Observational clinical follow-up of oocyte cryopreservation using a slow-freezing method with 1,2-propanediol plus sucrose followed by ICSI

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BACKGROUND: The value of oocyte cryopreservation remains controversial. Two major problems exist: poor survival and injury to the oocyte meiotic spindle after freezing and thawing. METHODS: For slow oocyte cryopreservation, we used 1.5 mol/l 1,2-propanediol and 0.3 mol/l sucrose. We waited 3 h after thawing for possible recovery of the meiotic spindles before performing ICSI. RESULTS: Forty-three women undergoing IVF or ICSI cycles cryopreserved some or all of their harvested oocytes; of these, 20 thawed their cryopreserved oocytes for personal use and one for donation. The survival rate of oocytes after thawing was 75%, with 67% of oocytes fertilizing normally after ICSI. All 21 cycles (100%) resulted in fertilization and embryo transfers. Seven pregnancies (33%) resulted. Four women delivered five babies with normal karyotypes. Three conceptions are ongoing. Compared to 38 cycles of frozen–thawed embryos at the pronuclear stage in the same period, the percentages of survival, pregnancy and implantation were similar. Additionally, four unmarried women with white blood cell diseases underwent oocyte freezing before preconditioning treatment for haematopoietic stem cell transplantation. CONCLUSIONS: This protocol achieved reproducible success of survival, fertilization and pregnancy for freezing and thawing of human oocytes. The 3 h post-thaw incubation could permit restoration of the meiotic spindles, thus facilitating normal fertilization.

Key words: cryopreservation/human oocytes/ICSI
metaphase II of oocytes. The meiotic spindle is crucial for the events following fertilization, including completion of meiosis, second polar body formation, migration of pronuclei, and formation of the first mitotic spindle (Schatten et al., 1985).

Using the fluorescent staining of tubulin, we examined the patterns of meiotic spindles of vitrified mouse oocytes immediately after dilution and at 1, 2 and 3 h intervals after dilution. We found that the freeze–thaw-injured spindle recuperated in a time-dependent process (Chen et al., 2001). After 2 or 3 h of incubation, the spindle patterns significantly improved, as did fertilization outcomes. Recently, Rienzi et al. (2004) used polscope analysis to observe meiotic spindles of human oocytes after slow freezing and rapid thawing. Immediately after thawing, the spindle was visible in only 36% of oocytes, but it disappeared in all of the thawed oocytes during the subsequent washing steps. Nevertheless, the spindle reappeared in all surviving oocytes after 3 h of incubation at 37°C in culture medium.

Freezing and thawing of oocytes could result in premature release of cortical granules and hardening of zona pellucida that may deter entry of a spermatozoon (Chen et al., 2003). ICSI has been used to overcome this problem in recent years (Porcu et al., 2000). In September of 2001, we started an oocyte cryopreservation programme using 1.5 mol/l PROH and 0.3 mol/l sucrose (Fabbri et al., 2001) and performing ICSI 3 h after thawing. We were especially interested in the recovery of the meiotic spindle. Here, we report our experience with oocyte cryopreservation as an integral part of our IVF programme.

**Materials and methods**

**Patients**

Nineteen infertile couples undergoing IVF or ICSI treatments, who did not wish to undergo embryo freezing for various ethical and religious reasons, cryopreserved some of their harvested oocytes. Twenty-two couples undergoing IVF froze excess oocytes for future use or possibly to donate their oocytes to other infertile couples. Two couples had unexpected ejaculatory failure on the day of oocyte retrieval, and the oocytes were frozen for use at a later date. In addition, women with benign ovarian tumour (n = 1) or malignant disease (n = 4) who hoped to preserve their fertility before surgery, chemotherapy or radiation therapy could undergo oocyte retrieval and cryopreservation, as could women who feared that time on their biological clock was becoming too short (n = 5).

This study protocol was approved by the Institutional Review Board of National Taiwan University Hospital. All women participating in the study signed an approved consent form prior to treatment.

