Viability of partially damaged human embryos after cryopreservation

Etienne Van den Abbeel, Michel Camus, Linda Van Waesbergh, Paul Devroey and André C. Van Steirteghem

Centre for Reproductive Medicine, University Hospital and Medical School, Dutch-speaking Brussels Free University (Vrije Universiteit Brussel), Laarbeeklaan 101, B-1090 Brussels, Belgium

1To whom correspondence should be addressed

In our centre, embryos are judged to have survived cryopreservation if at least half of the initial number of blastomeres remain intact. Therefore both fully intact and partially damaged embryos are transferred. The aim of this study was to investigate the viability of partially damaged human embryos after cryopreservation. We retrospectively analysed the implantation and in-vivo development of embryos which were either fully intact or had lost some blastomeres after cryopreservation. Oocytes were collected following stimulation with the gonadotrophin-releasing hormone (GnRH)-agonist Buserelin and human menopausal gonadotrophin. Supernumerary multicellular embryos with not more than 20% of their volume filled with anucleate fragments were frozen on day 2 or day 3 of the cycle using a slow cooling procedure with dimethylsulphoxide as the cryoprotectant. Following slow thawing, 431 fully intact embryos were transferred in 314 embryo transfer procedures and 488 partially damaged embryos were transferred in 327 such procedures. The percentage of gestational sacs with fetal heartbeat obtained after transfer of fully intact embryos was almost three times higher than that after transfer of partially damaged embryos (11.4 versus 3.5%). Forty-five children (birth rate 10% per embryo transfer) were born after transfer of fully intact embryos and 14 after transfer of embryos from which some blastomeres had been lost following cryopreservation.

In conclusion, although children have been delivered after transfer of partially damaged embryos, the aim of a cryopreservation programme must be to obtain fully intact embryos after thawing.

Key words: cryopreservation/embryo/human/viability

Introduction

Human embryo cryopreservation is now a widespread reliable and flexible routine procedure which has become an integral part of assisted reproductive technology. It allows the storage of supernumerary embryos, thus providing the possibility of increasing the cumulative pregnancy rates from a given treatment cycle. As this procedure has now been available for more than 10 years, it is now possible to study the results of human embryo cryopreservation over a long period of time. This has allowed us to identify a number of factors, some of which are controversial, which are involved in the success or failure of cryopreservation: these are the embryo developmental stage (Cohen et al., 1988; Hartshorne et al., 1990), the morphological appearance of the embryos before freezing (Camus et al., 1989), the hormone supplementation in the frozen-thawed embryo transfer cycle (Van der Auwera et al., 1994) and the ovarian stimulation procedure used in the oocyte collection cycle (Testart et al., 1990; Van der Elst et al., 1996).

Some 35–45% of cryopreserved human multicellular embryos are found to be fully intact after thawing and dilution of the cryoprotectants (Mandelbaum, 1990). Some authors mention that embryos that have been partially damaged are viable provided at least half of the initial number of blastomeres remain intact (Lasalle et al., 1985; Freeman et al., 1986). On the basis of these observations, we defined survival after cryopreservation of multicellular human embryos as the percentage of embryos that maintained at least half of the initial number of blastomeres intact (Van Steirteghem et al., 1987). As a result, it is common practice in our centre for frozen-thawed fully intact embryos to be used together with partially damaged ones for subsequent transfer.

In a study at our centre, it was found that the implantation of cryopreserved embryos depended on the stimulation protocol used in the oocyte collection cycle (Van der Elst et al., 1996). The percentage of fetal heartbeats per transferred frozen-thawed embryo was lower when the gonadotrophin-releasing hormone (GnRH)-agonist Buserelin in association with human menopausal gonadotrophin (HMG) was used as a stimulator than when clomiphene citrate was used in association with HMG. Interestingly, we found that the distribution of fully intact embryos and damaged embryos transferred differed widely between the two different stimulation procedures. Many more fully intact embryos were transferred in the group stimulated with clomiphene citrate and HMG. Hartshorne et al. (1990) found that thawed embryos with one or more blastomeres damaged during freezing had the same capacity to produce pregnancies as did those with all blastomeres intact. This finding was based on the observation of six implantations (two of which aborted) in four pregnancies, where they were sure that the pregnancies could have originated only from non-intact embryos. This was confirmed by Testart et al. (1990). In his analysis no clear definition was given of implantation rate. Furthermore, no information was given as to what stimulation regimen was used in the oocyte collection cycles.

