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Prognostic factors for the success rate of embryo freezing

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To find some prognostic factors for the outcome of frozen–thawed cycles, we have retrospectively analysed all frozen pre-embryos that were thawed during 1993 and 1994 at two in-vitro fertilization (IVF) units in Sweden. Supernumerary pre-embryos were frozen from 551 oocyte retrievals and these resulted in 660 frozen–thawed cycles which lead to 623 thawed embryo transfers. The outcome of these transfers was 137 clinical pregnancies with a pregnancy rate of 22% per frozen–thawed embryo transfers. Women <40 years of age had a higher birth rate than those 40 years, 19 and 5% respectively ($P < 0.01$). Transfers with two and three pre-embryos resulted in pregnancy rates of 23 and 27%, respectively, compared with 14% for transfer of one embryo. A pregnancy resulting from the initial embryo transfers had a predictive value for results of the subsequent frozen–thawed cycle. Embryo grade and cleavage stage at the time of freezing was important for the survival of the frozen–thawed pre-embryos. The pregnancy rate was not influenced by the cleavage stage, but a tendency toward a lower pregnancy rate was seen for the embryos with lower grading. To conclude, cryopreservation seems to be beneficial in women <40 years of age, who have supernumerary pre-embryos of good quality for freezing and of which at least two can be transferred.

Key words: cell stage/cryopreservation/embryo quality/in-vitro fertilization/pregnancy rate

Introduction

Cryopreserved embryos have been used in assisted reproductive technique programmes for achieving pregnancies for nearly 15 years (Trounson and Mohr, 1983). However, the pregnancy rate is surprisingly low compared to fresh embryos (American Society for Reproductive Medicine, 1995). This may depend on several factors, such as the fresh embryo quality, the woman's age, the cause of infertility and the protocols for embryo freezing. Nevertheless, freezing supernumerary pre-embryos is important since it simplifies the subsequent cycles and lowers the costs per birth. Moreover, the increased availability of fresh pre-embryos has increased due to modern ovarian stimulation protocols using gonadotrophin-releasing hormone agonists (GnRHa) (Hughes *et al.*, 1992), much improved culture media and the fact that fewer pre-embryos are transferred to avoid multiple pregnancies. However, in a recent study of ovarian stimulation with a long protocol, GnRHa had a negative effect on the survival and implantation rate for the frozen–thawed embryos (Van der Elst *et al.*, 1996). Therefore, cryopreservation has become essential and now it is necessary to find the best prognostic factors for selecting embryos for freezing.

Such factors are the morphology (embryo quality) (Scott *et al.*, 1991; Schalkoff *et al.*, 1993), the number of blastomeres (Lassalle *et al.*, 1985; Testart *et al.*, 1986), the outcome of the fresh embryo transfer cycle (Toner *et al.*, 1991; Lin *et al.*, 1995), and the day of freezing (Testart *et al.*, 1986; Cohen *et al.*, 1988).

The in-vitro fertilization (IVF) cycles resulting in embryo freezing vary between laboratories (American Society for Reproductive Medicine, 1995). The reason for this is not clear but it may be due to differences in the acceptance of the embryo quality suitable for freezing. Since the cost for a frozen–thawed cycle is approximatley one-third of that for a fresh IVF cycle in Sweden, a lower pregnancy rate can be accepted economically. By studying IVF units with different criteria of embryo grading for freezing, it is possible to analyse retrospectively embryos with a wide range of quality at the time of freezing. Consequently it should be possible to find the minimal requirements for obtaining a pregnancy rate that gives a cost per birth comparable with that for fresh embryo transfers.

The objective of the study was to analyse which pre-embryo quality and cleavage stage could be of predictive value for the survival of the pre-embryos and for increasing the pregnancy rate in the frozen–thawed cycles. Furthermore it should be determined whether the outcome of the fresh embryo transfer cycles has an influence on the results of the subsequent frozen–thawed cycles.