**Controlled ovarian stimulation (COS) and oocyte retrieval**

Each woman underwent COS with a long or short protocol of GnRH agonist (buserelin; Hoechst, Germany) or a GnRH antagonist (Cetrotide; Serono, Italy). FSH (Puregon; Organon, The Netherlands) and hMG (Serono) were used for ovarian stimulation. Follicular growth was monitored using vaginal or abdominal ultrasonography and serum estradiol (E2) detection. 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 or laparoscopy. The oocyte–cumulus complexes were cultured in human tubal fluid (HTF) medium with 15% heat-inactivated maternal serum and incubated at 37°C in an atmosphere of 5% CO2 in air.

**Cryopreservation of part or all of the oocytes**

For infertile couples, six to 12 oocytes were generally used for IVF or ICSI. The additional mature oocytes were cryopreserved. For couples having unexpected ejaculatory failure or unmarried women, all of the mature oocytes were frozen. The cumulus cells were dissected using 23 G needles followed by brief exposure (30 s) to hyaluonidase (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 2–3 h post-retrieval.

**Preparation of pretreatment, freezing, and dilution solutions**

The solutions for cryopreservation and dilution were prepared using Dulbecco’s phosphate-buffered saline (DPBS) (Gibco, USA) plus 20% maternal serum. 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 thawing procedures, the oocytes were thawed in stepwise dilution solutions with 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 pretreatment solution and kept at room temperature (22–24°C) for 10 min. Then the oocytes were transferred to the freezing medium. After the 15 min of equilibration, the oocytes were loaded into a 0.25 ml plastic straw (IMV, France) and transferred into an automated Kryo 10 series III biological vertical freezer (Planer Products Ltd, UK). The initial chamber temperature was 20°C, then 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 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 min of temperature stabilization, the straws were transferred into liquid nitrogen tanks and stored until thawing.

**Treatment cycles with frozen–thawed oocytes**

For thawing cycles, endometrial preparation involved the natural ovulatory cycle or the hormonal replacement cycle. For the protocol of hormonal replacement cycle, the female received oral estradiol valerate (2 mg) with incremental dosages 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 tested and endometrial thickness was measured using vaginal ultrasonography. If the endometrial thickness was ≥8 mm, with no evidence of ovulation, progesterone in oil (50 mg daily) was administered i.m. The cryopreserved oocytes were thawed and ICSI was performed at the day of starting progesterone administration. If pregnancy was achieved, the hormonal replacement was carried out to 12 weeks of gestation.

To thaw, the straws were air-warmed for 30 s and then immersed in a 30°C water bath for 40 s until all traces of ice disappeared (Fabbri et al., 2001). The contents of the straws were expelled in 1.0 mol/l PROH plus 0.3 mol/l sucrose solution and the oocytes
were equilibrated for 5 min. Then the oocytes were 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% maternal serum 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 pelludica and plasma membrane and refractive cytoplasm.

**Fertilization using ICSI**

ICS was performed using the surviving oocytes. The ICSI procedures were described in detail previously (Chen et al., 1996). Oocytes were examined 16–18 h post-ICSI. Those with two pronuclei (2PN) and a second polar body were identified as being normally fertilized. The oocytes with a single pronucleus (1PN) or three pronuclei (3PN) were considered abnormally fertilized. The digyny was defined as presence of 3PN or 2PN without extrusion of the second polar body for oocytes after ICSI treatment (Flaherty et al., 1998).

**Embryo transfer and follow-up of pregnancy**

The cleaving embryos on day 2 or 3 after fertilization were transferred into the uterine cavity transvaginally. Intramuscular administration of progesterone in oil (50 mg daily) was used for a luteal supplementation. Fourteen days after transfer, the serum β-hCG level was evaluated. Clinical pregnancy was defined as presence of the gestational sac in utero by ultrasound at 6 weeks of gestation. The subsequent development of a fetal heartbeat was defined as an ongoing pregnancy. The ongoing pregnancies were closely monitored.

**Statistics**

The percentages for survival, implantation, clinical pregnancy and ongoing pregnancy were calculated for the frozen–thawed cycles. These parameters were compared between the groups of oocyte freezing and embryo freezing in the same periods using χ2 or Fisher’s exact test. The mean ± SD of women’s ages and number of embryos transferred were compared between the groups using the Mann–Whitney test. Differences were considered significant at \( P < 0.05 \).

