

Effect of developmental stage of embryo at freezing on pregnancy outcome of frozen–thawed embryo transfer

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BACKGROUND: The study aim was to investigate the impact of the developmental stage of embryos on pregnancy outcome of frozen embryo transfer (FET). **METHODS:** The survival rates of embryos after thawing and pregnancy outcome following FET were compared retrospectively between three cryopreservation strategies utilizing either zygote, day 2 or day 3 embryo freezing. **RESULTS:** A total of 4006 embryos was analysed in 1657 thaw cycles. The highest ($P < 0.0001$) survival rate (all cells survived) was observed for zygotes (86.5%), followed by day 2 (61.7%) and day 3 (43.1%) embryos. FET was performed in 1586 (95.7%) of all thaw cycles, resulting in overall clinical pregnancy and implantation rates of 20.7 and 14.2% respectively. The delivery rate per transfer was 16.5%, and live birth rate per transferred embryo 11%. There were no significant differences in clinical pregnancy, implantation, delivery and birth rates between frozen zygote, day 2 and 3 embryo transfers. However, an elevated miscarriage rate was observed in the day 3 group (45%) compared with zygotes (21.3%; $P = 0.049$) and day 2 embryos (18.3%; $P = 0.004$). The overall efficacy of FET (birth rate per thawed embryo) was 7.3%. The efficacy was lower in day 3 group (4.2%) than in the zygote (7.1%; $P = 0.082$) and day 2 (7.6%; $P = 0.027$) groups. **CONCLUSIONS:** The developmental stage of embryos at freezing has a profound effect on their post-thaw survival, but seems to have little effect on rates of clinical pregnancy, implantation, delivery and birth after FET. The elevated miscarriage rate for day 3 frozen embryo transfers may be caused by damage during freeze–thaw procedures. The low survival rate and elevated miscarriage rate were both responsible for a reduced overall efficacy for day 3 FET when compared with zygotes and day 2 embryos.

Key words: cryopreservation of embryos/frozen embryo transfer/ICSI/IVF

Introduction

A well-established frozen embryo transfer (FET) programme may substantially increase the cumulative pregnancy rates of IVF and ICSI procedures (Bergh *et al.*, 1995; Lurie *et al.*, 2001). Freezing and storing of surplus embryos also allows the number of replaced embryos in both fresh and frozen embryo transfers to be reduced, thereby diminishing the risk of multiple pregnancies (Schnorr *et al.*, 2001; Tiitinen *et al.*, 2001). Additionally, all embryos could be cryopreserved if the woman has a risk of developing ovarian hyperstimulation syndrome (Tiitinen *et al.*, 1995). However, careful consideration of all clinical and embryological factors influencing the outcome of FET is a prerequisite for a successful programme.

Clinical factors important in the success of FET include the aetiology of infertility (Wang *et al.*, 2001), the age of the woman (Schalkoff *et al.*, 1993; Wang *et al.*, 2001), the type of ovarian stimulation used in the oocyte collection cycle (Van der Elst *et al.*, 1996) and the outcome of fresh embryo transfer (Lin *et al.*, 1995). The effects of embryological parameters on pregnancy rate after FET have also been addressed in several

studies. The pregnancy rate following FET has been shown to be related to the number of blastomeres and morphological appearance of embryos prior to freezing (Hartshorne *et al.*, 1990; Schalkoff *et al.*, 1993), the extent of embryo damage after thawing (Edgar *et al.*, 2000), and the resumption of post-thaw blastomere divisions (Van der Elst *et al.*, 1997). Embryos have been successfully cryopreserved at zygote (Cohen *et al.*, 1988), cleavage (Lassalle *et al.*, 1985) and blastocyst (Cohen *et al.*, 1985; Fehilly *et al.*, 1985) stages, using various freezing protocols with either dimethylsulphoxide (DMSO) (Mohr and Trounson, 1985), 1,2-propanediol (PROH) (Lassalle *et al.*, 1985) or glycerol (Cohen *et al.*, 1985) as cryoprotective agents. To date, few attempts have been made to compare the success rates of FET between different cryopreservation strategies utilizing either zygote or cleavage-stage embryo freezing, but these have resulted in contradictory conclusions (Demoulin *et al.*, 1991; Horne *et al.*, 1997; Kattera *et al.*, 1999; Senn *et al.*, 2000). Therefore, the current retrospective study was devised to evaluate the impact of developmental stage of embryos on the pregnancy outcome of FET.

Materials and methods

Study period and patients

The current study included all patients undergoing IVF or ICSI treatment at the Infertility Clinic of the Family Federation of Finland in Helsinki between 1993 and 2001, and with an FET between 1997 and 2001.

