Comparison of clinical outcome after cryopreservation of embryos obtained from intracytoplasmic sperm injection and in-vitro fertilization

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The impact of intracytoplasmic sperm injection (ICSI) on cryopreserved zygotes and embryos was evaluated by comparing embryo survival and implantation between embryos derived from ICSI and those derived from standard insemination procedures. The study included patients whose excess zygotes and embryos were cryopreserved between September 1993 and December 1994 and who subsequently underwent a frozen embryo transfer. Embryo survival, clinical pregnancy rates per transfer and pregnancy outcome were compared. Three hundred and thirty eight cryopreservation cycles, during which 1471 embryos were cryopreserved, were included in this study. Of those, 961 were derived from oocytes fertilized by insemination in vitro and 510 were derived from oocytes fertilized by ICSI. A total of 690 of the embryos (451 in the insemination group and 239 in the ICSI group) have since undergone a thaw cycle. The embryo survival rates were similar between the two groups (70.5 and 73.2%, insemination and ICSI respectively) and were not significantly affected by the stage at cryopreservation. There was no significant difference in pregnancy rates per transfer (31.8 and 32.3%), the preclinical pregnancy loss rate (16.7 and 23.8%), or the clinical miscarriage rate (16.7 and 23.8%) between the insemination and the ICSI groups respectively. It is concluded that ICSI does not have an adverse impact on the survival and successful implantation of cryopreserved and thawed embryos.

Key words: cryopreservation/intracytoplasmic sperm injection/in-vitro fertilization

Introduction

Embryo cryopreservation is a well established technique in most in-vitro fertilization (IVF)–embryo transfer clinics. It allows for storage and later transfer of supernumerary embryos obtained during IVF treatment. With the introduction of the revolutionary technique of intracytoplasmic sperm injection (ICSI) for treatment of couples with male factor infertility, a large number of couples whose prognosis for successful IVF treatment was poor can now be treated successfully. Given the high fertilization and implantation rates which can be achieved in the experienced centres using ICSI (Van Steirteghem et al., 1994; Palermo et al., 1995), many supernumerary embryos are now available for cryopreservation in male factor patients. Although extensive literature has been gathered regarding various cryopreservation techniques, there is a paucity of information regarding effects of cryopreservation on human embryos with perforated zonae. Four studies to date have addressed this issue; one evaluated the impact of subzonal insemination (SUZI) on subsequent embryo survival but only one pregnancy was achieved from cryopreserved–thawed SUZI embryos in that study (Obajasu et al., 1994). Another study (Van Steirteghem et al., 1994) which evaluated the impact of ICSI raised a concern that pregnancies from cryopreserved–thawed embryos derived from ICSI may carry an increased risk of preclinical and clinical pregnancy loss. Two subsequent studies evaluated the impact of microsurgical fertilization on outcome and thaw of pronuclear stage embryos only (Al-Hasani et al., 1996; Hoover et al., 1997). In the study reported here the survival rates and implantation potential of microsurgically fertilized embryos and those fertilized by standard insemination techniques are compared at the pronuclear stage and at the cleaved stage. Pregnancy and neonatal outcomes between the two groups are also compared.

Materials and methods

Patient characteristics, ovulation induction protocol and embryo data

The study population consisted of patients who underwent IVF or ICSI between September 1993 and December 1994 and who in addition to having a fresh embryo transfer had supernumerary embryos which were cryopreserved. Ovarian stimulation protocol was performed as previously described (Benadiva et al., 1995). Briefly, stimulation protocol involved luteal phase pituitary down-regulation with leuprolide acetate. Once down-regulation was confirmed, treatment with human menopausal gonadotrophin (HMG) and/or follicle-stimulating hormone (FSH) was initiated. When at least two lead follicles attained a mean diameter of 17 mm, human chorionic gonadotrophin (HCG) was administered. Transvaginal ultrasound-guided oocyte retrieval was performed 34–35 h following HCG administration. Cryopreservation of supernumerary zygotes and embryos was routinely offered to all patients during this time period and a specific consent was signed by all patients agreeing to embryo storage. Cryopreservation was performed in 338 cycles; in 217 of these cycles fertilization was accomplished using standard insemination procedures, and in the remaining 121 cycles ICSI was performed. In total, 1471 embryos were cryopreserved and of these 47% (n = 690) have since undergone a thaw cycle. After thawing, all viable embryos were transferred to the patients. Viability was defined as post-thaw cleavage of the zygotes, or survival of at least 50% of the
blastomers in the embryos. The rates of embryo survival, implantation per embryo transferred, miscarriage and delivery were recorded for all patients. A subsequent comparison of these parameters between embryos arising from in vitro insemination procedures and those arising from ICSI was made.