Materials and methods

A retrospective analysis was made of the 660 IVF cycles performed with frozen–thawed embryos from 551 couples during the period from January 1, 1993 to December 31, 1994. Of these cycles, 180 were carried out at Carl von Linné Kliniken Uppsala (Clinic I) and 480 at Fertilitetscentrum (Clinic II), Göteborg, Sweden. At Clinic I, embryo freezing was done in 30% of the cycles, whereas at Clinic II the corresponding figure was 50%, due to an acceptance of lower embryo quality for freezing. The grading of the embryo quality, as descibed below, did not differ between technicians and the clinics. Other parameters such as patient characteristics, stimulation protocol

and culturing conditions did not differ between the clinics. The median age of the women treated was 34 years (range 21–45) and the indications for the IVF cycles were tubal factor (n = 322), unexplained infertility (n = 112), endometriosis (n = 46), male factor (n = 141), ovulatory disorders (n = 7), mixed (n = 26), cervical factor (n = 2), unknown (n = 4). For the cycles from which the supernumerary pre-embryos were yielded, only long protocol with GnRHa in combination with human menopausal gonadotrophins (HMG) or follicle stimulating hormone (FSH) were used.

In women with regular menstrual periods, the thawed pre-embryos were replaced in spontaneous cycles (n = 456). Women with irregular or anovulatory cycles, or women who had difficulties in detecting the luteinizing hormone (LH) surge with a self-monitoring urinary test despite regular cycles, had the transfer of thawed pre-embryos in a stimulated cycle. The following stimulation protocols were used in the relevant transfer cycles: clomiphene citrate (n = 21), gonadotrophins (n = 49), GnRHa combined with gonadotrophins (n = 68) and oestrogen/progesterone (n = 29). In spontaneous and clomiphene cycles, the LH surge was identified by urinary or serum LH measurements. The times for embryo thawing and embryo transfer were determined according to calculations described by Cohen *et al.* (1988). In the cycles stimulated with gonadotrophins, with or without GnRHa, embryo transfers were performed 4 days after the administration of human chorionic gonadotrophin (HCG).

Pre-embryos were graded before freezing as follows: grade I = even blastomeres without any fragmentation, grade II = blastomeres of equal or unequal size and minor (<25% of the blastomere's surface) cytoplasmic fragments or blebs, grade III = blastomeres of equal or unequal size and medium cytoplasmic fragments (25–50% of the blastomere surface), grade IV = blastomeres of unequal size and major cytoplasmic fragments (>50% of blastomere volume). Grading of the pre-embyos was performed similarly at both clinics. Blind grading of the pre-embryos was done by the technicians to check uniform classification.

A total of 2162 pre-embryos was frozen, of which 2135 were graded according to the description above. We investigated the relationship between embryo grading and pregnancy rate. In this analysis transfers involving one embryo, and transfers in women >40 years were excluded due to the already well-known poor outcome for these groups (Schalkoff *et al.*, 1993; Lin *et al.*, 1995). Moreover, to find whether there was a relationship between cleavage stage at the time of freezing and pregnancy rate of the thawing cycle, only transfers with at least two pre-embryos with good quality (grade I and/or II) were considered in this study.

Pre-embryo freezing and thawing utilized 1,2-propanediol and sucrose, according to methods described by Testart *et al.* (1986). The goal was to transfer up to three embryos when available. Only the pre-embryos with two blastomeres and where ⩾50% of the blastomeres had survived were transferred. In a few cycles (n = 17), embryos with only one normal blastomere were transferred according to patient wishes. These cycles were excluded in the presentation and analysis of the results except in Table I. Up to a maximum of three thawed pre-embryos was transferred using the same technique as for fresh embryos, and clinical pregnancies were verified by ultrasound.

Differences in pregnancy rates were analysed by χ^2-test or Fisher's exact test. When necessary, contingency tables with analysis of trends in proportion were used.

Results

The overall clinical results are described in Table I. Of the frozen–thawed cycles, 94% resulted in transfers. Excluding those from transfers involving pre-embryos with only one blastomere (n = 17), women ⩾40 years (63 cycles) had a pregnancy and birth rate/embryo transfer of 11 and 5% respectively. The corresponding figures for women <40 years (543 cycles) were 24 and 19%, respectively ($P < 0.01$). A similar pregancy rate was obtained in spontaneous and stimulated cycles: 24% (106/444) and 19% (31/162) respectively.