IVF and ICSI procedures

The ovarian stimulation regimen and oocyte collection have remained essentially unchanged during the years 1993 to 2001, and have been described extensively elsewhere (Söderström-Anttila *et al.*, 1996). Briefly, the patients underwent pituitary down-regulation with a GnRH agonist which was commenced in the midluteal phase of the previous menstrual cycle. Between 1993 and 1996, when suppression was achieved, ovarian stimulation was performed using either hMG or highly purified FSH. Recombinant FSH was mainly used for ovarian stimulation between 1997 and 2001. When two or more follicles reached the size of ≥ 17 mm diameter, hCG (Pregnyl[®]; Organon, or Profasi[®]; Laboratories Serono) was administered, and transvaginal oocyte pick-up (OPU) was performed 36 h later. A similar ovarian stimulation regimen was used in patients who had the supernumerary embryos frozen at either 1, 2 or 3 days after insemination or ICSI.

The culture medium used for oocytes and embryos was Universal-IVF medium (U-IVF; Medi-cult, Denmark). Semen samples were prepared with a discontinuous gradient method using Percoll[®] (Pharmacia, Sweden) or PureSperm (Nidacon International AB, Sweden). Insemination or microinjection was carried out 4–6 h after OPU as described previously (Moilanen *et al.*, 1999; Salumets *et al.*, 2002). The oocytes were checked for the presence of pronuclei and polar bodies 16–18 h after insemination or ICSI. Zygotes were transferred to fresh medium (U-IVF) and incubated in an open-culture system without oil for a subsequent 24 or 48 h period. The quality of embryos was evaluated at 44–48 h (day 2 embryo transfers) or 68–72 h (day 3 embryo transfers) after insemination or ICSI. Routine examination of embryo quality included the number of blastomeres, the degree of fragmentation, the uniformity of blastomeres, and the presence of multinucleated blastomeres. Embryo morphology was scored as follows: grade 1, no fragments and equal blastomeres; grade 2, <20% fragmentation; grade 3A, unequal blastomeres and/or 20–35% fragmentation; grade 3B, unequal blastomeres and/or 35–50% fragmentation; and grade 4, >50% fragmentation. Fresh embryo transfer was carried out either on day 2 or 3 after insemination or ICSI.

Embryo freezing

The developmental stage of embryos at freezing was dependent upon the day of OPU as no freezing was performed on Saturdays and Sundays. Thus, in OPUs performed on Thursdays, fertilization was checked on Fridays and one or two zygotes were left in culture to be transferred on Saturday, while surplus zygotes were frozen on Friday (20–22 h after insemination/ICSI). The cryopreservation of zygotes did not rely on a quality assessment, but rather all supernumerary fertilized oocytes were frozen. The majority of OPUs were scheduled on Mondays, Tuesdays and Wednesdays. In these IVF and ICSI procedures, both embryo transfer and freezing were accomplished 2 days after oocyte retrieval (44–48 h after insemination/ICSI). For OPUs performed on Fridays, embryos were cultured for 3 days, and embryo transfer and freezing carried out on Mondays (day 3; 68–72 h after insemination/ICSI). The freezing strategy of cleavage-stage embryos differed from the zygotes as only good quality embryos, exhibiting morphology grades of 1 to 3A, were selected for freezing.

Cryopreservation was carried out using an automated Kryo 10 series II biological freezer (Planer Products Ltd, UK), following a

slow-freeze protocol using PROH (Sigma, USA) as a cryoprotectant (Lassalle *et al.*, 1985). An identical freezing programme was used for zygotes and day 2 or 3 embryos. The freezing solution was made up in phosphate-buffered saline (PBS) (Gibco, Life Technologies, UK) supplemented with 20% (v/v) human serum (Finnish Red Cross, Finland). Embryos were first incubated in 1.5 mol/l PROH freezing solution at room temperature (RT) for 10 min, and then transferred to a 1.5 mol/l PROH and 0.2 mol/l sucrose (Sigma) freezing solution. Thereafter, one to three embryos were loaded into plastic ministraws (0.25 ml; Paillette Souple, Industrie de la Médecine Vétérinaire, France) and the freezing programme was executed as follows. Embryos were placed in the freezing machine at 18.0°C, cooled at $-2.0^\circ\text{C}/\text{min}$ to -8.0°C , held at -8.0°C for manual seeding (10 min), cooled at $-0.3^\circ\text{C}/\text{min}$ to -30.0°C , and then at $-30.0^\circ\text{C}/\text{min}$ to -150.0°C , before being plunged into liquid nitrogen.