In this study, therefore, the aim was to investigate the effect
of blastomere damage in human embryos after cryopreservation in a large group of patients.

The patients were uniform as regards the stimulation procedure used in the oocyte collection cycle, the freezing procedure used and the selection criteria for the embryos for freezing. We investigated the implantation rate and the subsequent development of the implanted embryos.

Materials and methods

Patients studied

A total of 1434 transfers of cryopreserved embryos were performed between January 1986 and December 1992 with a mean of 1.5 embryos per transfer. Of these transfer cycles, 844 were included in this study. All the patients involved had their embryos frozen with dimethyl sulphoxide in a slow-freezing and slow-thawing protocol. Ovarian stimulation in the oocyte retrieval cycle had been carried out using a down-regulation protocol combining the gonadotrophin-releasing hormone agonist (GnRHα) Buserelin acetate (Suprefact® nasal spray, Hoechst AG, Frankfurt, Germany) with HMG (Humeon, Organon, Oss, The Netherlands) (Smits et al., 1988a, b). The cryopreserved embryos were multicellular embryos which had been supernumerary at day 2 or day 3 of the oocyte retrieval cycle.

Transfer cycles excluded from the study were those in which embryos had been obtained after gamete intra-Fallopian transfer (n = 120), those involving zygote intra-Fallopian transfer (n = 66), those where stimulation in the collection cycle was carried out using clomiphene citrate in association with HMG (n = 190) and those in which cryopreservation of the supernumerary embryos had been carried out using the cryoprotectants 1,2-propanediol–sucrose (n = 214).

Embryos for freezing

Oocyte retrieval was carried out by vaginal ultrasound-guided oocyte pick-up. In-vitro fertilization (IVF) was carried out as described by Staessen et al. (1993). About 16–18 h after insemination, the oocytes were observed under an inverted microscope and were inspected for the presence of two pronuclei. Embryonic development was assessed 24 h later, i.e. on day 2 after insemination. The embryos were categorized according to developmental stage and to the presence of anucleate fragments: (A) excellent embryos showing no anucleate fragments, (B) good-quality embryos with less than 20% of their volume filled with anucleate fragments and (C) fair-quality embryos with more than 20% and less than 50% of their volume filled with anucleate fragments. The two or three morphologically best embryos up to type C were selected for transfer in the oocyte collection cycle. If on day 2 or day 3 of the oocyte collection cycle, excellent or good-quality embryos were surplus to requirements, they were cryopreserved by a slow freezing protocol with dimethyl sulphoxide. At day 2 of the oocyte collection cycle, the developmental stage of the frozen embryos varied from the 2-cell to the 8-cell stage. The majority of the embryos were frozen at the 3- to 4-cell stage (65% of the embryos). At day 3 of the oocyte collection cycle, embryos to be frozen were at least at the 5-cell stage.

Slow freezing with dimethyl sulphoxide

Freezing-thawing solutions were always made up in HEPES-buffered Earle’s medium supplemented with 0.4% w/v human serum albumin (HSA) (Staessen et al., 1994), further referred to as HEPES-medium. Dimethyl sulphoxide (DMSO) was from Sigma (D26250) (Bornem, Belgium).

Embryos for freezing were first equilibrated in HEPES-medium with 0.75 M DMSO for 10 min at 22°C. They were then transferred to a properly labelled glass ampoule (Wheaton, Polylab, Brussels) containing 500 µl of HEPES-medium and 1.5 M DMSO.