**Results**

Forty-three couples undergoing IVF or ICSI cycles cryopreserved part or all of their oocytes. Forty-one couples served part or all of their oocytes. Fourty couples had fresh transfer. The mean age of these women was 32 ± 4 years (range, 20–40 years). The mean number of embryos transferred was 3.8 ± 1.3 (1–5). Seventeen pregnancies (41%) were achieved including 13 singletons and four sets of twins. The implantation rate was 14%. One pregnancy aborted due to blighted ovum (6%).

The survival, cleavage and pregnancy for cryopreserved oocytes after thawing, in comparison with embryos cryopreserved at the pronuclear (PN) stage in the same period, are summarized in Table I. Twenty-one couples thawed oocytes (20 for their own use and one for anonymous donation). The mean (±SD) age of the women was 32 ± 5 years (range 20–40). In every cycle we had at least one oocyte surviving from freezing–thawing with subsequent ICSI and fertilization. An average of 3.5 ± 1.4 embryos (1–5) was transferred per cycle. Seven pregnancies (33%) were achieved including six singletons and one set of twins. We did not routinely perform amniocentesis for pregnancies derived from cryopreserved oocytes. To date, four women have delivered five babies. Chromosomal analysis of umbilical cord blood from all five babies, collected at delivery, revealed normal karyotypes. Three conceptions are ongoing. One 35 year old pregnant woman received amniocentesis at the gestational age of 18 weeks that revealed 46,XX. No miscarriage was encountered in this patient series. By calculating the number of gestational sacs divided by the number of embryos transferred, the implantation rate was 11%. The implantation referring to the number of oocytes thawed would be a more sensitive index in oocyte freezing. In this series, the percentage of implantation on the basis of the number of oocytes thawed was 5%.

During this period, we performed 38 cycles of frozen–thawed embryo transfer at the pronuclear stage. The mean age of these women was 34 ± 4 years (range 24–40 years) and the mean number of embryos transferred was 4.0 ± 1.4 (1–6). Neither differed from the group using frozen–thawed oocytes. The percentages of survival, pregnancy and implantation for frozen–thawed pronuclear embryos were similar to those with frozen–thawed oocytes (\( P > 0.05 \)). Twelve pregnancies (32%) were achieved including 10 singletons and two sets of twins. Five women had delivered their babies. Five conceptions are ongoing. Two pregnancies aborted due to blighted ovum (16.7%).

Among unmarried woman, one had her oocytes cryopreserved due to recurrent ovarian endometrioma, while five women cryopreserved their oocytes due to fear of time running out on their biological clock. The mean age of these women was 38 ± 7 years (range 28–46 years). The baseline serum FSH level was 9.5 ± 5.1 mIU/ml (5.0–16.4 mIU/ml). Three women aged < 38 years used a long protocol of GnRH agonist, and three aged > 40 years received a short protocol. While the mean number of oocytes cryopreserved was 10 ± 6 (range 3–17), the younger group had a significantly greater number of oocytes cryopreserved (15 ± 3) than did the older group (6 ± 3) (\( P < 0.05 \)).

**Table I.** The survival, cleavage, and pregnancy for cryopreserved oocytes after thawing, in comparison with embryos cryopreserved at the pronuclear (PN) stage in the same period.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Oocyte freezing (n = 21)</th>
<th>2PN freezing (n = 38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32 ± 5</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>Thawed number</td>
<td>159</td>
<td>215</td>
</tr>
<tr>
<td>Survival after thawing</td>
<td>119 (75)</td>
<td>170 (79)</td>
</tr>
<tr>
<td>Damage after ICSI</td>
<td>6 (5)</td>
<td>3PN</td>
</tr>
<tr>
<td>2PN (normal fertilization)</td>
<td>80 (67)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>1PN</td>
<td>5 (4)</td>
<td></td>
</tr>
<tr>
<td>Cleavage</td>
<td>73 (91)</td>
<td>158 (93)</td>
</tr>
<tr>
<td>Mean embryos transferred</td>
<td>3.5 ± 1.4</td>
<td>4.0 ± 1.4</td>
</tr>
<tr>
<td>Clinical pregnancy</td>
<td>7 (33)</td>
<td>12 (32)</td>
</tr>
<tr>
<td>Multiple pregnancy</td>
<td>1 (14)</td>
<td>2 (17)</td>
</tr>
<tr>
<td>Implantation</td>
<td>8 (11)</td>
<td>14 (9)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.