Table I. Clinical results (including 17 transfers involving pre-embryos with only one blastomere)

No. of patients	551
No. of frozen–thawed cycles	660
No. of embryo transfers	623
No. of clinical pregnancies	137
per started cycle	21%
per embryo transfer	22%
No. of deliveries	128
per started cycle	19%
per embryo transfer	21%

Table II. The distribution of pre-embryos according to grading at the time of freezing and survival rate

Grade	No. of pre-embryos frozen	No. of pre-embryos surviving (⩾2 cells)	Survival rate (%)
I	717	572	80
II	1222	867	71
III	185	105	57
IV	11	5	45

Survival rate with test of trends in proportions ($P < 0.001$).

Again excluding results from one-blastomere pre-embryos, the pregnancy rates for the cycles with one, two or three frozen–thawed pre-embryos transferred were 14% (15/105), 23% (64/283) and 27% (58/218), respectively. A higher pregnancy rate was obtained when three embryos were transferred compared to one embryo ($P < 0.05$), but there was no difference between two and one embryo transfers ($P = 0.07$).

Of the women who became pregnant after a prior fresh embryo transfer, a pregnancy rate of 29% (33/112) was obtained in the subsequent freeze–thaw cycle. The corresponding figures for those who did not conceive after the fresh embryo transfers was lower, 21% (100/486) ($P < 0.05$). Six oocyte retrievals, from which all the fresh pre-embryos were frozen because of the risk of developing ovarian hyperstimulation syndrome, resulted in eight frozen–thawed transfers after which four pregnancies were obtained.

The survival rates for the different grading categories are presented in Table II. There was a significant decreasing trend in survival rate with increasing grading at the time for freezing ($P < 0.001$).

The graded embryo quality, as related to the pregnancy rate, is presented in Table III. In cycles when at least two embryos were transferred of which the two best embryos were of grades I and I, I and II or II and II, a similar pregnancy rate was achieved. Compared to this, pregnancy rates were lower in cycles where the two best embryos were of grades II and III or III and III, although the difference was not significant.

The survival rates for the different cleavage stages are presented in Table IV. Pre-embryos with an even number of

Table III. Pregnancy rate (pregnancies/cycles) according to embryo grade. Only cycles with transfer of at least two embryos, of which both were graded, are included. The grading presented refers to the two best pre-embryos

Grade	Pregnancy rate (%)
I and I	28 (44/157)
I and II	21 (24/116)
I and III	50 (1/2)
II and II	26 (49/190)
II and III	11 (2/18)
III and III	13 (1/8)

The values in parentheses are pregnancies/frozen−thawed embryo transfer.

Table IV. Survival rate related to cleavage stage at the time of freezing

	Cleavage stage (no. of cells)					
	2	3	4	5	6–7	8
No. of embryos frozen	573	233	1192	115	45	4
No. of embryos survived	414	153	901	62	31	3
Survival rate (%)	72	66	76	54	69	75

Survival rate for 2, 4 and 8 cells vs 3, 5, 6 and 7 cells: $P < 0.0001$.

Table V. Pregnancy rate according to the number of blastomeres at freezing when at least two embryos of good quality (grade I and/or II) with equal number of blastomeres were transferred (1993 and 1994)

No. of blastomeres	No. of cycles	No. of pregnancies	Pregnancy rate (%)
2	27	7	26
3	7	2	29
4	174	45	26
5	3	2	66
6	1	0	0

blastomeres had higher survival rates as compared to those with an uneven number of blastomeres ($P < 0.001$).

The cleavage stage at the time of freezing for those embryos with good quality (grades I and II) that survived had no influence on the outcome (Table V). When only pre-embryos with one blastomere were transfered no pregnancy occurred ($n = 17$).

Discussion

To date, studies that have dealt with cryopreservation in assisted reproduction treatment cycles have included rather moderate numbers of cycles, making it difficult to draw definite conclusions about prognostic factors. Therefore, our investigation of 660 cycles allows a more thorough statistical evaluation of prognostic factors.