Embryo thawing and replacement

The straws with frozen zygotes and cleaved embryos were removed from liquid nitrogen, exposed to RT (30 s) and immersed in a water bath at 30°C (30 s). Zygotes and cleaved embryos were first incubated in a series of decreasing PROH concentrations (1.0 mol/l for 5 min and 0.5 mol/l for 5 min) in the thawing solution [0.2 mol/l sucrose and 20% (v/v) human serum in PBS], next in the thawing solution only (10 min), and finally in sucrose-free thawing solution at 37°C (10 min), before being transferred to the culture medium.

FET was performed either in a natural or in down-regulated hormone replacement cycle. In natural cycles, the embryo transfer was timed by monitoring for spontaneous ovulation using vaginal ultrasound scanning and urinary LH. Vaginal micronized progesterone (Lugesteron[®]; Leiras, Finland) was used for luteal support.

Zygotes and cleaved embryos were evaluated twice, immediately after thawing and prior to transfer. The surviving zygotes with morphologically normal membranes, clear cytoplasm and no breaches of the zona pellucida were cultured for 24 h before transfer. The proportion of cleaved zygotes in the present study (94%) was similar to that observed for fresh zygotes (93.7%) (Salumets *et al.*, 2001). Cleaved embryos were classified as either fully intact (100% cells survived), partially damaged ($\geq 50\%$ cells survived) or degenerated (<50% cells survived) after thawing. Cleavage-stage embryos were predominantly transferred on the day of thawing, and only a small fraction of day 2 embryos were transferred after 24 h culture. Cleavage-stage embryos were accepted for transfer if they retained $\geq 50\%$ of blastomeres intact after thawing. A maximum of two frozen embryos was transferred, irrespective of the number of available embryos.

A positive serum hCG test (>10 mIU/ml) conducted 16 days after embryo transfer confirmed pregnancy. The clinical pregnancy was documented by the presence of a gestational sac(s) with or without fetal heart beat on transvaginal sonography, ~3 weeks later. The clinical pregnancy rate was determined by dividing the number of clinical pregnancies by the total number of embryo transfers. In the calculation of implantation rate, the number of gestational sacs was divided by the number of embryos transferred. Miscarriage was defined as a spontaneous abortion prior to 20 weeks gestation. Delivery rate was specified as a ratio between deliveries and embryo transfers, while birth rate was described as the number of children born per number of embryos transferred. The overall efficacy of FET was represented as birth rate per embryo thawed.

Statistical analysis

Comparisons between proportions were performed using a χ^2 -test. Data were provided as mean (\pm SD) and compared using a two-tailed unpaired Student's *t*-test. Correlations were estimated using linear

Table I. Effect of developmental stage of embryos on their cryosurvival

Proportion of cells survived (%)	No. of zygotes (%)	No. of day 2 embryos (%)	No. of day 3 embryos (%)
100	486 (86.5) ^{a,b}	1935 (61.7) ^c	134 (43.1)
≥50	–	550 (17.6) ^d	77 (24.8)
<50	76 (13.5) ^{a,b,c}	648 (20.7) ^c	100 (32.1)

Zygotes and day 2 or 3 embryos were categorized into three classes based on the proportion of cells which survived: fully intact (100% cells survived); partially damaged (≥50% cells survived); and degenerated (<50% cells survived).

^a Difference between zygotes and day 2 embryos ($P < 0.0001$).

^b Difference between zygotes and day 3 embryos ($P < 0.0001$).

^c Difference between day 2 and day 3 embryos ($P < 0.0001$).

^d Difference between day 2 and day 3 embryos ($P = 0.002$).

^e Degenerated zygotes.

regression analysis and characterized by Pearson's correlation coefficient (r). A P -value < 0.05 was considered statistically significant.

Results

Effect of developmental stage on outcome of thawing

A total of 4006 embryos was thawed in 1657 cycles. Among the thawed embryos, 562 were zygotes and 3444 were cleaved embryos. The large majority (91%) of cleavage-stage embryos were frozen on day 2 ($n = 3133$), and the remainder ($n = 311$) on day 3. The effect of the developmental stage of the zygotes and cleaved embryos on survival after thawing is summarized in Table I. The zygotes were compared either with fully intact cleaved embryos (100% cells survived) or degenerated embryos (<50% cells survived). The highest ($P < 0.0001$) survival rate was observed for zygotes (86.5%), followed by day 2 (61.7%) and day 3 (43.1%) embryos. The proportion of partially damaged (≥50% cells survived) embryos was lower ($P = 0.002$) on day 2 (17.6%) than on day 3 (24.8%). A significantly ($P < 0.0001$) lower percentage of degenerated embryos was found for zygotes (13.5%) than for day 2 (20.7%) and day 3 (32.1%) embryos.

