

# The presence of a sponsoring embryo in a batch of poor quality thawed embryos significantly increases pregnancy and implantation rate\*

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**Objective:** To evaluate quantitatively the effect of one good-quality (sponsoring) embryo in a batch of low-quality thawed embryos on the implantation and pregnancy rates (PR).

**Design:** Retrospective analysis of data.

**Setting:** Tertiary care center IVF clinic affiliated with a university medical school.

**Patient(s):** Between March 1988 and April 1995, 392 IVF patients underwent a total of 440 thawing and ET cycles of 1,436 multicellular embryos.

**Main Outcome Measure(s):** Implantation, clinical pregnancy, and multiple pregnancy rates.

**Result(s):** In the absence of sponsoring embryos in the thawed batch of embryos, a PR of 9.8% with an implantation rate of 3.1% was achieved. In the presence of a single sponsoring embryo, the PR nearly doubled (18.2%), with a significantly higher implantation rate of 7.0%. Only singleton pregnancies were achieved in the absence of sponsoring embryos compared with 21.7% multiple pregnancies in the single sponsoring embryo group.

**Conclusion(s):** The presence of a sponsoring embryo in a batch of poor quality thawed embryos is an important factor that significantly increases pregnancy and implantation rates. The optimal strategy for planning batches of multicellular frozen embryos is to include at least one sponsoring embryo in each batch when possible. We speculate that the sponsoring embryo may favorably influence the chances of low-quality embryos to undergo successful implantation. (Fertil Steril® 1997;67:711–6. © 1997 by American Society for Reproductive Medicine.)

**Key Words:** Cryopreservation, human embryos, sponsoring embryo, IVF

Various factors have been found to affect the outcome of human embryo cryopreservation. The more significant factors include the number of thawed embryos replaced (1), ovulation-induction regimen in the replacement cycle (2), embryo cleavage stage at

freezing (1, 3, 4), and embryo quality (5). It is well established that prefreeze embryo quality, judged by its morphological appearance, is important for survival after thawing. Good-quality frozen-thawed embryos are an important factor in achieving high pregnancy rate (PR) (6) whereas poor-quality embryos are more likely to be damaged during the cryopreservation process (7). As a result of these facts many IVF programs have adopted the policy of freezing only good-quality multicellular embryos. However, other IVF groups, including ours, prefer to immediately transfer the three highest-quality embryos and freeze all the remaining embryos, as long as their morphological evaluation demonstrates at least two apparently viable blastomeres, with no signs of massive degeneration or damaged zona. It is not clear whether cryopreserving large numbers of poor-to-moderate-quality embryos contributes to

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the overall PR and what is the optimal strategy for grouping these embryos at the freezing stage. Furthermore, since Schalkoff et al. (6) demonstrated the beneficial effect of good-quality embryos on the pregnancy outcome, another important question arises: does the presence of good-quality embryos improve the implantation of the poor-quality ones? We conducted our study to answer these questions by analyzing the effect of one good-quality embryo, the "sponsoring embryo," in a batch of poor-quality thawed embryos, on implantation rates and PRs.

## MATERIALS AND METHODS

### Patients

From March 1988 until April 1995, a total of 440 thawing and replacement cycles were performed in 392 IVF patients at the Rambam Medical Center. Ovarian stimulation in the IVF cycle was accomplished through the use of hMG (Pergonal; Teva, Petah Tiqva, Israel) alone or in combination with FSH (Metrodin; Teva). In 38% of the IVF cycles, we used a desensitizing protocol of GnRH agonist (buserelin acetate, Superfact; Hoechst, Frankfurt, Germany) or D-Trp<sub>6</sub>-LHRH (Decapeptyl; Ferring, Malmo, Sweden). The mean age of the patients when embryo freezing was done was 31.4 years. Of the 440 thawing cycles, 321 were completed in women who were  $\leq 34$  years of age at the embryo freezing, 78 were completed in women 35 to 39 years old, and 41 cycles were completed in women  $\geq 40$  years old (mean age 41.5 years).

### Embryo Replacement and Freezing Policy

Two days after insemination, the embryos were evaluated by experienced embryologists and graded according to their morphological appearance (see Embryo Quality Grading below). The embryo replacement policy during the study period was to transfer immediately the three best-quality (highest grade) with additional one to two lower-grade embryos, depending on the total number of embryos, their quality, the patient's age, and the results of previous WF cycles. All multicellular embryos in excess were assigned for freezing. Cleaving embryos were not frozen if they appeared degenerated and/or were retarded  $>24$  hours in their development and/or contained only one blastomere with or without cytoplasmic fragments.