In our study, pregnancy and birth rates per thawed embryo transfer of 22 and 21% were obtained (Table I), and these are similar to the rates reported by others (Toner *et al.*, 1991; Lin *et al.*, 1995; Kondo *et al.*, 1996). However, in contrast to our results, Van der Elst *et al.*, (1996) found a much lower pregnancy rate with frozen embryos originating from GnRH–HMG protocols. The reason for this discrepancy is difficult to explain. Although a birth rate of 21% after transferring thawed embryos is lower than that for fresh embryos, this outcome after cryopreservation is acceptable since the costs are also much lower than for fresh IVF–embryo transfer.

Maternal age seemed also to be one of the most important prognostic factors for the pregnancy outcome of cryopreservation cycles, which has also been shown by Schalkoff *et al.* (1993). This is also in agreement with results obtained with fresh embryo tranfers (Piette *et al.*, 1990). Thus, due to the low birth rate (5%), cryopreservation of pre-embryos from women >40 years seems not to be cost-effective.

The outcome, with regard to pregnancy rate, of the fresh cycles in our study seemed to be of predictive value for the outcome of subsequent frozen–thawed cycles. This was shown previously by Toner *et al.* (1991) and Lin *et al.* (1995). However, in the study by Toner *et al.* (1991) the only significant difference found was in implantation rates, whereas in the study by Lin *et al.* (1995) a threefold difference in pregnancy rate was found. Despite the fact that our results showed a significantly lower pregnancy rate in the thawed cycle for the group who did not conceive in the fresh cycle, it is an acceptable rate when considering the simplicity and low costs of frozen–thawed cycles. Such a conclusion is perhaps not applicable to the results found in the study by Lin *et al.* (1995) where the pregnancy rate in the same group was only 8.5%.

The importance of morphological grading of pre-embryos at cryopreservation for their survival has, to the best of our knowledge, never been investigated in such a large number of embryos ($n = 2135$) as in this study. Grades I and II of the pre-embryos were associated with a higher survival rate than those embryos with lower grading (III and IV). In a small study ($n = 193$) by Testart *et al.* (1987), the same tendency was seen. Other investigaters have only related grading to pregnancy rate (Cohen *et al.*, 1988; Schalkoff *et al.*, 1993; Kondo *et al.*, 1996). This was also supported in our study where we found a tendency towards a decreased pregnancy rate with lower grading (Table III). Thus, grading at the time of freezing seems to be an important prognostic factor for the survival of the pre-embryos and probably also for pregnancy rate after thawed transfers. Therefore, our suggestion is to freeze only pre-embryos of good quality (grades I and II).

Our study clearly indicates that development stage (number of cells) at the time for freezing is of importance for survival at thawing (Table IV). Pre-embryos at an exponential stage (2-, 4-, 8-cell) survived better than those at an intermediate cleavage stage (3-, 5-, 6-, 7-cell), an observation also made by Lassalle *et al.* (1985) and Testart *et al.* (1987), who speculated that this difference might be related to the mitotic rest and membrane stability of blastomeres. Despite there being a difference in pre-embryo survival rate between different development stages, an acceptably high rate of survival was achieved for the intermediate cleavage stages to justify freezing.

The relationship between cell stage and pregnancy rate has rarely been investigated. In a study including 76 one-embryo transfers by Testart *et al.* (1987), a higher pregnancy rate was obtained for pre-embryos frozen at the 4-cell stage than for all other stages combined. In a study by Cohen *et al.* (1988),

a tendency towards a lower pregnancy rate was found after transferring thawed embryos that had been frozen at the 2-cell stage compared to other stages. In contrast to this, our study showed that the cleavage stage had no influence on pregnancy rate when only two or three embryos of good quality were transferred. The reason for this selection of transfers was to minimize the influence of embryo grading and the negative effect of only one-embryo transfer on the results. Although the survival rates were statistically lower for intermediate cell stages, no difference in pregnancy rate could be discerned. Therefore, our opinion is that all embryos of good quality should be frozen irrespective of the cell stage.

In conclusion, our analysis of 660 frozen–thawed cycles showed that the prognostic factors of importance to a good outcome from transferring supernumerary frozen/thawed embryos were maternal age, the outcome of prior fresh IVF cycle(s), the number of thawed embryos transferred and the morphological grade of the embryos.

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