**Insemination and cryopreservation techniques**

ICSI was performed as previously described (Palermo and Joris, 1993; Palermo et al., 1995). The parameters utilized for patient selection were as follows: history of fertilization failure with standard insemination procedures, <500 000 progressively motile spermatozoa in the ejaculate, or <1% normal morphology as defined by the Kruger criteria (Kruger et al., 1987). In all other cases standard insemination was performed with approximately 150 000 spermatozoa/ml.

Fertilization was assessed 16–18 h post insemination or micro-injection and was considered normal when two clear pronuclei and two distinct polar bodies were present. Patients with supernumerary zygotes (n ≥ 6) on day 1 post retrieval, who signed a cryopreservation consent, had their zygotes cryopreserved at this stage. In all cases, six or eight fertilized oocytes remained in culture to allow for embryo selection at the time of replacement. The exact number of zygotes maintained in culture was dependent on the patient’s age and prior IVF history. If any cleaved embryos not utilized for fresh transfer were of satisfactory quality, they were cryopreserved on day 2 or 3 of culture. The cryopreservation was carried out in a Planer R204® (Planer, Sunbury-on-Thames, UK) cell freezer utilizing the slow freeze protocol using 1.5 M 1,2-propanediol as a cryoprotectant (Cohen et al., 1988). The cooling rate was 2°C/min until –6°C seeding, then 0.3°C/min until ~36°C. At this temperature, the cooled embryos were placed in liquid nitrogen for storage. The same cryopreservation protocol was utilized for the 4-cell stage embryos as for the zygotes. Embryos at the 8-cell stage were cryopreserved using the slow cool/slow freeze protocol with 1.5 M dimethylsulphoxide as a cryoprotectant. The cooling temperature was 1°C/min until seeding at ~6°C, then at 0.3°C/min until ~80°C. All embryos were stored in liquid nitrogen at ~196°C until subsequent thaw. Embryos were thawed using the rapid thaw method (Cohen et al., 1998) by placing the embryos in a 30°C water bath for 20–35 s until the ice crystals were no longer seen. Zygotes were placed in culture and survival was defined as post-thaw cleavage. Cleaved embryos were examined under the inverted microscope at ×100 and those with at least 50% intact blastomers were deemed viable and thus used for transfer.

**Replacement of frozen-thawed embryos**

Embryos were transferred to the patient in either a natural cycle or a programmed cycle. In a natural cycle, patients were monitored for the onset of endogenous luteinizing hormone (LH) surge (day 14) and the transfer of embryos was performed on day 17. In a programmed cycle, patients were down-regulated with leuprolide acetate 1 mg daily starting in the mid-luteal phase of the preceding menstrual cycle. Subsequently endometrial development was achieved as previously reported (Davis and Rosenwaks, 1993) by administration of transdermal 17β oestradiol (Estraderm®; Ciba-Geigy, Summit, NJ, USA) at a gradually increasing dose to closely mimic the natural cycle. Progesterone 50 mg i.m. was administered once sonographic examination confirmed adequate endometrial development; the first day of progesterone treatment was arbitrarily designated as day 15 of the cycle. Embryo transfer was performed on day 17 and both oestradiol and progesterone treatment were continued throughout the luteal phase. If a pregnancy was achieved, steroid supplementation continued until 8 weeks post-transfer, and then was slowly decreased and discontinued by week 12. Pregnancy was defined as a spontaneous rise in a hCG concentration on at least two separate measurements, at least 10 days post-transfer. Clinical pregnancy implied a presence of an intrauterine gestational sac on an ultrasound performed at 7 weeks of gestation.

**Statistical analysis**

Statistical analysis was carried out using two tailed t-tests and χ² tests when appropriate. The significance level was set at P < 0.05.

**Results**

Of 1471 zygotes and embryos cryopreserved, 961 were obtained as a result of insemination and 510 resulted from injection of a single spermatozoon. The average age of the patients in the two groups did not differ significantly: 34.4 ± 4 years, IVF and 33.7 ± 4 years, ICSI. There was no difference between the two groups in the proportion of embryos cryopreserved at the zygote or the cleaved stage. In the insemination group 528 (54.9%) of the embryos were cryopreserved at the cleaved stage and 433 (45.1%) were zygotes. In the ICSI group 253 (49.6%) and 257 (50.4%) were embryos and zygotes respectively.