Ampoules were closed and transferred to a biological freezer (Kryo 10 series III, Planer, VEL, Leuven, Belgium) with a freezing chamber temperature of 4°C. The ampoules were kept at this temperature for 15 min. The temperature was then lowered to −7°C at 2°C/min at which point a 5 min pre-seeding hold was built in. Seeding was then performed manually by touching the ampoule with liquid nitrogen-cooled forceps at the level of the fluid meniscus. After another 5 min post-seeding hold at −7°C, the temperature was lowered to −80°C at the rate of 0.3°C/min. The ampoules were then further cooled to −110°C at the rate of 10°C/min and were finally plunged into liquid nitrogen (LN2). Ampoules were stored in LN2-filled containers (GT40, Air Liquide, Machelen, Belgium).

For thawing, the ampoules were removed from LN2 and immediately placed on crushed ice in a cold room at 4°C until ice crystals in the medium had disappeared. This took about 15 min. After this, the content of each ampoule was emptied into a Petri dish using a Pasteur pipette. Embryos were picked up under stereo microscope guidance using a finely pulled glass pipette and put in HEPES-medium containing 1 M DMSO for 10 min at 22°C. After three further dilution steps of 10 min at 22°C in HEPES-medium containing 0.75 M DMSO, 0.375 M DMSO and 0 M DMSO, the embryos were washed twice in HEPES-medium. Embryos were then inspected for survival under an inverted microscope at a magnification of ×200 before being cultured in B2 medium as described by Staessen et al. (1994). The evaluation criteria for survival of frozen-thawed embryos were as follows: at least half of the initial number of blastomeres were intact and there were no signs of zona pellucida damage. Damaged blastomeres were either lysed blastomeres, degenerated or dark blastomeres, or blastomeres where the membrane was no longer distinct. Transferable embryos were cultured for 2–4 h before subsequent transfer to the patients.

Frozen embryo transfer, pregnancy and implantation of transferred embryos

To study the influence of the presence of blastomere damage in cryopreserved embryos on their subsequent implantation, two types of transfer were evaluated: cryopreserved embryo transfers where only fully intact embryos (100% blastomere survival) were transferred and cryopreserved embryo transfers where only embryos with a maximum of 50% blastomere damage were transferred.

Frozen embryos were mainly transferred during the course of a natural cycle. Other transfer cycles involved the use of clomiphene citrate or human menopausal gonadotrophins in association with human chorionic gonadotrophin.

Serum HCG concentrations were determined on at least two independent occasions at least 10 days after embryo replacement. Biochemical pregnancy was defined as a significant increase in HCG concentration (>10 mIU per ml) between days 10 and 20 after luteinizing hormone surge. Clinical pregnancy was defined as the observation of a gestational sac with fetal heartbeat by means of echographic screening at 7 weeks of pregnancy. Embryo implantation rate was defined as the number of gestational sacs with fetal heartbeat observed by echographic screening at 7 weeks of pregnancy compared with the number of embryos transferred. The birth rate was defined as the number of children born per cryopreserved embryo transferred.

Statistics

The χ2-test was used to compare pregnancy and embryo implantation rates between transfers done under different conditions. The mean
### Results

#### Outcome of embryo transfer according to survival after cryopreservation

We analysed 844 transfer cycles with cryopreserved embryos. There were 314 transfer cycles with fully intact embryos (group 1) and 327 transfer cycles with only embryos with damaged blastomeres (group 2). Other transfer cycles were mixed transfers of fully intact embryos together with partially damaged ones (203 cycles). The outcome of embryo transfer is given in Table I. The mean age of the patients did not differ between the two groups; 32.5 years in group 1 and 32.9 years in group 2. Neither did the distribution of the different indications for infertility differ between the two groups. The percentage of positive HCG measurements was significantly higher in group 1 than in group 2 ($P < 0.005$). From 28 positive HCG measurements in group 2, 12 pregnancies were biochemical pregnancies. There were only nine biochemical pregnancies out of 52 positive HCG measurements in group 1 ($P < 0.05$). As a result, the percentage of clinical pregnancies was significantly higher in group 1 than in group 2 ($P < 0.0001$). Where three fully intact embryos could be transferred, 37.5% of HCG measurements per transfer were positive.

