Freezing within 2 h from oocyte retrieval increases the efficiency of human oocyte cryopreservation when using a slow freezing/rapid thawing protocol with high sucrose concentration

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BACKGROUND: A number of factors influence the success of oocyte cryopreservation and subsequent ICSI. The aim of the present study is to establish the ideal time, after oocyte retrieval, for human oocyte cryopreservation via slow freezing/rapid thawing protocol with 0.3 M sucrose concentration in cryoprotectant solution (SF/RT 0.3 M).

METHODS: Retrospective study with 75 patients on the clinical outcome of 93 oocyte thawing cycles divided into three groups. Group A: freezing within 2 h from oocyte retrieval. Group B: freezing between 2 and 3 h from retrieval. Group C: freezing after 3 h. RESULTS: The rate of best quality embryos was significantly higher (35.2%; P = 0.050) in Group A than in Group C (14.1%). Pregnancy and implantation rates were 39.1% (9/23) and 18.5% (10/54) in Group A. Nine clinical pregnancies per 124 thawed (7.3%) and 73 injected (12.3%) oocytes were observed in Group A versus 3 pregnancies per 174 thawed, 103 injected (1.7%, 2.9%, P = 0.046 and 0.0049) in Group B and 4 per 139 thawed, 88 injected (2.9%, 4.5%, NS) in Group C. The overall yield from oocytes cryopreserved within 2 h of retrieval was 8.1 implantations per 100 oocytes thawed. CONCLUSIONS: Embryo quality and clinical outcome of thawing cycles were significantly improved when oocyte cryopreservation by SF/RT 0.3 M was carried out within 2 h from oocyte retrieval.

Keywords: human oocyte cryopreservation; slow freezing; high sucrose concentration; embryo quality

Introduction

Human oocyte cryopreservation allows fertility to be preserved in women who intend to delay childbearing for family planning reasons or in those at risk of losing their gonadal function (e.g. in case of cancer before gonadotoxic therapies). It can also be used in addition to conventional IVF, representing an alternative to circumvent many of the ethical issues associated with embryo cryopreservation. After the early successes using human cryopreserved oocytes, reported 20 years ago (Chen, 1986; Al-Hasani et al., 1987), for a long period this technology was not investigated further, being perceived as inefficient and probably unsafe. The publication of later validation studies optimizing various cryopreservation techniques (Gook et al., 1993, 1994, 1995a,b; Porcu et al., 1997; Kuleshova et al., 1999; Fabbri et al., 2001; Kuwayama et al., 2005) opened new perspectives on oocyte cryopreservation and led to the publication of several reports describing the clinical outcome of this interesting technology. Although, in Italy, the use of oocyte cryopreservation as a clinical tool represents the only alternative to embryo cryopreservation (forbidden by the national IVF law), in other situations, ‘oocyte cryo-banking’ could represent a more efficient approach in oocyte donor–recipient treatment. In spite of the increase in the clinical application of oocyte cryopreservation worldwide, some authors still consider oocyte cryopreservation an experimental technique that needs further studies on safety and efficiency (Jain and Paulson, 2006; Oktay et al., 2006). Human oocytes can be cryopreserved by two main methods: the slow-cooling computer-controlled protocol and the ultrarapid cooling (vitrification) protocol. The results of human oocyte cryopreservation using the slow freezing/rapid thawing (SF/RT) protocol with 1,2-propanediol (PROH) and high sucrose concentration (0.2 or 0.3 M) as cryoprotectants has shown a gradual improvement in efficiency over time, with live birth rates per transfer increasing during recent years (Jain and Paulson, 2006). Vitrification seems to be a very promising technique even if, being a
Materials and Methods

Study population

This retrospective study was carried out on 93 oocyte–thawing cycles performed from March 2004 to May 2007. The oocytes were cryopreserved from 75 patients with a mean age $\pm$ SE at the time of oocyte retrieval of 34.9 $\pm$ 0.7 years. All the women included in our cryopreservation programme were informed about the procedure and a written consent was obtained from each. Oocyte cryopreservation was usually carried out between 1 and 5 h after oocyte retrieval. We retrospectively observed the influence of the timing of freezing on outcome of oocyte–thawing cycles, and divided the thawing cycles into three groups: freezing within 2 h (Group A), between 2 and 3 h (Group B) or more than 3 h (Group C) from oocyte retrieval.