As of July 1996, 690 of the cryopreserved embryos and prezygotes (452 in the insemination and 243 in the ICSI group) had been thawed for replacement to the patients in 184 planned transfer cycles. In the insemination group, six out of 119 patients (5.0%) did not undergo an embryo transfer as no embryos survived the thaw. All planned transfer cycles were carried out in the ICSI group. There was no difference in the rates of embryo survival between the inseminated and the microsurgically fertilized embryos. Three hundred and eighteen embryos (70.5%) survived the thaw and the remaining 175 (73.2%) survived the thaw in the ICSI group (Table I). The rates of survival were similar for the pronuclear and early cleaved embryos. Table II shows the survival rates, implantation rates and pregnancy outcome information for the IVF and ICSI groups. The 178 transfers resulted in 57 pregnancies for an overall pregnancy rate of 32.0%. The average number of embryos transferred was similar between the two groups (2.8 versus 2.7 for insemination and ICSI groups respectively). There was no difference in the pregnancy rate per transfer between inseminated–thawed and ICSI–thawed embryos (31.8 versus 32.3%). Additionally, there was no

**Table I. Embryo survival**

<table>
<thead>
<tr>
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<th>Insemination</th>
<th>ICSI</th>
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<tr>
<td>Maternal age at cryopreservation (years)</td>
<td>34.4 ± 4</td>
<td>33.7 ± 4</td>
</tr>
<tr>
<td>No. cycles with cryopreservation</td>
<td>217</td>
<td>121</td>
</tr>
<tr>
<td>No. embryos cryopreserved</td>
<td>961</td>
<td>510</td>
</tr>
<tr>
<td>– pronuclear stage</td>
<td>433</td>
<td>257</td>
</tr>
<tr>
<td>– cleaved stage</td>
<td>528</td>
<td>253</td>
</tr>
<tr>
<td>No. cycles with thaw</td>
<td>119</td>
<td>65</td>
</tr>
<tr>
<td>No. embryos thawed</td>
<td>451</td>
<td>239</td>
</tr>
<tr>
<td>– pronuclear stage</td>
<td>225</td>
<td>112</td>
</tr>
<tr>
<td>– cleaved stage</td>
<td>226</td>
<td>127</td>
</tr>
<tr>
<td>No. embryos survived thaw (%)</td>
<td>318 (70.5)</td>
<td>175 (73.2)</td>
</tr>
<tr>
<td>– pronuclear stage (%)</td>
<td>165 (73.3)</td>
<td>85 (75.9)</td>
</tr>
<tr>
<td>– cleaved stage (%)</td>
<td>153 (67.7)</td>
<td>90 (70.9)</td>
</tr>
</tbody>
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ICSI = intracytoplasmic sperm injection.
difference between the two groups in implantation rates per embryos transferred. The rate of preclinical and clinical pregnancy loss was 33.3% in the insemination group and 47.6% in the ICSI group and the difference was not statistically significant. The delivery rate per transfer was the same between the two groups.

Delivery records were reviewed to elucidate the rates of birth defects in the two groups. In the insemination group one child was delivered as a product of a twin gestation with a prenatally diagnosed omphalocele, ectopic liver and cordis. Additionally, one set of twins was delivered prematurely at 24 weeks gestation secondary to maternal complications. Both infants suffered severe perinatal complications as a result of extreme prematurity. Two out of the 14 singleton pregnancies in the insemination group started out as twin gestations. In one of the pregnancies a spontaneous loss of one of the twins occurred in the first trimester; in the other, an elective pregnancy reduction from twins to a singleton was performed. None of the children delivered in the ICSI group were found to have major or minor birth defects. One therapeutic abortion was performed in this group, when trisomy 21 was diagnosed on a routine amniocentesis, which was performed for advanced maternal age.

**Discussion**

Cryopreservation of human cells was first successfully attempted in 1949 (Polge et al., 1949). Since then, rapid improvements in cryopreservation techniques have been achieved, largely due to a clinical need for cryopreservation of human embryos. ICSI was introduced into clinical practice in 1992 (Palermo et al., 1992) and has gained wide acceptance for treatment of male factor infertility patients. Several reports have been published regarding the impact of microsurgical fertilization on subsequent embryo survival after thawing (Van Steirteghem et al., 1994; Al-Hasani et al., 1996; Hoover et al., 1997). These reports include data regarding cryopreservation at the zygote stage (Al-Hasani et al., 1996; Hoover et al., 1997) and cleaved stages (Van Steirteghem et al., 1994); however, the last report raises concerns regarding high miscarriage rates in pregnancies resulting from ICSI–frozen–thawed embryos.