A total of 1409 embryos were transferred in 844 transfer procedures, including 668 fully intact embryos (47.4%) and 741 embryos with damaged blastomeres (52.6%). Overall, the embryo implantation rate was 6.5% (90 cases of fetal heartbeat after transfer of 1409 embryos).

The implantation of transferred embryos according to survival after cryopreservation is shown in Table II.

A total of 431 fully intact embryos were transferred in 314 embryo transfer procedures (group 1) and 488 damaged embryos were transferred in 327 embryo transfer procedures (group 2). The morphological quality of the embryos before freezing in group 1 did not differ from that in group 2. The embryo implantation rate was significantly higher in group 1 than in group 2 ($P < 0.0001$). In group 1, 45 children were delivered; in group 2, 14 children were delivered ($P < 0.0001$).

The influence of the number of blastomeres lost on the implantation of cryopreserved embryos is shown in Table III, in which we analysed the implantation of embryos synchronously dividing before freezing. As can be seen from Table III, a clear reduction in embryo implantation rate per single embryo transfer occurred when only one blastomere was lost after cryopreservation ($P < 0.001$). (all developmental stages together). No clinical pregnancies were obtained when one blastomere was lost from a 2-cell stage embryo or from a 4-cell stage embryo.

A description of the outcome of the 28 positive HCG measurements obtained after transfer of partially damaged embryos is given in Table IV.

#### Discussion

The aim of this study was to determine whether damaged human embryos had the same potential for implantation as fully...
Are partially damaged cryopreserved human embryos viable?

Table IV. Description of the embryos transferred and the pregnancy outcome in 29 pregnancies obtained after transfer of damaged embryos only

<table>
<thead>
<tr>
<th>Subject number</th>
<th>Number of blastomeres surviving in transferred embryos</th>
<th>Outcome of pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial number of blastomeres present</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2/4 2/4</td>
<td>biochemical pregnancy</td>
</tr>
<tr>
<td>2</td>
<td>2/4 3/6</td>
<td>one child</td>
</tr>
<tr>
<td>3</td>
<td>2/4 3/4</td>
<td>biochemical pregnancy</td>
</tr>
<tr>
<td>4</td>
<td>6/8 4/6 4/6</td>
<td>miscarriage</td>
</tr>
<tr>
<td>5</td>
<td>4/5 7/8 5/8</td>
<td>two children</td>
</tr>
<tr>
<td>6</td>
<td>6/8</td>
<td>biochemical pregnancy</td>
</tr>
<tr>
<td>7</td>
<td>5/6</td>
<td>one child</td>
</tr>
<tr>
<td>8</td>
<td>5/8</td>
<td>one child</td>
</tr>
<tr>
<td>9</td>
<td>3/4 3/4</td>
<td>one child</td>
</tr>
<tr>
<td>10</td>
<td>7/8</td>
<td>one child</td>
</tr>
<tr>
<td>11</td>
<td>2/4 2/4</td>
<td>biochemical pregnancy</td>
</tr>
<tr>
<td>12</td>
<td>5/6</td>
<td>one child</td>
</tr>
<tr>
<td>13</td>
<td>1/8 6/8 6/8</td>
<td>one child</td>
</tr>
<tr>
<td>14</td>
<td>3/4 4/5</td>
<td>biochemical pregnancy</td>
</tr>
<tr>
<td>15</td>
<td>2/3</td>
<td>miscarriage</td>
</tr>
<tr>
<td>16</td>
<td>8/11 2/4</td>
<td>one child</td>
</tr>
<tr>
<td>17</td>
<td>7/8 7/8 7/8</td>
<td>biochemical pregnancy</td>
</tr>
<tr>
<td>18</td>
<td>6/8 5/8</td>
<td>biochemical pregnancy</td>
</tr>
<tr>
<td>19</td>
<td>1/4</td>
<td>biochemical pregnancy</td>
</tr>
<tr>
<td>20</td>
<td>4/6</td>
<td>one child</td>
</tr>
<tr>
<td>21</td>
<td>4/6</td>
<td>one child</td>
</tr>
<tr>
<td>22</td>
<td>4/8 1/4</td>
<td>biochemical pregnancy</td>
</tr>
<tr>
<td>23</td>
<td>7/8 4/8 3/4</td>
<td>one child</td>
</tr>
<tr>
<td>24</td>
<td>7/8 4/8</td>
<td>biochemical pregnancy</td>
</tr>
<tr>
<td>25</td>
<td>7/8</td>
<td>biochemical pregnancy</td>
</tr>
<tr>
<td>26</td>
<td>3/6</td>
<td>miscarriage</td>
</tr>
<tr>
<td>27</td>
<td>3/4 1/4</td>
<td>one child</td>
</tr>
<tr>
<td>28</td>
<td>4/8</td>
<td>one child</td>
</tr>
</tbody>
</table>

