

Factors Affecting Success in an Embryo Cryopreservation Programme

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Abstract

*Embryo cryopreservation is a vital part of any assisted conception programme. We analysed our experience with respect to various clinical factors which may influence success and help modify our own practice. Factors studied were endometrial preparation, number of embryos transferred, woman's age at embryo freezing and endometrial thickness. The 212 cycles analysed represented all our frozen embryo-transfers from 1994 to 1996. Four cycles were excluded because of incomplete data. Statistical analysis was done with Student's *i*-test, Fisher's exact test and chi-square test for trend.*

The clinical pregnancy rate rose yearly and it was 12.3% per transfer in 1996. The most important clinical factor appeared to be the type of endometrial preparation. Natural cycles resulted in a pregnancy rate of 17.7%, almost twice hormone-replacement cycles. When transfer was on days 14 to 16 of the natural cycle, the pregnancy rate reached 33%. Other factors that were suggestive of success were younger age at embryo freezing, transferring at least 2 embryos and endometrium thickness ≥ 1 mm. The natural cycle gave the best pregnancy rate in our hands and is our method of choice for ovulatory women with normal cycle lengths. For all other women, hormonal preparation is needed but our protocols need refinement. Our initial performance is encouraging and embryo cryopreservation has certainly enhanced our overall success rate.

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Key words: *Frozen embryo, Hormone-Replacement, In vitro fertilization, Natural cycle, Pregnancy*

Introduction

The first pregnancy resulting from the thaw and transfer of cryopreserved pre-embryos was reported in 1983 by Trounson¹ and his team, in Melbourne, Australia. This technological breakthrough in infertility treatment has been successfully repeated since then and cryopreservation of excess embryos now forms an integral part of any assisted reproductive programme. By providing the possibility for a further embryo transfer, it increases the cumulative pregnancy rate and reduces cost to the patient as additional transfer cycles are produced without having to repeat ovarian stimulation.^{2,3} In addition, it may reduce the risks of high-order multiple births by allowing the clinician to transfer only a few embryos in the fresh cycle and freezing the rest of the embryos for future chances.

Frozen/ thawed embryos have been replaced in a natural cycle in ovulatory women. The success of these cycles depends upon precise synchronisation. The timing of the embryo thawing must match the stage of the endometrial development to the age of the *in vitro*-developed embryo. Five to six per cent of natural cycles are cancelled because of poor endometrial development in

patients with regular menstrual cycles.⁴ The high incidence of ovulatory dysfunction amongst IVF and GIFT patients also defines a population for whom the natural cycle approach cannot be used. For these patients, a hormonally controlled cycle using exogenous steroids to prepare the endometrium is required. Other factors that have been reported to influence success include the number of embryos transferred, endometrial thickness and characteristics on ultrasound scanning, age of the women at freezing and laboratory factors.

To this end, a retrospective study was undertaken to ascertain which clinical factors influenced success for frozen embryo transfers as determined by the initial three years of our experience, and to suggest proposals for improvement.

Materials and Methods

From January 1994 to December 1996, 216 frozen embryo transfers (FET) were performed at KKIVF, Department of Reproductive Medicine, KK Women's and Children's Hospital, Singapore. Strict criteria were observed to freeze suitable embryos and also in determining whether the embryos were viable for transfer

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after the thawing procedure. During the study period, 20 (8.5%) cycles were cancelled because of failure of all embryos to survive the freeze/thaw procedure. These were part of the initial 236 cycles that started off. Four case-records were either incomplete or not available and thus excluded from the study. The remaining 212 cycles analysed represented 33% of all our fresh and frozen assisted conception cycles for the study period.

Cryopreservation and Thawing Method

A "slow-cooling" method using Propanediol⁵ and a computerised program of freezing (Programmable Planner KRYO 10 Series II Controlled Rate) was used. Only suitable embryos were frozen and they had to be generally uniform in appearance with less than 30% fragmentation (or between grades 1 to 5, on a scale of 9 with 9 being the worst grade and 1 being the best grade), and in early cleavage stages (day 2 after oocyte retrieval). The embryos were stored in batches of one, two or three per straw.

A "rapid-thaw" method using sucrose solution and propanediol was used.⁵ After thawing, the embryos were placed in pre-equilibrated culture media and checked for intactness of blastomeres. Only embryos with more than 50% of their blastomeres intact were deemed to have survived and allowed for embryo transfer. Embryo transfers were done by a small group of 4 clinicians.

Regimen

Women who were ovulatory had FET during natural cycles (51 cycles, 24.1%). Women with anovulation and polycystic ovaries were placed in the remaining group given hormone replacement therapy (HRT). Age was not a factor in selection for either type of cycle.