### Embryo Quality Grading

The classification method was based on a modification of the grading system described by Veeck (8), using a scale of 1 to 5: grade 1 embryos had barely defined blastomeres with major or complete frag-

mentation; grade 2 embryos had irregular or uneven blastomeres with moderate to heavy fragmentation; grade 3 embryos has uneven blastomeres with minor cytoplasmic fragments; grade 4 embryos had regular and even blastomeres with minor cytoplasmic fragments; and grade 5 embryos had regular and even blastomeres and no cytoplasmic fragments. A sponsoring embryo was defined as one having grade 3 or higher, with two to six blastomeres in a 2-day embryo, or at least six blastomeres in a 3-day embryo.

### Freezing and Thawing Procedures

Embryo freezing and thawing were performed according to the two well-established methods previously described by Freemann et al. (9) and Lassalle et al. (10), with some modifications. We have been using these two different freezing protocols based on their early reports showing that the dimethyl sulfoxide (DMSO) protocol gave the best results for 6- to 10-cell embryos (day 3) whereas the propanediol protocol gave a higher survival rate for 2-day embryos compared with 3-day embryos. Briefly, day 3 embryos were frozen in 1.5 M DMSO in modified Dulbecco's phosphate-buffered saline (PBS) supplemented with 10% human cord serum. Dimethyl sulfoxide equilibration occurred at room temperature in incremental steps of 10 minutes, until a final concentration of 1.5 M was reached. Embryos then were loaded into 1.0 mL Nunc cryovials (Nunc, Roskilde, Denmark) and frozen in a Planer biologic freezer (Planer Products, London, United Kingdom), by cooling from room temperature to  $-6^{\circ}\text{C}$  at  $2^{\circ}\text{C}/\text{min}$ . Seeding was induced manually and maintained at  $-6^{\circ}\text{C}$  for 30 minutes. Embryos were cooled further to  $-32^{\circ}\text{C}$  at  $0.3^{\circ}\text{C}/\text{min}$ , and then to  $-35^{\circ}\text{C}$  at  $0.1^{\circ}\text{C}/\text{min}$ , and then to  $-80^{\circ}\text{C}$  at  $0.3^{\circ}\text{C}/\text{min}$ , and from  $-80^{\circ}\text{C}$  to  $-110^{\circ}\text{C}$  at  $10^{\circ}\text{C}/\text{min}$  before storage in liquid nitrogen. For thawing, embryos were transferred from liquid nitrogen to  $-80^{\circ}\text{C}$  and warmed at  $8^{\circ}\text{C}/\text{min}$  to  $4^{\circ}\text{C}$  in the biologic freezer, followed by stepwise removal of DMSO. The embryos were evaluated, graded, and placed in culture for 1 to 4 hours before transfer to the patient.

Day 2 embryos were exposed to a graded series (0.5 M, 1 M, 1.5 M) of propanediol in modified PBS or HEPES-human tubal fluid with 20% human cord serum. Embryos then were loaded into Nunc cryovials containing 1.5 M propanediol and 0.2 M sucrose and frozen in the Planer freezer. Embryos were cooled from room temperature to  $-6.5^{\circ}\text{C}$  at  $2^{\circ}\text{C}/\text{min}$ , held for 10 minutes after seeding, and cooled to  $-30^{\circ}\text{C}$  at  $0.3^{\circ}\text{C}/\text{min}$ . After holding for 5 minutes at  $-30^{\circ}\text{C}$ , embryos were cooled to  $-180^{\circ}\text{C}$  at  $50^{\circ}\text{C}/\text{min}$  and plunged into liquid nitrogen for storage. For thawing, the vials were immersed in a  $37^{\circ}\text{C}$  water

bath for a few minutes until all ice disappeared. The cryoprotectant then was removed in a stepwise fashion by decreasing the propanediol concentrations at 5-minute intervals. The embryos then were evaluated, graded, and transferred after 1 to 4 hours in culture.

### Post-thaw Replacement Cycles

Patients returned for thawing and ET 2 to 37 months after freezing. In 315 cycles, ET was timed during a natural, nonmedicated cycle. For oligo-ovulatory patients, endometrial preparation was achieved by ovulation induction (clomiphene citrate or hMG) in 56 cycles and by a programmed cycle with exogenous hormonal replacement in 69 cycles. For the programmed cycle, 2 mg of E<sub>2</sub> valerate were given for the first 4 days, followed by 4 mg (days 5 to 8) and 6 mg (days 9 to 12). From day 13, the E<sub>2</sub> valerate dose was reduced to 2 mg with 100 mg/d P in oil. Embryo transfer was done on the 3rd or 4th day of P, according to the embryo's age.

In the natural cycles, timing of ovulation was determined with serial daily blood tests of LH, E<sub>2</sub>, and P. The exact time for ET was calculated to be 48 or 72 hours (according to the embryo's age) postovulation, as determined from the onset of the spontaneous LH surge or hCG injection. Embryo transfer was timed for -20 to +10 hours from this exact time and the embryos were thawed and evaluated 2 to 4 hours before transfer. Our policy was to thaw up to four embryos, if at least two of them were of good prefreeze quality (grade 3 or higher), up to five embryos if only one sponsoring embryo was available, and up to six embryos if all embryos were of poor quality (grade 2). Natural cycle transfers were supplemented with 50 to 100 mg P in oil during the luteal phase.