Percentage of implantation: no. of gestational sacs/no. of embryos transferred.

Differences non-significant by χ2 or Mann-Whitney tests.

**Slow freezing of human oocytes and ICSI 3 h post-thaw**

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Four women with white blood cell diseases (one with lymphoma, one with chronic myelocytic leukaemia, one with acute myelocytic leukaemia, and the other with acute lymphocytic leukaemia) were referred by haematologists for preservation of fertility. All of them were in remission after several courses of chemotherapy. Before preconditioning treatment with chemotherapeutic agents and total body irradiation for haematopoietic stem cell transplantation (HSCT), COS and oocyte freezing were performed. The mean age of these women was 24 ± 3 years (range 21–29 years). One patient received a long protocol of GnRH agonist and three a short protocol. The baseline serum FSH level was 9.3 ± 1.6 mIU/ml (7.9–11.1 mIU/ml). The number of oocytes cryopreserved was 13 ± 8 (7–24).

Discussion

Our data indicated that slow freezing of oocytes in 1.5 mol/l PROH plus 0.3 mol/l sucrose and performing ICSI at 3 h post-thaw resulted in favourable outcomes for survival (75%), fertilization (67%), cleavage (91%) and pregnancy (33%). All 21 (100%) frozen–thawed cycles resulted in fertilization and embryo transfer. No fetal loss occurred. Fosas et al. (2003) used a Fabbri’s regime to freeze and thaw oocytes from young women for donation to infertile couples, with a modification of the thawing procedures. The oocytes stayed in the rehydration solutions at room temperature for 12.5 min, compared with the 30 min illustrated by Fabbri et al. (2001). They reported a survival rate of 90% and a fertilization rate of 73%. Four pregnancies (57%) were produced after seven transfers. Taken together, these results demonstrated that the slow-freezing method developed by Fabbri et al. (2001) achieved reproducible success for oocyte cryopreservation.

Delayed insemination of mature oocytes results in compromised embryos and development failure (Gook et al., 1994). We performed oocyte freezing within 2–3 h post-retrieval. After thawing, the oocyte must be fertilized in a timely manner or it will undergo apoptosis. However, inseminating oocytes immediately after thawing when there is serious spindle disorganization adversely affects fertilization events and increase the rate of digyny (Eroglu et al., 1998). Therefore, choosing the optimum time interval between oocyte thawing and insemination is critical for normal fertilization. Considering both aspects of oocyte ageing and spindle recovery (Chen et al., 2001; Rienzi et al., 2004), we selected 3 h of post-thaw incubation before performing ICSI. The low digyny (2%), high normal fertilization and cleavage, and favourable pregnancy outcomes appeared to indicate that this strategy was appropriate.

Timing of insemination of human oocytes after thawing varies in the literature, ranging from 1 to 6 h (Quintans et al., 2002; Boldt et al., 2003; Fosas et al., 2003; Yoon et al., 2003). Fosas et al. (2003) shortened the rehydration procedures at room temperature to 12.5 min and performed ICSI at 1 or 2 h after thawing. Although the normal fertilization rate was 73%, the percentage of zygotes with 3PN appeared higher (6%). Al-Hasani et al. (1987) reported a high polyploidy rate of 15%. However, the timing of insemination was not specified in the article. A high pregnancy loss from cryopreserved human oocytes was reported by Quintans et al. (2002) using Na-depleted medium and performing ICSI 4–6 h after thawing. The reason was unclear; however, they did not mention the time interval between oocyte retrieval and initiation of freezing.

If the couples had completed a family, the oocytes could be a precious source for donation. After one of our patients underwent screening for infectious diseases, the couple anonymously donated their excess oocytes to an infertile couple without compensation. This is especially important for countries such as Taiwan that authorize donation of oocytes, but not embryos, to infertile couples. In addition, cryopreservation of excess sibling oocytes for patients receiving IVF has a possible advantage of circumventing unexpectedly low fertilization rates or fertilization failures (Chen et al., 2004). One couple with tubal factor infertility had an extremely low fertilization rate for IVF, although the husband had normal semen parameters. They did not achieve pregnancy with the fresh cycle. Fortunately, the couple had a successful pregnancy using frozen–thawed oocytes and ICSI.