Monitoring of natural cycles was started 10 days after the onset of menstruation via transvaginal ultrasound assessment of follicular growth. Subsequent scans were at 2 to 3 days intervals to identify the dominant follicle. When the diameter of the dominant follicle reached >15 mm, daily morning urinary LH testing with a commercially available kit (Clear Plan[®]) was started. Once ovulation was confirmed by the LH surge and vaginal ultrasonography (endometrial thickness also measured), FET was planned for 3 days after the LH surge.

All patients on the HRT cycles received daily estradiol valerate (Progynova) from the 4th day of their menstrual period. The initial dosage was 2 mg/day for five days, followed by 4 mg/day for another five days. Transvaginal ultrasound assessment of endometrial thickness was started on the 10th day of estradiol valerate treatment. The dosage of estradiol valerate was increased by 2 mg/day, every 5 to 7 days, until the endometrial assessment showed adequate thickness. The uterus was visualised in the longitudinal plane showing the depth of the

uterus. The maximum endometrial thickness between the echogenic interfaces of the myometrium and endometrium, perpendicular to the axis of the uterus was measured twice and averaged. All the scans were performed with a 5mH₂ vaginal transducer by a team of 3 registrars. When the endometrial thickness reached at least 8 mm, progesterone suppositories, 400 mg every 12 hours was started and FET scheduled for 2 days later. No cycles were cancelled because of poor endometrial development and FET proceeded on when there was no further change in the endometrial growth despite prolonged steroid treatment in a few of the cycles.

Embryo Transfer

A maximum of three embryos was thawed initially. If only one embryo survived the procedure and there were more embryos available for thawing, these were thawed in order to be able to transfer 2 or 3 embryos. If there was only one embryo frozen, this was thawed and if it survived, transferred in a FET cycle before going on to any more stimulated cycles (our department policy at this time).

Luteal Phase

The luteal phase was supported with twice daily 400 mg progesterone pessaries for both types of cycles. In HRT cycles, a fixed daily dose of 4 mg estradiol valerate was continued, from the day of FET. Serum β -hCG was performed between 16 and 18 days after embryo transfer. Clinical pregnancy was confirmed by presence of a gestational sac on vaginal ultrasonography 4 to 5 weeks after ET. All pregnant patients continued on hormonal support as in their luteal phase until at least 12 weeks gestation. Ongoing pregnancy and delivery information was obtained directly from the hospital birth records. For statistical analysis, two-sample t-test, Fisher's exact test and chi-square analysis were used as necessary. A *P* value of <0.05 was considered as statistically significant.

Results

Of all the 212 completed FET cycles, 26 (12.3%) achieved pregnancy. Three of these (11.5%) were biochemical pregnancies and are excluded from the final analysis. Eight (30.8%) were miscarriages, one (3.8%) was an ectopic pregnancy and 14 (53.8%) delivered live-born babies. This resulted in a 10.8% clinical pregnancy rate and 6.6% overall live birth rate.

Table I shows the pregnancy outcome by the type of cycle. There were 9 (17.7%) clinical pregnancies in the group with natural cycle FET. In the HRT group, 14 (8.7%) clinical pregnancies were achieved. The pregnancy rate for the natural cycle group was thus almost twice that of the HRT group. There was also a lower live birth rate (*P* = 0.002) and higher pregnancy loss rate (*P* = 0.04) in the HRT cycle group.

TABLE I: COMPARISON BETWEEN NATURAL AND HORMONE REPLACEMENT THERAPY (HRT) CYCLES

Variable	Natural cycle	HRT Cycle	P value *
Number of cycles	51	161	
Age at freezing†(y)	32.9 ± 0.51	33.5 ± 0.30	ns
Embryos per transfer?	2.4 ± 0.09	2.5 ± 0.05	ns
Cycle day of transfer?	18.3 ± 0.43	18.6 ± 0.31	ns
Endometrial thickness?	10.4 ± 0.29	9.4 ± 0.12	s
Clinical pregnancy rate	17.7% (9/51)	8.7% (14/161)	ns
Live birth rate	15.6% (8/51)	3.7% (6/161)	s
Pregnancy loss rate	11.1% (1/9)	57.1% (8/14)	s

ns: not significant; s: significant

* Significance assumed at $P < 0.05$

† Values given as mean ± SEM (standard error of mean)

Comparison between the natural cycle and the HRT group showed that the two groups were similar with respect to age at freezing, number of embryos transferred and cycle day of FET. We also looked at the influence of the woman's age (at the time of embryo freezing). As expected, embryo freezing at a younger age was associated with pregnancy success (Fig. 1) but this trend did not reach significance.