An inverse correlation (Figure 1) was found ($r = -0.9$; $P = 0.003$) between the proportion of fully intact zygotes or cleaved embryos and the total number of cells/blastomeres. The fraction of partially damaged embryos showed a positive correlation ($r = 0.8$; $P = 0.014$) with the cell number, while the incidence of totally degenerated embryos did not show any significant relationship ($r = 0.2$; $P = 0.718$) with the number of cells.

Effect of developmental stage on pregnancy outcome of FET

Between 1997 and 2001, a total of 1586 FETs was performed from 697 IVF and 322 ICSI cycles. Significantly more oocytes were recovered from patients who had supernumerary embryos cryopreserved on day 2 (15.3) than on day 1 (zygote stage) (13.4; $P = 0.005$) or day 3 (12.8; $P = 0.002$). Embryos were successfully thawed and transferred in 95.7% of all freeze-thaw cycles. The average age of all patients was 34 ± 4.3 years (range 22.2–44.3). With an average transfer of 1.7 embryos, the

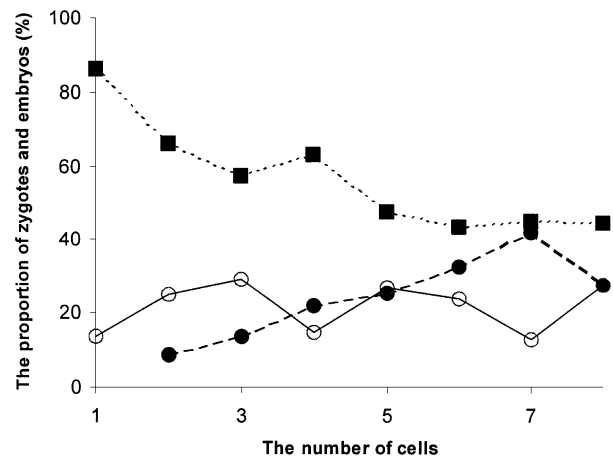


Figure 1. The impact of cell number of zygotes and cleaved embryos on their cryosurvival. (filled square), fully intact (100% cells survived): $y = 81.2 - 5.4x$; $r = -0.9$; $P = 0.003$; (filled circle), partially damaged (≥50 cells survived): $y = 2.4 + 4.4x$; $r = 0.8$; $P = 0.014$; (open circle), degenerated (<50% cells survived): $y = 19.8 + 0.4x$; $r = 0.2$; $P = 0.718$.

overall clinical pregnancy and implantation rates were 20.7% (329/1586) and 14.2% (373/2635) respectively. All FETs resulted in 262 deliveries (16.5% per embryo transfer) and the birth of 291 children (11% per embryo transferred).

The analysis included 234 transfers with zygotes, 1243 transfers with day 2 embryos, and 109 transfers with day 3 embryos. The clinical parameters and pregnancy outcome of FET in all three groups are detailed in Table II. There were no significant differences between groups in mean patient age and number of embryos transferred, nor in the clinical pregnancy or implantation rates. A significantly elevated miscarriage rate was, however, observed in the day 3 transfer group (45%) compared with zygotes (21.3%; $P = 0.049$) and day 2 embryos (18.3%; $P = 0.004$). The delivery rate for frozen zygotes (15.8%) was similar to that observed for day 2 frozen embryos (17.2%). Although a lower delivery rate was found for day 3 transfers (10.1%) than for zygotes and day 2 embryos, these differences were not statistically significant. The birth rates per embryo transferred were comparable in all three groups.

The overall efficacy of cryopreservation procedure for each developmental stage was expressed as the birth rate per embryo thawed (Table II). A similar overall efficacy was observed for zygote (7.1%) and day 2 (7.6%) embryo freezing. The birth rate per embryo thawed for day 3 (4.2%) freezing was significantly ($P = 0.027$) lower when compared with day 2 freezing.

Discussion

There are two embryological key factors that contribute to the success of FET, namely the survival of embryos after thawing and the developmental competence of frozen-thawed embryos. In the present study, the effect of the developmental stage of embryos on FET outcome in an unselected group of patients attending the authors' clinic between 1997 and 2001 was analysed.