intact embryos. We retrospectively analysed cryopreserved embryo transfers where either fully intact multicellular embryos or damaged multicellular embryos were transferred. The stimulation procedure used in the oocyte collection cycle was GnRH-agonist buserelin in association with HMG. Cryopreservation used a slow cooling, slow thawing procedure with DMSO as the cryoprotectant. We found that the birth rate per embryo transferred was three times higher when fully intact embryos were transferred than when damaged embryos were transferred (10.4 versus 2.9%).

In a mouse model, biopsy at the 4- or 8-cell stages and removal of 1/4, 1/8 or 2/8 has no effect on blastocyst formation, implantation or postimplantation development after transfer to pseudopregnant recipients (Monk and Handyside, 1988; Wilton et al., 1989; Liu et al., 1993). In humans, there has as yet been no direct testing of whether biopsy at the 4- or 8-cell stage has an effect on further in-vitro or in-vivo development. Hardy et al. (1990) concluded that removal of one or two cells at the 8-cell stage, while reducing the cellular mass, does not adversely affect the preimplantation development of biopsied embryos in vitro and suggested that this approach might be used for preimplantation diagnosis of genetic defects. Since then, several pregnancies have been described after transfer of biopsied human embryos and genetic diagnosis of biopsied blastomeres (Harper, 1996). More recently Balakier and Cadesky (1997) found that when 2-cell stage human embryos containing a mononucleated and a binucleated blastomere were transferred to patients, birth of healthy babies as well as spontaneous abortions occurred. This suggests that the one normal blastomere is totipotent. They also suggest that occasional transfers of defective embryos can be considered because these sometimes develop normally.

Rülicke and Autenried (1995), studying the potential of 2-cell mouse embryos to develop to term despite partial damage after cryopreservation, found that, although full in-vivo development to term of damaged embryos is possible, the in-vivo developmental rate of a damaged group was only 26% compared to 53% in the intact group. In contrast to the results described for biopsied embryos, our results in the present study as well as those of Rülicke and Autenried seem to prove that the coexistence of damaged blastomeres and intact blastomeres within mammalian embryos reduces their further chances of developing to term.