Cycles with endometrial thickness greater than 10 mm had the highest pregnancy rate of 15.5% (Fig. 2). The success rate in this group was more than twice that for the group with the thinnest endometrium of less than 8 mm. However, this difference did not reach statistical significance.

With regard to the timing of the embryo transfer, we assumed the first day of the menstrual cycle as day 1 for the natural cycle group. Table II shows that the best results were obtained when FET was on day 14 to 16 of the cycle ($P = 0.019$). When ovulation was delayed and FET was correspondingly later in the cycle, the chances of pregnancy decreased, with no pregnancies when FET was on day 20 or later ($P = 0.042$).

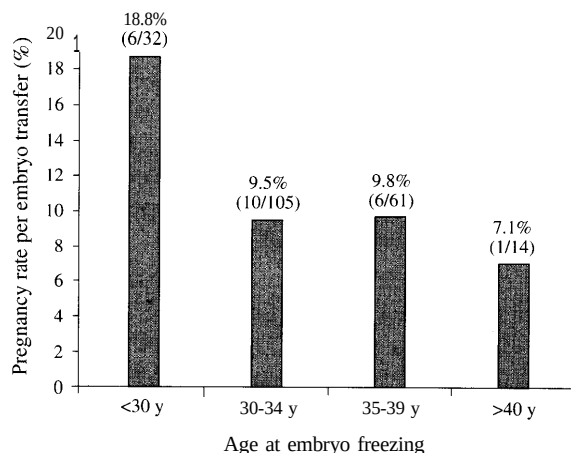


Fig. 1. Pregnancy rate in relation to age at freezing.

For HRT cycles, day 1 was arbitrarily taken as the day of starting steroid therapy. There was no "best" timing for the FET and this would be expected because our department policy was to continue HRT until an adequate endometrial thickness was obtained.

In Table III, 113 cycles (53.3%) had three embryos transferred whilst 77 (36.3%) had two embryos transferred. In 21 cycles (9.9%), only one surviving embryo was available for transfer. The pregnancy rate was best when at least 2 or 3 embryos were available for transfer. The only pregnancy that occurred in the group with one-embryo transfers, turned out to be a biochemical pregnancy and was thus not included.

TABLE II: PREGNANCY RATE BY CYCLE DAY OF EMBRYO TRANSFER

Day of ET	Natural cycle		HRT cycle	
	n	Pregnancy rate*	n	Pregnancy rate†
Id13	0		70	8.6%
d14 – d16	18	33.3%	34	8.8%
d17 – d19	17	17.6%	31	9.7%
≥d20	16	0.0%	26	7.7%
Total	51	17.7%	161	8.7%

ET: embryo transfer; HRT: hormone replacement therapy

* chi-square test for trend for natural cycles showed $P = 0.01$ (significant)† chi-square test for trend for HRT cycles showed $P =$ not significant

TABLE III: PREGNANCY RATE BY NUMBER OF EMBRYOS PER EMBRYO TRANSFER (ET)

No. of embryos per ET	n	Pregnancy rate	P value
1	21	0.0%	ns
2	77	13.0%	ns
3	113	11.5%	ns
4	1	0.0%	ns

ET: embryo transfer; ns: not significant

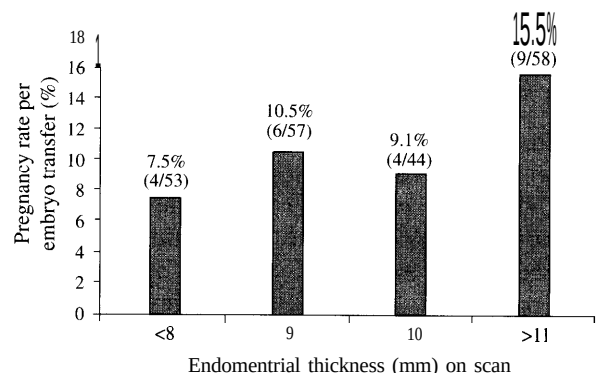


Fig. 2. Pregnancy rate in relation to endometrium thickness.

Table IV shows our centre's performance over the three consecutive years. If "one-embryo" transfers were to be taken as "suboptimal" cycles, in the light of the above data, the proportion of so-called "suboptimal" cycles decreased significantly over the study period. This improvement in performance was accompanied by an improving pregnancy rate, live birth rate and decreasing pregnancy losses.

