferred to 0.5 mol/l PROH plus 0.3 mol/l sucrose solution for an additional 5 min and then into a 0.3 mol/l sucrose solution for 10 min. Final dilutions were completed in PBS solution plus 20% SSS for 20 min (10 min at room temperature and 10 min at 37°C). The oocytes were placed in HTF culture medium and incubated for 3 h. Oocytes were defined as having morphologically survived if the cells possessed an intact zona pellucida and plasma membrane as well as refractive cytoplasm.
Fertilization using conventional IVF or ICSI

The frozen–thawed oocytes were fertilized by IVF or ICSI according to the semen parameters (World Health Organization, 1999) or previous fertilization results of IVF. When the semen analysis revealed normal parameters or the previous fertilization rate for IVF was ≥50%, IVF was performed. Otherwise, ICSI was carried out. Oocytes were examined 16–18 h after IVF or ICSI. Those with two pronuclei (2PN) and a second polar body were identified as normally fertilized. Those oocytes with a single pronucleus (1PN) or three pronuclei (3PN) were considered as abnormally fertilized.

Embryo transfer and follow-up of pregnancy

Cleaving embryos on day 2 or 3 after fertilization were transferred into the uterine cavity transvaginally. Fourteen days after transfer, the serum β-hCG level was evaluated. Clinical pregnancy was defined as the presence of gestational sac in utero by ultrasound at 6 weeks of gestation. The subsequent development of a fetal heartbeat was defined as an ongoing pregnancy.

CG staining of frozen–thawed oocytes

Two couples who had achieved successful pregnancies in fresh cycles donated their cryopreserved oocytes for research on CG. Eighteen frozen oocytes were thawed, of which 15 (83%) survived. Three hours after thawing, oocytes were briefly treated with acid Tyrode’s solution (pH 2.3) to remove the zona pellucida. The zona-free oocytes were fixed for 15 min in freshly prepared 4% paraformaldehyde (Fan et al., 2002). They were permeabilized by treatment with 0.2% Triton X-100 at room temperature for 15 min, and then blocked in 1% bovine serum albumin for 1 h.

For the staining of CG (Van Blerkom and Davis, 1994), oocytes were incubated with biotinylated lectin (LCA; 5 μg/ml, Molecular Probes, USA) overnight. After extensive washing, oocytes were incubated with fluorescein isothiocyanate (FITC)–streptavidin (2 μg/ml) for 30 min. The meiotic stage of each oocyte was confirmed by its chromatin configuration through staining with 20 μg/ml Hoechst 33258. Oocytes were examined under a confocal laser scanning microscope equipped with an argon–krypton laser (MRC-2100, Bio-Rad, USA).

Statistics

The percentages of surviving oocytes, fertilization, implantation, clinical pregnancies and ongoing pregnancies were calculated. These parameters were compared between the groups using χ² or Fisher’s exact test. The mean ± SD of women’s ages and number of embryos transferred were compared using the Mann–Whitney test between the groups. Differences were considered significant at \( P < 0.05 \).

Results

Since June 2003, 28 infertile couples undergoing IVF (n = 16) or ICSI (n = 12) treatments cryopreserved a proportion of their oocytes. All couples had fresh transfer. The mean age of these women was 30 ± 2 years (range, 25–33 years). The mean number of embryos transferred was 3.0 ± 1.0. Eleven pregnancies (39%) were achieved including eight singletons and three sets of twins. The implantation rate was 17%. One pregnancy was aborted due to a blighted ovum (9%).

Twelve infertile couples underwent 15 treatment cycles using cryopreserved oocytes. Three couples used their own frozen oocytes for three cycles. Nine couples received donation of cryopreserved oocytes for 12 cycles from nine couples who had achieved successful pregnancies in the fresh cycles. The indications for receiving donated oocytes included physiological menopause in four, premature ovarian failure in two, poor ovarian reserve in two, and an abnormal karyotype in the wife in one.

The results of survival of oocytes after freezing–thawing, fertilization, and pregnancy following IVF or ICSI using personal or donated oocyte are presented in Table I. Three couples with their own cryopreserved oocytes used IVF for insemination because they had normal semen parameters and had a normal fertilization rate in the previous fresh IVF cycles. Among those receiving donated oocytes, three couples with normal semen used IVF for insemination and six couples underwent ICSI due to abnormal semen parameters.