Mammalian embryos have been described as having a remarkable capacity to regulate for reduction of cellular mass at cleavage stages. In the mouse, reduced cell numbers are compensated by inner cell mass (ICM) lineage soon after implantation (Lewis and Rossant, 1982). Furthermore, Handyside et al. (1990) found that the reduction in cell numbers at the blastocyst stage in both 7/8 and 6/8 biopsied embryos was distributed equally between trophectoderm (TE) and ICM so that the ratio of ICM/TE cells was not altered. An explanation for the lower implantation potential of damaged cryopreserved embryos might be that blastocysts do not contain an ICM or that the ICM/TE ratio is dramatically disturbed. This might explain the higher biochemical pregnancy rates in transfer cycles of embryos with damaged cryopreserved embryos. We also think, however, that the presence of a damaged blastomere has a negative influence on the further development of the intact ones. This situation is clearly different from that of biopsied embryos where some blastomeres have been completely removed. Further evidence for a toxic effect from the damaged blastomere(s) came from the observation that the loss of one blastomere was in itself sufficient to compromise dramatically the further development of the cryopreserved embryo (this study). The toxic effect seems to be more pronounced at earlier developmental stages (2-cell stage, 4-cell stage), than at later developmental stages (8-cell stage). More evidence for a toxic effect from the damaged blastomere(s) has been reported. Alikani et al. (1993) studied the effects of degeneration of blastomeres on the developmental fate of mouse embryos. Micromanipulation techniques were used to destroy one or two blastomeres of a 4-cell embryo. Destruction of two blastomeres was detrimental, as only 3.2% hatched. An increase in hatching, however, was achieved after repair of the half embryo by microsurgical removal of the degenerate material (72% hatched).

Our study cannot confirm the statements by Hartshorne et al. (1990) and Testart et al. (1990), that damaged embryos have the same potential to implant as fully intact embryos, most probably for the reasons already mentioned in the introduction (low numbers of embryos investigated, unclear selection criteria both for the embryos to be cryopreserved and for those transferred in the oocyte retrieval cycle, unclear definition of implantation rate).

In the present study, the overall cryopreserved embryo
implantation rate was 6%. Although in the literature implantation rate per transferred embryo has not always been clearly defined, our results are comparable to other published figures (Lornage et al., 1990; Hartshorne et al., 1990; Mandelbaum, 1990; De Ziegler and Frydman, 1990). In this study, 47% of surviving embryos were found to be fully intact. For embryos obtained after GnRH-agonist treatment, this figure is comparable to that previously reported (Hartshorne et al., 1990; Testart et al., 1990; Lornage et al., 1990).

Several factors are known to be involved in the destruction of blastomeres during freezing and thawing procedures (Ashwood-Smith et al., 1988): intracellular ice formation, osmotic stress and physical factors. Many of these problems could be avoided by vitrification, in which solidification occurs not by ice-crystal formation but by the formation of a glassy state (Fahy et al., 1984). Interestingly, Ali et al. (1995) demonstrated that after vitrification of human embryos in an ethylene-glycol-based solution, fully intact embryo survival rates of 100% were obtained. The safety of this method needs, however, to be further evaluated. Also, 1-cell human embryos survive the cryopreservation procedure better, yielding more fully intact embryos appropriate for transfer than when multicellular embryos are frozen (Hartshorne et al., 1990). However, when 1-cell-stage embryos are frozen, the number of embryos available for fresh transfer is limited and the fresh cycle is not optimized. It remains to be proved that this approach has no impact on the potential of a single oocyte collection cycle to lead to birth(s).

In conclusion, although this and previous studies have shown that children have been born after transfer of partially damaged cryopreserved embryos, including those which retained <50% of the initial number of blastomeres (Mohr et al., 1985; Veiga et al., 1987; Hartshorne et al., 1990; Testart et al., 1990; Mandelbaum, 1990), the aim of a cryopreservation programme should be to yield fully intact embryos. The lower implantation rate of cryopreserved embryos derived from GnRHa-HMG stimulated cycles compared with those derived from clomiphene citrate-HMG stimulated cycles can be explained by the lower number of fully intact embryos transferred.

Acknowledgements
The authors wish to thank Mrs Sabrina Vitrier and Mrs Marleen Van der Linden for skilful technical assistance and for help in data collection. Mr F. Winter of the Language Education Centre is kindly acknowledged for correction of the English text. This study was supported by the Belgian National Fund for Scientific Research (grant no. 3.0036.85 and grant no. 3.0181.95).

References

Received on December 12, 1996; accepted on July 1, 1997