Table II. Effect of developmental stage on pregnancy outcome after frozen–thawed embryo transfer (FET)

Parameter	Developmental stage		
	Zygotes	Day 2 embryos	Day 3 embryos
Mean (\pm SD) age (years)	34.1 \pm 3.8	34 \pm 4.3	33.3 \pm 4.2
No. of embryo transfers	234	1243	109
No. of embryos transferred	393	2066	176
Average (\pm SD) number of embryos transferred	1.7 \pm 0.5	1.7 \pm 0.5	1.6 \pm 0.5
No. of clinical pregnancies (% per embryo transfer)	47 (20.1)	262 (21.1)	20 (18.3)
No. of implantations (% per embryo transferred)	55 (14)	296 (14.3)	22 (12.5)
No. of miscarriages (% per clinical pregnancy)	10 (21.3) ^a	48 (18.3) ^b	9 (45)
No. of deliveries (% per embryo transfer)	37 (15.8)	214 (17.2) ^c	11 (10.1)
No. of twin deliveries (% per delivery)	3 (8.1)	24 (11.2)	2 (18.2)
No. of children born (% per embryo transferred)	40 (10.2)	238 (11.5)	13 (7.4)
Birth rate per embryo thawed (%)	7.1 ^d	7.6 ^e	4.2

^aDifference between zygotes and day 3 embryos ($P = 0.049$).

^bDifference between day 2 and day 3 embryos ($P = 0.004$).

^cDifference between day 2 and day 3 embryos ($P = 0.056$, not significant).

^dDifference between zygotes and day 3 embryos ($P = 0.082$, not significant).

^eDifference between day 2 and day 3 embryos ($P = 0.027$).

The present study included 4006 frozen zygotes and cleaved embryos from 1657 freeze–thaw cycles. The highest rate of embryo survival (all cells survived) was shown for zygotes (86.5%), followed by day 2 (61.7%) and day 3 (43.1%) embryos, while the opposite was found for degenerated (<50% cells survived) embryos. Traditionally, embryos have been considered as surviving if they retain at least half of their initial number of blastomeres intact after thawing (Mandelbaum *et al.*, 1998). Using this criterion, a significantly ($P < 0.0001$) higher survival rate was still observed for zygotes (86.5%) than for day 2 (79.3%) and day 3 (67.9%) embryos.

The effectiveness of zygote (Veeck *et al.*, 1993; Damario *et al.*, 2000) and cleavage-stage embryo (Mandelbaum *et al.*, 1998; Edgar *et al.*, 2000; Wang *et al.*, 2001) cryopreservation in terms of embryo survival has been the subject of several studies. However, only a few studies have directly compared the results of different freezing strategies utilizing either zygote or cleavage-stage embryo cryopreservation, and these have yielded highly controversial results (Demoulin *et al.*, 1991; Horne *et al.*, 1997; Kattera *et al.*, 1999; Senn *et al.*, 2000). The findings of the present study support the conclusions of others (Senn *et al.*, 2000), who reported a better survival rate for zygotes (80.4%) than for day 2 embryos (71.8%). Other studies comparing the survival rates of zygotes and day 2 embryos have, however, demonstrated either similar results for zygotes (74.4%) and day 2 embryos (77.4%) (Horne *et al.*, 1997) or better results for day 2 embryos (73.9%) than for zygotes (64.4%) (Kattera *et al.*, 1999). The present results are in agreement with those of others—that the post-thaw survival of day 2 embryos is better than that of day 3 embryos (Mandelbaum *et al.*, 1987).

The view that the survival of embryos after thawing is highly related to their developmental stage was further confirmed by the inverse correlation between the extent of cryodamage after thawing and the number of blastomeres (see Figure 1). Lower survival rates with increasing cell number have also been demonstrated by others, and have been thought to be due to the increased total surface area of all cells (Hartshorne *et al.*,

1990). In the present study, embryos were cultured only in Universal-IVF medium. Therefore, the question of whether the cryosurvival of day 3 embryos can be improved by using sequential culture media remains to be answered by future studies.

In the present study, the rates of clinical pregnancy (20.1%), delivery (15.8%) and birth (10.2%) for frozen zygotes were comparable with those reported previously (Miller and Goldberg, 1995; Senn *et al.*, 2000). The combined clinical pregnancy (20.9%), delivery (16.6%) and birth (11.2%) rates for day 2 and 3 embryos were also comparable with those of other studies (Mandelbaum *et al.*, 1998; Wang *et al.*, 2001). The comparison of pregnancy and implantation rates revealed no significant differences between zygote, day 2 and day 3 frozen embryo transfers. Although a lower delivery rate per embryo transferred (10.1%) and birth rate per embryo transferred (7.4%) were reported for day 3 embryos than for zygotes (15.8%, 10.2%) and day 2 embryos (17.2%, 11.5%) the differences were not statistically significant.