Controlled ovarian stimulation, oocyte retrieval and selection

Controlled ovarian stimulation was achieved using GnRH analogues in combination with a graded menotropin administration, as previously described by Filicori and Cognigni (2001). Transvaginal ultrasound guided oocyte retrieval was performed 35 h after ovulation induction with 10 000 IU hCG. After retrieval oocytes were cultured for between 1 and 5 h at 37°C in an atmosphere of 6% CO₂ before the complete removal of cumulus mass and corona cells by enzymatic digestion with recombinant hyaluronidase (SynVitro® Cumulase® and MediCult, Jyllinge, Denmark) and by gentle mechanic aspiration with plastic pipettes (Denuding Flexipet™ Cook, Eight Mile Plains Queensland, Australia). The denuded oocytes were then evaluated to assess their nuclear maturation stage. The oocytes that had released the first polar body (metaphase II—MII) underwent a strict selection by morphological features ( zona pellucida thickness, perivitelline space size, oocyte shape, cytoplasm colour and granularity, presence of vacuoles and first polar body morphology) under an inverted microscope with Hoffman modulation contrast. We classified as ‘high quality’ those which were colourless and of regular shape, with regular zona pellucida and small perivitelline space without debris, homogeneous cytoplasm and no vacuoles or granulations (Xia, 1997; De Sutter et al., 1996; Ebner et al., 2003). Among the ‘high quality’ oocytes, the presence of an intact, round or ovoid polar body with smooth surface was considered as a selection criterion (Ebner et al., 2000). Immediately after decumulation and quality evaluation, the three best available MII oocytes were inseminated by ICSI, according to the Italian law regulating ART. Only the supernumerary MII oocytes reaching our own ‘high quality’ standards were cryopreserved.

Cryopreservation protocol

The cryopreservation protocol consisted of a SF/RT method. Oocyte freezing and thawing solutions (OocyteFreeze™–OocyteThaw™ MediCult, Jyllinge, Denmark) contained Dulbecco’s phosphate buffered saline (PBS) supplemented with human serum albumin, alpha- and betaglobulins, and PROH and sucrose as cryoprotectants.

Freezing procedure

After washing in a PBS solution, the oocytes were equilibrated for 10 min at room temperature in 1.5 M PROH and then transferred into the loading solution of 1.5 M PROH and 0.3 M sucrose. Between one and three oocytes were loaded in plastic straws (Paillette Cristal 133 mm; Cryo Bio System, Paris, France) and transferred into an automated biological vertical freezer (Kryo 360–1.7, Planer, Sunbury, UK). The cooling process was initiated reducing chamber temperature from 20°C to $-7°C$ at a rate of 2°C/min. Ice nucleation was induced manually at $-7°C$. After a hold 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 at stabilization temperature, the straws were transferred into liquid nitrogen and stored for later use.

Thawing procedure

The straws were air-warmed for 30 s and then immersed in a 30°C water bath for 40 s. The cryoprotectant was removed at room temperature by stepwise dilution of PROH in the thawing solutions: the contents of the straws were expelled in 1.0 M PROH and 0.3 M sucrose solution, and the oocytes were equilibrated for 5 min. The oocytes were then transferred into 0.5 M PROH and 0.3 M sucrose solution for 5 min and then into 0.3 M sucrose solution for 10 min before the final dilution in PBS solution for 20 min (10 min at room temperature and 10 min at 37°C). The oocytes were finally cultured at 37°C in an atmosphere of 6% CO₂ in air for 3 h before ICSI.