The mean age of females receiving donated oocytes (41 ± 7 years) was significantly older than that of females using their own cryopreserved oocytes (31 ± 2 years, \( P < 0.05 \)). The percentages of normal fertilization (29/35, 83% versus 31/38, 82%), abnormal fertilization (3/35, 9% versus 2/38, 5%) and cleavage (25/29, 86% versus 29/31, 94%) of frozen–thawed

<p>| Table I. Results of cryopreserved oocytes inseminated with IVF or ICSI for personal use or donation |
|---------------------------------|-------------------|---------------------|---------------------|</p>
<table>
<thead>
<tr>
<th>Patients</th>
<th>Personal use with IVF</th>
<th>Receiving donation with IVF</th>
<th>Receiving donation with ICSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 3)</td>
<td>(n = 3)</td>
<td>(n = 6)</td>
<td></td>
</tr>
<tr>
<td>Age (years) (mean ± SD)</td>
<td>31 ± 2</td>
<td>39 ± 11</td>
<td>42 ± 4*</td>
</tr>
<tr>
<td>Cycles</td>
<td>3</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Thawed oocytes</td>
<td>16</td>
<td>22</td>
<td>43</td>
</tr>
<tr>
<td>Survival</td>
<td>15 (94)</td>
<td>20 (91)</td>
<td>38 (88)</td>
</tr>
<tr>
<td>2PN</td>
<td>12 (80)</td>
<td>17 (85)</td>
<td>31 (82)</td>
</tr>
<tr>
<td>1PN</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>3PN</td>
<td>1 (7)</td>
<td>2 (10)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Degeneration</td>
<td>0 (0)</td>
<td>1 (5)</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Cleavage</td>
<td>10 (83)</td>
<td>15 (88)</td>
<td>29 (94)</td>
</tr>
<tr>
<td>Embryos transferred</td>
<td>3.0 ± 1.0</td>
<td>3.0 ± 1.7</td>
<td>3.4 ± 0.9</td>
</tr>
<tr>
<td>Clinical pregnancies</td>
<td>1 (33)</td>
<td>2 (50)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Multiple pregnancies</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Implantation</td>
<td>2 (22)</td>
<td>2 (17)</td>
<td>4 (15)</td>
</tr>
<tr>
<td>Abortions</td>
<td>0 (0)</td>
<td>1 (50)</td>
<td>1 (25)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages
*\( P < 0.05 \), compared to the group of personal use.
PN = pronuclear.

3392
Frozen oocytes of infertile couples help other couples

Oocytes using IVF were similar to those using ICSI. The mean number of embryos transferred was not different between the three groups. Differences in the pregnancy and implantation rates were also not distinct.

Overall, the survival rate of oocytes from freezing–thawing was 90% (73/81). The fertilization and cleavage rates were 82% (60/73) and 90% (54/60) respectively. In every cycle, we had at least one oocyte surviving from freezing–thawing with subsequent fertilization. The mean number of embryos transferred was 3.2 ± 1.0. Seven pregnancies (47% per cycle) were achieved including six singletons and one set of twins. The implantation rate was 17%. Five women had delivered six healthy babies. Two pregnancies were aborted due to a blighted ovum (28%).

The immunostaining examination for CG of the frozen–thawed oocytes (15/15) did not reveal evidence of the premature release of CG (Figure 1).

Discussion

This pilot study achieved successful pregnancies from donated surplus oocytes that had been cryopreserved during assisted reproduction treatment cycles of infertile couples. To freeze excess oocytes can be helpful not only for couples themselves but also for other infertile couples. Using the regime developed by Fabbri et al. (2001), we reproduced the success of oocyte cryopreservation in terms of percentages of survival (90%), fertilization (82%), and pregnancy per cycle (47%) that were comparable to those reported by Fosas et al. (2003) and Chen et al. (2005). The implantation rate of embryos derived from frozen oocytes (17%) was similar to that of fresh embryos. This new strategy for oocyte-sharing programme can save redundant oocytes in assisted reproduction treatment cycles.