TABLE IV: CLINICAL PERFORMANCE BY YEAR OF EMBRYO TRANSFER

Year	n	Suboptimal cycle*	Clinical pregnancy rate†	Live birth rate†	Pregnancy losses†
1994	26	27.0%	7.7%	3.8%	50.0%
1995	80	11.1%	10.0%	6.3%	37.5%
1996	106	3.8%	12.3%	7.5%	38.5%

* chi-square test for trend showed $P = 0.004$ (significant)

† chi-square test for trend showed $P =$ not significant

Discussion

Various attempts have been made to improve the success of frozen embryo transfers (FET) since the first successful pregnancy in 1983. Our specific aim was to try to identify any factor(s) which may influence the success of our embryo cryopreservation programme, and thus make proposals for improvements.

In our study, the type of endometrial preparation was a factor influencing success. The natural cycle group had almost twice the pregnancy rate of the HRT group, with significantly better live birth rate and lower pregnancy losses. In contrast, several other studies had similar pregnancy rates with both types of cycles.^{2,4,6} Our results also showed that the most optimal period for performing the embryo transfer in the natural cycle group was on days 14 to 16. In 31% of all the natural cycles, embryo transfer was performed later than day 19, and there were no pregnancies at all in this group. This implied that the retarded follicular growth and hence, retarded endometrium changes, may be detrimental for implantation. Successful outcome is also dependent on accurate synchronisation of the cycle. We used a combination of serial urinary LH tests and ultrasound assessments. From our results, we feel that this method is sufficiently accurate and yet not too cumbersome or invasive.

In the HRT group, there was no particular factor which predisposed to a favourable outcome. We need to improve our method of preparing the endometrium and this may include trials of rendering the women agonadal with a gonadotrophin-releasing-hormone agonist,⁷ further adjusting of the estradiol doses, using transdermal estradiol patches and so forth before finding an optimal regimen for our centre.

At the San Fernando Valley Fertility and Reproductive Centre in California, two general approaches are used to achieve good pregnancy rates from frozen embryo cycles, either a natural thaw cycle or a programmed one with hormonal replacement with ovarian suppression. Quinn et al⁸ observed that their pregnancy rates were similar after transfer of thawed embryos whether the embryos were transferred in a natural menstrual cycle or one that has been regulated with gonadotrophin-releasing hormone analogue (GnRHa) suppression and oestrogen/progesterone replacement. However this applied only when the patients had regular menstrual cycles with no other abnormalities. However, they found that patients with oligomenorrhoea or a luteal phase defect, had performed better using the programmed cycle. They used leuprolide acetate from midluteal phase to achieve ovarian suppression. This was followed by oral estradiol in increasing doses for two weeks, whereby intramuscular progesterone was added and embryo transfer timed for two days later.

Frederick of University of California Irvine Medical Centre⁹ also used a hormone replacement cycle similar to ours (without gonadotrophin-releasing hormone analogue suppression) but continued steroid replacement until the endometrium reached ≥ 10 mm thickness with a triple layer, before they begun intramuscular progesterone injection. In their study group of 54 patients, they achieved a very high pregnancy rate of 41% with an average of 4.4 embryos transferred per cycle. It is important to note that this researcher did not mention how many cycles were cancelled when they did not achieve the optimum endometrium criteria as above. Also, they transferred a much higher average number of embryos as compared to us (the limit in Singapore being 3).

The group in Norfolk used all three types of replacement protocol-natural cycles, HRT with GnRHa, HRT without GnRHa-and achieved similar pregnancy rates for all three protocols. However, for their HRT programme, they were different from us in that they used transdermal oestrogen patches and they reduced the dosage of oestrogen from two days before the embryo transfer. In addition, they used daily intramuscular progesterone right until the first trimester. The disadvantage of using intramuscular injections is of course the cost, pain and time needed by the patient to go for daily injections, especially if they conceive as the treatment needs to be continued until the corpus luteum reaches autonomy.

Yee et al¹⁰ also compared a "simple" artificial hormone replacement regimen with two other protocols that incorporate GnRHa-pituitary downregulation: leuprolide with transdermal estrogen patches and leuprolide with oral micronized estradiol. In their "simple" HRT regimen, they only utilised oral micronized estradiol followed by progesterone replacement. Their clinical

pregnancy rates were 13.7% and 11.4% for the two protocols using GnRHa and 13.5% for the “simple” HRT regimen. They concluded that the simple sequential HRT regimen resulted in an endometrium suitable for frozen embryo transfers and that pituitary downregulation was not necessary. A French group¹¹ also concurred with Yee’s findings¹⁰ that controlled preparation of the endometrium can be achieved with exogenous estradiol and progesterone without prior GnRHa in women with functioning ovaries. This is not only a viable simpler regimen but avoids the cost and side effects of GnRHa. Hence, every centre seems to have a different approach to optimising their HRT cycles. We concur with Yee’s group¹⁰ from California that a simple programme would be most acceptable to patients provided it gives a satisfactory pregnancy rate. As such, for women who have long but ovulatory cycles, we will now consider the possibility of either adjusting the doses used in their HRT programmed cycle, inducing ovulation with small doses of Clomiphene citrate or perhaps, carrying out trials with the various preparations of oestrogen and progesterone.