Survival evaluation, ICSI and embryo culture

After a 3 h post-thaw culture, the three best surviving oocytes, according to the previously described parameters, were inseminated by ICSI, as allowed under the Italian IVF act. The evaluation of survival was carried out by inverted microscope with Hoffman modulation contrast and thawed oocytes were considered to have survived in absence of negative characteristics: dark or contracted ooplasm, vacuolization,
cytoplasmic leakage, abnormal perivitelline space and cracked zona pellucida. The surviving oocytes were selected prior to ICSI following our own ‘high quality’ standards. Amongst the ‘high quality’ thawed oocytes, the presence of an intact polar body was considered as a selection criterion; as far as possible, we avoided injecting thawed oocytes presenting an atretic polar body or the so-called ‘ghost polar body’: an empty membrane without cytoplasm (La Sala et al., 2006). Fertilization and embryo development were examined by inverted microscope. Embryos were graded 1–5 (1 best–5 worst), with grade 1 assigned to the best quality embryos containing equally sized symmetrical blastomers with no fragmentation, according to the criteria previously described by Veeck (1999). The embryo development rating (EDR) as described by Cummins et al. (1986) was calculated to define the growth rate of transferred embryos obtained from thawed oocytes. The formula for calculating the EDR was as follows: EDR=(TE/TO) × 100 (TE, time expected; TO, time observed). The ideal EDR is 100; this value is obtained when a hypothetical ‘normally’ growing embryo is at the 2-cell stage at 33.6 h, at the 4-cell stage at 45.5 h and at 8-cell stage at 56.4 h.

Endometrial preparation and embryo transfer
Preparation of endometrium for the embryo transfer involved the natural ovulatory cycle or the hormonal replacement cycle. The hormonal replacement cycle was performed by the administration of estradiol valerate (Progynova, Bayer-Schering Pharma, Milan, Italy—2 mg, by mouth daily) for 9–15 days with a steadily increasing dose (2–16 mg) depending on the patient, or with or without pituitary desensitization induced by GnRH agonist (Enantone, Takeda, Rome, Italy or Decapeptyl, Ipsen, Milan, Italy—3.75 mg, one depot administration, starting from Day 21 of the previous menstrual cycle). An endometrial thickness ≥8 mm was considered to be optimal for performing an embryo transfer. Progesterone administration (Prontogest, Amsa, Rome, Italy—100 mg, i.m.) was started the day of oocyte thawing. Embryo transfer was carried out after 2 (Day 2) or 3 days (Day 3) from oocyte thawing and ICSI. In one case (Group B), the embryo transfer was carried out after 6 days (Day 6—blastocyst stage) from thawing and ICSI. Treatment with progesterone and estradiol valerate (Progynova, Bayer-Schering Pharma, Milan, Italy or Decapeptyl, Ipsen, Milan, Italy—3.75 mg, one depot administration) was continued for 14 days after embryo transfer, until the pregnancy rate per thawed and injected oocyte was significantly lower (34.1 ± 0.7) in Group C than in Group B (36.0 ± 0.6, P = 0.018) (Table I).

Clinical outcome
Sixteen clinical pregnancies were obtained after 83 embryo transfers (pregnancy rate per transfer: 19.3%) with 19 embryos implanted out of 198 transferred (9.6% implantation rate). Overall, the abortion rate was 31.2% (5/16): two patients had a gestational sac without the FHB and one pregnancy was ectopic. Ten healthy babies were born (four singletons, three twins) and one singleton pregnancy is still ongoing (Table I). The highest pregnancy and implantation rates of 39.1% (9/23) and 18.5% (10/54), respectively, were seen in Group A, when compared with 9.1% and 6.8% of Group B (3/33–5/73) and to 14.8% and 5.6% of Group C (4/27–4/71). The abortion rates were 22.0% (Group A), 0.0% (Group B) and 75.0% (Group C). The results in terms of the technique’s efficiency were: 9 clinical pregnancies per 124 thawed (7.3%) and 73 injected (12.3%) oocytes were observed in Group A versus 3 per 174–103 (1.7–2.9%) of Group B (P = 0.046, 0.049) and 4 per 139–88 (2.9–4.5%) of Group C (Table II). Thus, the pregnancy rate per thawed and injected oocyte was significantly higher when oocytes were cryopreserved within 2 h from oocyte retrieval. The overall implantation rate per thawed