In the present study, no differences were found in the pregnancy and implantation rates between zygotes and day 2 embryos. In contrast to this, others have reported better pregnancy and implantation rates after zygote than day 2 FET (Demoulin *et al.*, 1991; Senn *et al.*, 2000). In one of these studies (Demoulin *et al.*, 1991), the pregnancy and implantation rates for zygotes were 17.9 and 10.7%, and for day 2 embryos 5.5 and 4.7% respectively. The surprisingly low pregnancy and implantation rates for day 2 FET reported in these studies might indicate that factors other than the developmental stage of the embryos were responsible for such results. By contrast, another group (Kattera *et al.*, 1999) showed that supernumerary IVF embryos frozen on day 2 yielded a significantly higher pregnancy rate (22.8%) than did frozen zygotes (14.8%). To date, a comparison has been made between the outcome of day 2 and 3 FET in only one small study which included 185 FETs, and a significantly better pregnancy rate was demonstrated for day 2 than for day 3 FET (Mandelbaum *et al.*, 1987). The differences between the results

of these studies could be attributed to a range of factors including: the type of ovarian stimulation regimen used; the criteria applied for embryo selection for freezing; and, most importantly, the embryo freezing and thawing protocols used.

In the present study, a higher miscarriage rate (45%) was found in the day 3 FET group compared with other groups. In general, the rate of miscarriage has been suggested to be higher in frozen than in fresh embryo transfer pregnancies (Aytoz *et al.*, 1999). On the other hand, the miscarriage rate for fresh day 2 and 3 embryo transfers has been shown to be similar (Dawson *et al.*, 1995; Carrillo *et al.*, 1998). One explanation for an elevated miscarriage rate observed in the day 3 FET group might be that patients who had OPU on Fridays were the poor responders and therefore exhibited lower IVF performance (Toner *et al.*, 1991). However, the high numbers of oocytes retrieved from those patients (mean 12.8 per woman) excludes this possibility. Furthermore, the analysis of fresh day 2 and 3 embryo transfers performed in the present authors' clinic between 2000 and 2002 revealed similar rates for pregnancy (29.3 versus 29.6%) and miscarriage (19.5 versus 16.7%) in these two groups. When this evidence is combined, it seems much more likely that the elevated miscarriage rate for day 3 FET group observed in the present study was caused by the embryos being damaged during the freeze–thaw procedure. The high proportion (24.8%) of partially damaged ($\geq 50\%$ cells survived) embryos may be a reason for the increased level of pregnancy losses after day 3 FET, though this finding should be elucidated by further studies.

The overall efficacy of FET can be expressed as the birth rate per embryo thawed (Van der Elst *et al.*, 1995), and this was lower in the day 3 group (4.2%) than the day 2 group (7.6%). Although there was a trend towards a better birth rate per embryo thawed for zygotes (7.1%) than for day 3 embryos, the difference was not statistically significant ($P = 0.082$). The low survival rate and elevated miscarriage rate were both responsible for a reduced overall efficacy for day 3 FET when compared with zygotes and day 2 embryos. According to previously published reports, PROH is most frequently used in embryo cryopreservation (Mandelbaum *et al.*, 1998; Edgar *et al.*, 2000), though others have indicated better survival and implantation rates after cleavage-stage embryo cryopreservation with DMSO rather than PROH (Van den Abbeel *et al.*, 1988; Van der Elst *et al.*, 1995). In the present authors' clinic, PROH is used to cryopreserve both zygotes and cleavage-stage embryos. Thus, it might be speculated that the results would differ had DMSO rather than PROH been used for the freezing of cleavage-stage embryos.

To the best of the present authors' knowledge, this is the first study to compare the outcome of zygote, day 2 and day 3 embryo cryopreservation in which identical freezing and thawing protocols were used for both zygotes and cleaved embryos. The results suggest that the developmental stage of an embryo has a major impact on its survival after freezing and thawing, with the best survival rate being found for zygotes, followed by day 2 and day 3 embryos. In addition, the number of cells in an embryo was inversely correlated with its survival. By contrast, the developmental stage did not influence rates of clinical pregnancy, delivery and birth after FET. The higher

incidence of miscarriage found after the transfer of day 3 frozen embryos may have been related to damage caused to the embryo during the cryopreservation process. Taking into consideration the higher survival and lower miscarriage rate, a better overall efficacy was observed for zygotes and day 2 embryos compared with day 3 embryos.