Men with documented sperm procurement problems are recommended for sperm cryopreservation before treatment. In this patient series, two couples decided to freeze retrieved oocytes due to ejaculation failure on the day of oocyte retrieval. These two men were able to obtain sperm samples by masturbation in the previous treatments of intrauterine insemination. Ejaculation failure is an uncommon event that has been estimated to occur in 0.2% of oocyte retrieval cycles (Emery et al., 2004). Nevertheless, Emery et al. (2004) suggested the use of invasive sperm recovery in such situations. They thought that cryopreservation of oocytes was unsuitable because it was still at an experimental stage. With the improvement of outcome of oocyte freezing, this notion may change. Porcu et al. (1997) achieved the first birth of a healthy baby with oocytes frozen with PROH and inseminated with ICSI, performed in a case of failure to produce sperm on the day of oocyte retrieval. The same happened with the first pregnancy from frozen oocytes and testicular sperm (Porcu et al., 1999). Oocyte cryopreservation could become a viable alternative option in this situation.

For cancer patients, their age and drug regimen for treatment of their specific disease (type, duration and dose) determines their ovarian reserve after treatment (Falcone et al., 2004). The methods and timing for performing fertility preservation are important issues and deserve further investigation (Porcu et al., 2004; Sonmez and Oktay, 2004). Four patients with white blood cell diseases underwent oocyte freezing in our study. All of them were in remission after several courses of chemotherapy. They were referred by haematologists prior to HSCT because the preconditioning regimens for ablation of the pre-existing bone marrow, including chemotherapeutic agent and total body irradiation, were highly gonadotoxic (Couto-Silva et al., 2001). Three received a short protocol of GnRH agonist and gonadotrophin treatment and one a long protocol. With these protocols, oocyte retrieval was possible in a time lapse ranging from
2 to 4 weeks. The short protocol may be feasible for the cancer patients before HSCT because of shorter administration of medication and compromised ovarian reserve after chemotherapy. Five unmarried women concerned about their ‘biological clock’, cryopreserved oocytes for use later in life. Because age is related to quality and number of oocytes retrieved, we suggest preserving oocytes before 38 years old.

In our centre, we use the protocol of 1.5 mol/l PROH and 0.1 mol/l sucrose for cryopreservation of pronuclear or 2–8-cell embryos (Ho et al., 1992). The embryos cryopreserved at the pronuclear stage were randomly allocated, unlike those at the cleavage stage that are usually frozen after selection for fresh transfer of those with better morphology. The percentages for survival, pregnancy and implantation for frozen–thawed pronuclear embryos were similar to those of frozen–thawed oocytes. These findings are consistent with the results from Boldt et al. (2003). In addition, Yang et al. (2002) found that the parameters from thawed oocytes and embryos derived from sibling oocytes were also similar. Consequently, with various modifications of the conventional slow-freezing method, the outcomes of oocyte freezing have been improved and are comparable to those of embryo freezing.

In this series of cases, maternal serum was used to supplement the medium. Worldwide, the preferential option is to use human serum albumin or a synthetic serum replacement. Therefore, we are going to use human serum albumin for supplementing the medium in the subsequent application. In this report, the average of 3.5–4 embryos transferred per cycle was high. In the past, the recommended number of embryos transferred was four in our institute, with an implantation rate of ~15–20% for fresh embryos. At present, restriction of number of embryos transferred is critical to prevent multiple pregnancies. Recently, a guideline for three embryos is proposed in Taiwan. Furthermore, an average of one or two is regarded as our upcoming goal.

Recently, Borini et al. (2004) reported a lower survival rate of 37% for cryopreserved oocytes using a slow-freezing protocol with 0.1 mol/l sucrose. The regime developed by Fabbri et al. (2001) with increased sucrose concentration (0.3 mol/l) was thought to dehydrate the oocytes more adequately and reduce intracellular ice formation, improving survival. ICSI was performed using oocytes after 3 h of post-thaw incubation, with restoration of the microtubular spindle. This protocol achieved reproducible success for survival, fertilization and pregnancy for cryopreserved human oocytes. Because of comparable survival and pregnancy rates, oocyte freezing could be an alternative to embryo freezing for couples with religious or ethical concerns. Although the case numbers are small, our preliminary results may encourage greater clinical application of oocyte cryopreservation with greater success. Oocyte freezing merits being an integral part of IVF centres.

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