In this study we compared survival and implantation potential of embryos resulting from standard insemination techniques and ICSI. Furthermore, we evaluated whether cryopreservation at the zygote stage or the early cleavage stages has a significant effect on post-thaw embryo survival. Our data indicate that the fertilization method does not significantly impact on embryo survival. High rates of post-thaw survival were demonstrated in both ICSI and ‘inseminated’ embryos (73.2 and 70.5% respectively). In addition we were able to demonstrate that independent of the developmental stage of the embryo at the time of cryopreservation (zygote or early cleaved), satisfactory cryopreservation results were obtained.

The impact of the embryo’s developmental stage on its survival of the freeze–thaw process has long been debated in the literature. Some investigators advocate cryopreservation in the pronuclear stage documenting pregnancy rates similar to those of the fresh cycle (Veeck et al., 1993), or showing improved survival over the early cleavage stages (Lassalle et al., 1985; Testart et al., 1987; Troup et al., 1990). Others show either no difference in survival between zygotes and early cleavage embryos (Cohen et al., 1988) or demonstrate improved survival for the early cleavage stages (Tucker et al., 1995). Our study shows that, independent of the fertilization technique utilized, the cleaved stage frozen–thawed embryos have similar survival rates to the pronuclear stage embryos.

Our cryopreservation policy has been to freeze supernumerary zygotes (in excess of six), to allow for embryo selection during the fresh transfer, and to freeze the viable cleavage stage embryos after fresh transfer selection is completed. Our data support this policy, as acceptable rates of post-thaw survival can be obtained in the cleavage stage embryos using our protocol. This policy could also result in higher cumulative pregnancy rates per ovarian stimulation by maximizing fresh cycle pregnancy rates, and allowing for the supernumerary embryos to be utilized in subsequent frozen embryo transfers.

The implantation rate per embryo was not affected by the fertilization method utilized, and 11.6% of embryos in the insemination group and 8.6% of embryos in the ICSI group implanted successfully. This difference was not statistically significant. The effect of the cleavage stage at the time of cryopreservation on subsequent implantation could not be assessed as mixed transfers of cleavage stage and thawed–cultured zygote stage embryos were performed in some of our patients.

A recommendation for cryopreservation of supernumerary embryos in patients whose oocytes were fertilized by ICSI can be made with confidence. It has been demonstrated that cryopreservation does not impact adversely on post-thaw implantation potential of high quality embryos (Fugger et al., 1988; Sellick et al., 1995), and our data and those of others (Van Steirteghem et al., 1994; Al-Hasani et al., 1996; Hoover et al., 1997) show that microsurgical fertilization does not seem to affect post-thaw implantation of embryos.

Concerns have been raised about high incidence of preclinical abortions (40.9%) after transfer of embryos cryo-
preserved after ICSI (Van Steirteghem et al., 1994). Others subsequently reported clinical miscarriage rates of 20–25% (Al-Hasani et al., 1996; Hoover et al., 1997). From our observations, it appears that the preclinical and clinical miscarriage rates are similar between patients whose cryopreserved embryos were fertilized by microsurgical techniques or insemination. In total, 47.6% pregnancies in the ICSI group and 33.3% pregnancies in the insemination group did not result in a delivery of at least one infant. This pregnancy loss rate, although seemingly high, is similar to the loss rate of 42% (64/152) reported to occur after spontaneous conception among fertile women (Miller et al., 1980). Therefore, the initial concerns regarding pregnancy loss after transfer of cryopreserved, sperm-injected embryos, may not have been warranted.

Few studies to date have evaluated the neonatal outcome of pregnancies achieved with cryopreserved/thawed embryos (Heijisbroek et al., 1995; Wada et al., 1994; Olivennes et al., 1996). However all reports published to date suggest that the outcomes are favourable. In our study, a total of 43 children was delivered between the two study groups. One child was noted to have a major birth anomaly: omphalocoele with an associated ectopic liver and cordis. No minor birth defects were noted. One pregnancy was terminated electively, because of trisomy 21 diagnosed on a routine amniocentesis performed because of advanced maternal age. The small number of children delivered in this study, and lack of long-term postnatal follow-up, does not permit any definite conclusions about the effects of cryopreservation on subsequent neonatal outcome. At the present time the procedure appears to be safe, and the risk of chromosomal abnormalities is in the same range as observed in fresh ICSI cycles (Palermo et al., 1996). Nevertheless, further long-term follow-up studies of children born as a result of this technology are clearly indicated.

In conclusion, pregnancies can be achieved from cryopreserved embryos derived from ICSI, with success rates similar to those expected from transfer of embryos derived from conventional in-vitro insemination techniques. No differences in miscarriage rates should be anticipated. Careful, ongoing follow-up of children conceived as a result of this technology should be instituted.

References


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