It is well-known that age of the women, or rather, age of the oocytes do influence the results of fresh stimulated IVF cycles.¹² However, there is limited information available on the effect of increasing age on frozen-thawed cycles, as older women tend to have fewer embryos available for freezing and thus, fewer FET cycles. A number of researchers have shown a decline in FET pregnancy rates for women aged 40 years and older at the time of freezing.^{3,6} This would not be surprising given the fact that older oocytes may be of poorer quality. In our study, age below 30 years at time of freezing, gave the highest pregnancy rate while age 40 years and above had the lowest. However, the difference was not statistically significant due to the small number of cycles in these groups.

There is also some uncertainty as to the effect of increasing the embryo numbers per transfer. Some researchers have shown that the pregnancy rate was significantly better when 3 or more embryos were transferred as compared to when fewer embryos were available.^{3,6,13} On the other hand, Sathanandan et al⁴ and Schalkoff et al¹⁴ did not find any difference in the pregnancy rates. Schalkoff proposed that this was especially the case when the patient had a “good-quality” or “sponsoring” embryo. We did not look at the embryo grade in this study. Our results support transferring at least 2 embryos that have “survived” the freeze-thaw procedure. We feel that it is important to note that none of the 21 cycles in the one-embryo group reached clinical pregnancy.

There have been many reports on the value of ultrasound assessment of the endometrium in assisted conception programmes. There is, however, controversy

regarding the degree of correlation between endometrium thickness and pregnancy. No association was noted by some researchers^{15,16} and others^{17,18} found a positive correlation. Al-Shawaf et al² and Abdalla et al¹⁹ were among the workers who found a decline in pregnancy rate when the endometrium was less than 8 mm in thickness. In our study, endometrial thickness correlated positively with pregnancy. The pregnancy rate of the group with the thinnest endometrium was half that of those with endometrium greater than 10 mm.

The period 1994 to 1996 represented our early experience, our centre having begun in March 1993. Thus, we also wondered whether the outcome had any relationship to the year of embryo transfer. There were increasing numbers of FET cycles performed, particularly those with 2 or more surviving embryos, and increase in pregnancy and live birth rates in 1996. Obviously, many factors could have accounted for the improvements. We would like to think that it was, in part, due to increasing clinical experience and laboratory skills, resulting in better oocyte and hence, embryo quality.

A look at a few other countries²⁰ which also set a 3-embryo limit on their embryo transfers in 1995, showed that our performance was not far off (Table V) and this is particularly encouraging. It was also encouraging to note that a third of all our embryo transfers were from the cryopreservation programme, an indirect reflection on the quality of our stimulated IVF programme, i.e. our stimulated IVF cycles were producing “quality” embryos that could survive the freeze-thaw procedure.

TABLE V: PREGNANCY OUTCOMES FOR EMBRYO CRYOPRESERVATION PROGRAMMES IN SELECTED COUNTRIES WHICH SET A 3-EMBRYO LIMIT²⁰

Country	No. of ART centres	Clinical pregnancy rate (per transfer)
Australia & New Zealand	29	14.5%
Belgium	8	9.7%
Germany	26	12.8%
Switzerland	7	19.3%
United Kingdom	69	14.9%
World Average	291	16.0%

ART: assisted reproductive technology

Conclusion

Embryo cryopreservation offers a significant advantage in assisted reproduction programmes. Embryos, which survive the freeze-thaw procedure, demonstrate satisfactory potential for pregnancy in our centre. With proper selection of patients, transfer in a natural cycle, especially on days 14 to 16, gave the highest pregnancy rate. In natural cycles, when ovulation occurred late (FET on day 20 or beyond) or when endometrial thickness was poor (≤ 8 mm), abandoning the cycle may need

to be considered. The overall outcome for HRT cycles in our study was poorer and we need to improve the uterine receptivity in this group. Transfer of at least 2 embryos was a positive factor. Our initial experience in embryo cryopreservation is very encouraging with yearly improvements in both the number of "optimal" cycles done and pregnancy success.

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