### Table I. Clinical results of ICSI using oocytes frozen at different times after retrieval.

<table>
<thead>
<tr>
<th></th>
<th>Total (93 thawing cycles)</th>
<th>Group A (26 thawing cycles)</th>
<th>Group B (37 thawing cycles)</th>
<th>Group C (30 thawing cycles)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean female age (SE) at oocyte retrieval</td>
<td>34.9 ± 0.4</td>
<td>34.4 ± 0.7</td>
<td>36.0 ± 0.6</td>
<td>34.1 ± 0.7</td>
<td>0.018*</td>
</tr>
<tr>
<td>Transfers performed</td>
<td>83</td>
<td>23</td>
<td>33</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Clinical pregnancies</td>
<td>16</td>
<td>9</td>
<td>3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Gestational sacs</td>
<td>19</td>
<td>10</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Clinical pregnancy rate per transfer</td>
<td>19.3%</td>
<td>39.1%</td>
<td>9.1%</td>
<td>14.8%</td>
<td>NS</td>
</tr>
<tr>
<td>Implantation rate</td>
<td>9.6%</td>
<td>18.5%</td>
<td>6.8%</td>
<td>5.6%</td>
<td>NS</td>
</tr>
<tr>
<td>Abortions</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Ectopic pregnancies</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gestational sacs without FHB</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Abortion rate</td>
<td>31.3%</td>
<td>22.0%</td>
<td>0.0%</td>
<td>75.0%</td>
<td>NS</td>
</tr>
<tr>
<td>Live births</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ongoing pregnancies</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* *Group B versus Group C.*
Laboratory results

A total of 437 oocytes were thawed, with a mean ± SE of 4.7 ± 0.1 per patient: 328 oocytes survived (survival rate: 75.1%), 264 were injected and 64 were discarded (oocytes not injected: 19.5%) after morphological selection. Two hundred and twenty-seven oocytes fertilized after ICSI (fertilization rate: 86%) and 198 cleaved (cleavage rate: 87.2%). Oocyte survival, fertilization and cleavage were comparable in all the groups (Table III). EDR was significantly higher (78 ± 3.9) in Group C than in Group B (70 ± 3.8, P = 0.040). The best quality embryo rate (Grade 1) in Group A was significantly higher (35.2%, P = 0.050) than in Group C (14.1%).

Discussion

The optimal time for insemination of a SF/RT oocyte is strictly dependent on the complete restoration of cellular function and in particular the organization of the meiotic spindle. When using a vitrification protocol at 37°C, Larman et al. (2007) showed the maintenance of the meiotic spindle in human oocytes. Thus, although fertilization can proceed without having to await spindle reformation after a 37°C vitrification/warming, an incubation of ~3 h is recommended prior to ICSI when using frozen/thawed oocytes via SF/RT, to allow spindle reappearance (Rienzi et al., 2004; Bianchi et al., 2005a). The timing of ICSI can affect embryo implantation: even though it is possible to achieve implantation of embryos derived from aged oocytes (Chen and Kattera, 2003), it has been shown that the developmental capacity of the oocyte declines 10 h after retrieval (Yanagida et al., 1998). Insemination of fresh oocytes between 37 and 41 h after hCG administration to trigger ovulation determines highest embryo implantation rate (Dozortsev et al., 2004). The metabolic ageing at ICSI of slow cooled oocytes thus depends on: (i) time of retrieval after hCG administration, (ii) pre-incubation before cryopreservation and (iii) post-thawing culture before insemination. In our study, oocyte retrieval was performed 35 h after hCG administration and thawed oocytes were injected after 3 h of post-thaw culture. Thus, in our study, the timing of insemination (i + ii + iii) was maximum 40 h (35 h + 3 h) for Group A, 41 h (35 ± 3 h) for Group B and more than 41 h (35 + 3 h) for Group C. When using a SF/RT protocol, the time of incubation between oocyte retrieval and cryopreservation is critical in order to avoid injecting 'aged' oocytes. In fact, in our study, ICSI was performed between 37 and 41 h from hCG administration in Groups A and B, whereas ICSI on oocytes with hypothetical lower implantation potential was carried out in Group C. Furthermore, it may be that the freezing procedure could influence cellular ageing. The excessive extension of the culture period before freezing could affect oocyte competence after cryopreservation, considering that in human oocytes the oolemma permeability characteristics change over time (Hunter et al., 1992). It has been demonstrated that insemination of oocytes cryopreserved several hours after retrieval was 4.3%; a trend towards better implantation (8.1%) was found in Group A, where cryopreservation was carried out within 2 h, but this is not statistically significant.
caused an elevated polyplody (Gook et al., 1994), an increased rate of second polar body retention (Gook et al., 1995a) and the development of abnormal embryos (Gook and Edgar, 2007). Several authors have reported a culture time >2 h before oocyte freezing via SF/RT (Chen et al., 2005; Borini et al., 2006a,b; Chamayou et al., 2006; De Santis et al., 2006; Levi Setti et al., 2006). Although the positive effect of shorter intervals before oocyte freezing was previously reported (Li et al., 2005; Bianchi et al., 2007), none of these studies have assessed this parameter systematically. Thus, we decided to assess the impact of different time lags between retrieval and oocyte freezing in our patients.