The quality of oocytes is an important factor of survival from cryopreservation (Fabbri et al., 2001). Oocytes from young females may have better tolerance to freezing and thawing (Fosas et al., 2003). In addition, the regulatory mechanisms controlling the assembly of the meiotic spindle of oocytes are significantly altered in older women, leading to a high prevalence of aneuploidy (Battaglia et al., 1996). Considering these points, we recruited women aged between 25 and 33 years for cryopreservation of surplus oocytes. They were undergoing assisted reproduction treatment due to tubal factor or severe male factor infertility, so they may possess better quality oocytes. In our series, the high survival rate of frozen–thawed oocytes and the favourable pregnancy rate may indicate that this strategy is probably appropriate.

Results in the literature regarding the premature release of CG of frozen–thawed mammalian oocytes are contradictory. Some authors found that exposure to cryoprotectants or cryopreservation of mouse oocytes led to premature release of CG (Vincent et al., 1990; George et al., 1992). The resulting zona hardening would deter the entry of sperm and subsequent fertilization. However, some authors did not observe a change of CG in frozen–thawed human oocytes (Gook et al., 1993; Jones et al., 2004; Van Blerkom and Davis, 1994). The reasons for these discrepancies are unclear, but may be related to the differences in species and the protocols for freezing and thawing. Using the regime of 1.5 mol/l PROH plus 0.3 mol/l sucrose, our results indicated that human oocytes did not apparently prematurely release CG after slow freezing and rapid thawing.

Kazem et al. (1995) reported a higher fertilization rate using ICSI for frozen–thawed oocytes, compared with IVF, though Gook et al. (1995) found a similar fertilization rate. Even so, ICSI has commonly been used as an effective technique to achieve fertilization for cryopreserved oocytes in recent years (Quintans et al., 2002; Boldt et al., 2003; Yoon et al., 2003; Borini et al., 2004; Chen et al., 2005). In our study, the percentages of normal fertilization, cleavage and pregnancy for frozen–thawed oocytes using IVF were similar to those using ICSI. This further indicates that changes in the zona pellucida from freezing and thawing could be minimal and had no significant effect on sperm penetration.

Chen et al. (2005) reported that the percentages of survival, pregnancy and implantation of frozen–thawed oocytes were similar to those of cryopreserved pronuclear embryos. Their findings are consistent with the results from Boldt et al. (2003), who compared frozen oocytes and cryopreserved cleaving embryos. Likewise, Yang et al. (2002) found that frozen–thawed oocytes achieved results comparable to their fresh sibling oocytes and cryopreserved embryos. Accordingly, with various modifications of the conventional slow freezing method, outcomes of oocyte freezing have been improved and are akin to those of embryo freezing. Oocyte freezing could provide a viable choice to circumvent ethical issues of embryo cryopreservation.

Fosas et al. (2003) recruited young females for oocyte donation and achieved pregnancies in infertile recipients using cryopreserved oocytes. We further proved the possibility of oocyte banking using redundant oocytes from assisted reproduction treatment cycles. This new oocyte-sharing strategy may prevent the wastage of surplus oocytes in assisted reproduction.

Figure 1. A frozen–thawed oocyte was stained immunocytochemically with biotinylated Lens culinaris lectin and fluorescein isothiocyanate–streptavidin to visualize cortical granules (CG) (green) and counterstained with Hoechst 33258 to visualize the chromosomes (blue). The CG were distributed adjacent to the plasma membrane (arrows). There was no apparent exocytosis. Scale bar = 30 μm.
treatment cycles. Compared to recruiting additional female donors, our proposal can reduce the costs as well as avoid the extra risks of ovarian hyperstimulation and oocyte retrieval. Our examination of CG of the frozen–thawed oocytes revealed no apparent premature exocytosis. Both IVF and ICSI achieved comparable percentages of fertilization and pregnancy. These findings may justify conventional IVF for frozen–thawed oocytes when the semen parameters are normal. However, the case number is still small, so additional studies to substantiate the ideals of oocyte banking are necessary.

References


Chen SU, Lien YL, Cheng YY, Chen HF, Ho HN and Yang YS (2001) Vitrification of mouse oocytes using closed pulled straws (CPS) achieves a high survival and preserves good patterns of meiotic spindles, compared with conventional straws, open pulled straws (OPS) and grids. Hum Reprod 16,2350–2356.


Submitted on May 18, 2005; resubmitted on June 26, 2005; accepted on July 5, 2005.