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References

- Aytoz, A., Van den Abbeel, E., Bonduelle, M., Camus, M., Joris, H., Van Steirteghem, A. and Devroey, P. (1999) Obstetric outcome of pregnancies after the transfer of cryopreserved and fresh embryos obtained by conventional in-vitro fertilization and intracytoplasmic sperm injection. *Hum. Reprod.*, **14**, 2619–2624.
- Bergh, C., Josefsson, B., Nilsson, L. and Hamberger, L. (1995) The success rate in a Swedish in-vitro fertilization unit: a cohort study. *Acta Obstet. Gynecol. Scand.*, **74**, 446–450.
- Carrillo, A.J., Lane, B., Pridman, D.D., Risch, P.P., Pool, T.B., Silverman, I.H. and Cook, C.L. (1998) Improved clinical outcomes for *in vitro* fertilization with delay of embryo transfer from 48 to 72 h after oocyte retrieval: use of glucose- and phosphate-free media. *Fertil. Steril.*, **69**, 329–334.
- Cohen, J., Simons, R.F., Edwards, R.G., Fehilly, C.B. and Fishel, S.B. (1985) Pregnancies following the frozen storage of expanding human blastocysts. *J. In-Vitro Fertil. Embryo Transfer*, **2**, 59–64.
- Cohen, J., DeVane, G.W., Elsner, C.W., Fehilly, C.B., Kort, H.I., Massey, J.B. and Turner, T.G., Jr (1988) Cryopreservation of zygotes and early cleaved human embryos. *Fertil. Steril.*, **49**, 283–289.
- Damario, M.A., Hammitt, D.G., Session, D.R. and Dumesic, D.A. (2000) Embryo cryopreservation at the pronuclear stage and efficient embryo use optimizes the chance for a liveborn infant from a single oocyte retrieval. *Fertil. Steril.*, **73**, 767–773.
- Dawson, K.J., Conaghan, J., Oстера, G.R., Winston, R.M. and Hardy, K. (1995) Delaying transfer to the third day post-insemination, to select non-arrested embryos, increases development to the fetal heart stage. *Hum. Reprod.*, **10**, 177–182.
- Demoulin, A., Jouan, C., Gerday, C. and Dubois, M. (1991) Pregnancy rates after transfer of embryos obtained from different stimulation protocols and frozen at either pronucleate or multicellular stages. *Hum. Reprod.*, **6**, 799–804.
- Edgar, D.H., Bourne, H., Speirs, A.L. and McBain, J.C. (2000) A quantitative analysis of the impact of cryopreservation on the implantation potential of human early cleavage stage embryos. *Hum. Reprod.*, **15**, 175–179.
- Fehilly, C.B., Cohen, J., Simons, R.F., Fishel, S.B. and Edwards, R.G. (1985) Cryopreservation of cleaving embryos and expanded blastocysts in the human: a comparative study. *Fertil. Steril.*, **44**, 638–644.
- Hartshorne, G.M., Wick, K., Elder, K. and Dyson, H. (1990) Effect of cell number at freezing upon survival and viability of cleaving embryos generated from stimulated IVF cycles. *Hum. Reprod.*, **5**, 857–861.
- Horne, G., Critchlow, J.D., Newman, M.C., Edozien, L., Matson, P.L. and Lieberman, B.A. (1997) A prospective evaluation of cryopreservation strategies in a two-embryo transfer programme. *Hum. Reprod.*, **12**, 542–547.
- Kattera, S., Shrivastav, P. and Craft, I. (1999) Comparison of pregnancy outcome of pronuclear- and multicellular-stage frozen-thawed embryo transfers. *J. Assist. Reprod. Genet.*, **16**, 358–362.
- Lassalle, B., Testart, J. and Renard, J.P. (1985) Human embryo features that influence the success of cryopreservation with the use of 1,2 propanediol. *Fertil. Steril.*, **44**, 645–651.
- Lin, Y.P., Cassidenti, D.L., Chacon, R.R., Soubra, S.S., Rosen, G.F. and Yee, B. (1995) Successful implantation of frozen sibling embryos is influenced by the outcome of the cycle from which they were derived. *Fertil. Steril.*, **63**, 262–267.
- Lurie, D., Check, J.H., Nazari, A., Choe, J.K. and Lee, G. (2001) Cumulative pregnancy rates after four embryo transfers of either fresh or frozen embryos. *Clin. Exp. Obstet. Gynecol.*, **28**, 148–152.