The critical parameters in cryopreserving human oocytes are the maintenance of cell integrity after thawing (survival) and the preservation of the cellular function (fertilization, cleavage and implantation). Regarding the post-thaw survival, the SF/RT method originally designed for embryo freezing (Lassalle et al., 1985) has been optimised by Fabbri et al. (2001), increasing the concentration of sucrose from 0.1 to 0.2 M (SF/RT 0.2) and 0.3 M (SF/RT 0.3) to achieve survival rates of 70% and 82%, respectively. In our study, the assessment of oocyte survival was performed 3 h after thawing. Our overall survival rate (75%) was comparable to that obtained in the other studies performed with SF/RT 0.3 protocol (Fosas et al., 2003; Chen et al., 2005; Li et al., 2005; Borini et al., 2006b; Chamayou et al., 2006; De Santis et al., 2006; La Sala et al., 2006; Levi Setti et al., 2006) and no significant difference between our three study groups was observed (Table III). The pooled results from reports using different freezing techniques were elegantly summarized and presented in Gook and Edgar’s (2007) latest review on human oocyte cryopreservation: very high cleavage rates were observed when injection was performed in around 6000 oocytes frozen with high sucrose concentration (93% with 0.2 M and 90% with 0.3 M). Vitrification protocols showed very promising fertilization (91%) and cleavage (92%) rates. The results of our study were at least comparable to the other studies using SF/RT with high sucrose concentration: our overall fertilization rate was 86% whereas the cleavage rate was 87%. These rates did not significantly vary among the three study groups, even though the embryo quality was influenced by the time of freezing: we observed a significant increase of top quality embryo rate (35%) by freezing the oocytes within 2 h from retrieval rather than freezing them after 3 h (14%, P = 0.050) (Table III). We observed that the growth rate (EDR) of embryos obtained by thawed oocytes in our study (74 ± 2.1) was lower than the hypothetical ‘normally’ growing embryo rate (100) theorized by Cummins et al. (1986). The retarded development of embryos obtained from oocytes cryopreserved in 0.3 M sucrose was previously reported by Borini et al. (2006b), who observed a 14% incidence of 4-cell stage embryos on Day 2, and by Bianchi et al. (2005b) who observed a 7% early cleavage rate at 25 h post-insemination. Nevertheless, in our study, a significantly higher mean EDR (78 ± 3.9) was seen in Group C than in Group B (70 ± 3.8, P = 0.040); female age was also lower in Group C than in Group B (P = 0.018), suggesting a relationship between female age and the development competence of cryopreserved oocytes.