- Mandelbaum, J., Junca, A.M., Plachot, M., Alnot, M.O., Alvarez, S., Debache, C., Salat-Baroux, J. and Cohen, J. (1987) Human embryo cryopreservation, extrinsic and intrinsic parameters of success. *Hum. Reprod.*, **2**, 709–715.
- Mandelbaum, J., Belaisch-Allart, J., Junca, A.M., Antoine, J.M., Plachot, M., Alvarez, S., Alnot, M.O. and Salat-Baroux, J. (1998) Cryopreservation in human assisted reproduction is now routine for embryos but remains a research procedure for oocytes. *Hum. Reprod.*, **13** (Suppl. 3), 161–174; discussion 175–177.
- Miller, K.F. and Goldberg, J.M. (1995) *In vitro* development and implantation rates of fresh and cryopreserved sibling zygotes. *Obstet. Gynecol.*, **85**, 999–1002.
- Mohr, L.R. and Trounson, A.O. (1985) Cryopreservation of human embryos. *Ann. N. Y. Acad. Sci.*, **442**, 536–543.
- Moilanen, J.M., Tulppala, M., Reima, I. and Hovatta, O. (1999) Fertilization, embryo quality, and cryosurvival in *in vitro* fertilization and intracytoplasmic sperm injection cycles. *J. Assist. Reprod. Genet.*, **16**, 17–23.
- Salumets, A., Hyden-Granskog, C., Suikkari, A.M., Tiitinen, A. and Tuuri, T. (2001) The predictive value of pronuclear morphology of zygotes in the assessment of human embryo quality. *Hum. Reprod.*, **16**, 2177–2181.
- Salumets, A., Suikkari, A.M., Mols, T., Söderström-Anttila, V. and Tuuri, T. (2002) Influence of oocytes and spermatozoa on early embryonic development. *Fertil. Steril.*, **78**, 1082–1087.
- Schalkoff, M.E., Oskowitz, S.P. and Powers, R.D. (1993) A multifactorial analysis of the pregnancy outcome in a successful embryo cryopreservation program. *Fertil. Steril.*, **59**, 1070–1074.
- Schnorr, J.A., Doviak, M.J., Muasher, S.J. and Jones, H.W., Jr (2001) Impact of a cryopreservation program on the multiple pregnancy rate associated with assisted reproductive technologies. *Fertil. Steril.*, **75**, 147–151.
- Senn, A., Vozzi, C., Chanson, A., De Grandi, P. and Germond, M. (2000) Prospective randomized study of two cryopreservation policies avoiding embryo selection: the pronucleate stage leads to a higher cumulative delivery rate than the early cleavage stage. *Fertil. Steril.*, **74**, 946–952.
- Söderström-Anttila, V., Foudila, T. and Hovatta, O. (1996) A randomised comparative study of highly purified follicle stimulating hormone and menopausal gonadotrophin for ovarian hyperstimulation in an oocyte donation programme. *Hum. Reprod.*, **11**, 1864–1870.
- Tiitinen, A., Husa, L.M., Tulppala, M., Simberg, N. and Seppala, M. (1995) The effect of cryopreservation in prevention of ovarian hyperstimulation syndrome. *Br. J. Obstet. Gynecol.*, **102**, 326–329.
- Tiitinen, A., Halttunen, M., Harkki, P., Vuoristo, P. and Hyden-Granskog, C. (2001) Elective single embryo transfer: the value of cryopreservation. *Hum. Reprod.*, **16**, 1140–1144.
- Toner, J.P., Philput, C.B., Jones, G.S. and Muasher, S.J. (1991) Basal follicle-stimulating hormone level is a better predictor of *in vitro* fertilization performance than age. *Fertil. Steril.*, **55**, 784–791.
- Van den Abbeel, E., Van der Elst, J., Van Waesberghe, L., Camus, M., Devroey, P., Khan, I., Smits, J., Staessen, C., Wisanto, A. and Van Steirteghem, A. (1988) Hyperstimulation: the need for cryopreservation of embryos. *Hum. Reprod.*, **3** (Suppl. 2), 53–57.
- Van der Elst, J., Camus, M., Van den Abbeel, E., Maes, R., Devroey, P. and Van Steirteghem, A.C. (1995) Prospective randomized study on the cryopreservation of human embryos with dimethylsulfoxide or 1,2-propanediol protocols. *Fertil. Steril.*, **63**, 92–100.
- Van der Elst, J., Van den Abbeel, E., Camus, M., Smits, J., Devroey, P. and Van Steirteghem, A. (1996) Long-term evaluation of implantation of fresh and cryopreserved human embryos following ovarian stimulation with buserelin acetate-human menopausal gonadotrophin (HMG) or clomiphene citrate-HMG. *Hum. Reprod.*, **11**, 2097–2106.
- Van der Elst, J., Van den Abbeel, E., Vitrier, S., Camus, M., Devroey, P. and Van Steirteghem, A.C. (1997) Selective transfer of cryopreserved human embryos with further cleavage after thawing increases delivery and implantation rates. *Hum. Reprod.*, **12**, 1513–1521.
- Veck, L.L., Amundson, C.H., Brothman, L.J., De Scisciolo, C., Maloney, M.K., Muasher, S.J. and Jones, H.W., Jr (1993) Significantly enhanced pregnancy rates per cycle through cryopreservation and thaw of pronuclear stage oocytes. *Fertil. Steril.*, **59**, 1202–1207.
- Wang, J.X., Yap, Y.Y. and Matthews, C.D. (2001) Frozen-thawed embryo transfer: influence of clinical factors on implantation rate and risk of multiple conception. *Hum. Reprod.*, **16**, 2316–2319.

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