Efficiency of oocyte freezing can be measured as implantation and pregnancy rates obtained per oocyte thawed or per embryo transferred (Oktay et al., 2006), though the latter provides a less direct assessment of the freezing technique. Furthermore, the number of oocytes frozen and the number of thawing cycles performed vary widely among published reports. Highest implantations per oocyte thawed were reported in thawing cycles of SF/RT high sucrose cryopreserved donor oocytes: 21/158 (13.3%) by Yang et al. (2002) and 10/81 (12.3%) by Li et al. (2005). The best clinical results in larger studies involving at least 50 thawing cycles (Porcu et al., 2000; Borini et al., 2006a,b; De Santis et al., 2006; La Sala et al., 2006; Levi Setti et al., 2006) were reported by Bianchi et al. (2007) with implantation and pregnancy rates per transfer of 13% and 21%, respectively, resulting in a 5.9% (24/403) implantation rate per thawed oocyte. In the wider studies with vitrification protocols, an implantation rate per thawed oocyte of 11.2% (12/107) was reported by Kuwayama et al. (2005), and of 11.8% (39/330) by Antinori et al. (2007). In our study, the overall pregnancy rate per transfer reported was 19.3%: 16 clinical pregnancies were obtained after 83 embryo transfers, with 19 implants on 198 transferred embryos (9.6% of implantation rate) and on 437 thawed oocyte (4.3%). Up to now, 10 healthy babies have been born thanks to our cryopreservation programme (Table I). Our data are comparable to the best results obtained in the wider studies on SF/RT (Bianchi et al., 2007). But, observing the results in Group A, in which 26 thawing cycles were performed with oocytes previously frozen within 2 h from retrieval, pregnancy and implantation rates were 39.1% (9/23) and 18.5% (10/54), with 8.1 implantations per 100 oocytes thawed (10/124). This implantation rate represents the highest efficiency rate ever reported in studies regarding homologous oocyte slow cooling method, only surpassed by results obtained in donor oocyte thawing programmes (Yang et al., 2002; Li et al., 2005), or with the latest vitrification protocols (Kuwayama et al., 2005; Antinori et al., 2007). These findings suggest that freezing oocytes via SF/RT 0.3 M within 2 h allows optimal clinical results to be achieved, comparable to those obtained with vitrification.

Embryo freezing is a well established technique applied in most ART centres; some authors maintain that an implantation rate per thawed oocyte of 5% or higher shows greater efficiency than embryo freezing (De Santis et al., 2007). Our findings indicate that oocyte cryopreservation via SF/RT, when performed with optimized techniques, can yield a clinical outcome at least comparable to the one achieved by embryo freezing.

In our study, the fact that the time of freezing significantly affects the efficiency of the slow cooling technique is clearly demonstrated by the increase in clinical pregnancies per thawed oocytes (9/124, 7.3%) and per injected oocytes (9/73, 12.3%) obtained when oocytes were frozen within 2 h rather than when they were frozen later (in Group B: 3/174, 1.7%, P = 0.046; 3/103, 2.9%, P = 0.049; Table II). It is also interesting that in this study, no live birth was obtained when oocytes were frozen after 3 h from retrieval (Group C, Table I). Furthermore, a significant increase in top-quality Grade 1 embryos (35%) was observed when the oocytes were
frozen within 2 h from retrieval rather than freezing them after 3 h (14%, P = 0.050) (Table III). In Groups A and B, in which oocytes had the same supposed high implantation potential due to the optimal time lag (from 37 to 41 h) between insemination and hCG administration (Dozortsev et al., 2004), the fact that significant differences in clinical pregnancy rates were observed seems to demonstrate that the slow-freezing procedure can influence cellular ageing.

In conclusion, our study demonstrated significant improvement in both embryo quality and thawing-cycle clinical outcome when oocyte cryopreservation by SF/RT 0.3 M was carried out within 2 h from oocyte retrieval. Thus, we conclude that the use of high sucrose concentration in cryoprotectant solution and timely (< 2 h) oocyte freezing can optimize oocyte cryopreservation and yield very encouraging clinical results.

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