### Pregnancy Determination

Serum quantitative  $\beta$ -hCG assay was performed 12 days after ET, and, if positive, transvaginal sonography was done 2 to 3 weeks later. In this study, pregnancy was defined as the presence of a distinct intrauterine gestational sac seen on ultrasound 4 to 6 weeks after ET (clinical pregnancy). Implantation rate was defined as the number of gestational sacs per number of embryos transferred.

A  $\chi^2$  statistical analysis was used to test the significance of differences between groups. A comparison was considered significantly different when  $P < 0.05$ .

## RESULTS

In all, 1,578 embryos were thawed and 1,436 embryos were replaced in 440 thawing cycles. In 18

cases no transfer of the frozen-thawed embryos was achievable due to embryo lysis. In 268 of 440 thawing cycles (61%), the embryos were frozen on day 2 (propanediol protocol) and, in 172 cycles (39%), on day 3 (DMSO protocol). Seventy-six clinical pregnancies were obtained, representing a pregnancy per transfer rate of 17.3%. There was no significant difference in PR between the propanediol and the DMSO protocols, (17.5% versus 16.8%;  $P = 0.95$ ) or between the three methods of endometrial timing-preparation for embryo replacement, (natural cycle 17.1%, programmed cycle 18.8%, ovulation induction 16.1%;  $P = 0.91$ ).

Of the 76 pregnancies, 4 were achieved in women who were  $\geq 40$  years of age when embryo freezing was done. This outcome represents a pregnancy per transfer rate of 9.8% for this age group compared with 14.1% in the 35-to-39-year-old age group and 19.0% in the group of women  $\leq 34$  years of age.

The correlation between PR and the number of sponsoring embryos in the thawing batch is given in Table 1. Importantly, PR nearly doubled (from 9.8% to 18.2%) when a single sponsoring embryo was included in the thawed and transferred batch compared with ETs without sponsoring embryos ( $P < 0.05$ ). Further increase in the number of sponsoring embryos resulted in higher PRs, up to 31.8% when three or more sponsoring embryos were transferred.

A similar trend was noted for the implantation rate (Table 2), which more than doubled (from 3.1% to 7.0%) when a single sponsoring embryo was included in the transferred batch, compared with transfers without sponsoring embryos ( $P = 0.01$ ). The implantation rate for batches with two sponsoring embryos was similar to that observed for batches with three or more sponsoring embryos (10.3% and 10.4%, respectively). In the latter group, only 41 of the transferred embryos (22%) were of low quality

**Table 1** Pregnancy Rate Versus Number of Sponsoring Embryos in the Thawing Batch

No. of sponsoring embryos	Total no. of ETs	Mean no. of embryos per ET	Clinical pregnancies	Pregnancy rate per ET
0	183	3.9	18	9.8
1	126	3.1	23	18.2*
2	87	3.8	21	24.1†‡
$\geq 3$	44	3.8	14	31.8§
Total	440	1,436/440	76	17.3

\*  $P < 0.05$  versus zero sponsoring embryos,  $\chi^2$  test.

†  $P < 0.005$  versus zero sponsoring embryos,  $\chi^2$  test.

‡ Not significant versus one sponsoring embryo,  $\chi^2$  test.

§  $P < 0.001$  versus zero sponsoring embryos,  $\chi^2$  test.

|| Not significant versus one sponsoring embryo,  $\chi^2$  test.

**Table 2** Implantation Rate Versus Number of Sponsoring Embryos in the Thawing Batch

No. of sponsoring embryos	No. of gestational sacs	No. of embryos transferred	Implantation rate*
0	18	563	3.1
1	28	402	7.0†
2	30	289	10.3‡§
≥3	19	182	10.4‡§
Total	95	1,436	6.6

\* Implantation rate = no. of gestational sacs per no. of embryos.

†  $P = 0.01$  versus zero sponsoring embryos,  $\chi^2$  test.

‡  $P < 0.001$  versus zero sponsoring embryos,  $\chi^2$  test.

§ Not significant versus one sponsoring embryo,  $\chi^2$  test.

compared with 95 embryos (33%) in the two sponsoring embryos groups.

The correlation between the number of sponsoring embryos in the thawing batch and multiple pregnancies is given in Table 3. All 18 clinical pregnancies achieved without sponsoring embryos were singletons. Multiple pregnancies were observed only in the presence of sponsoring embryos in the transferred batch. Moreover, in the group containing only one sponsoring embryo, there were five sets of twins out of 23 clinical pregnancies (21.7%;  $P < 0.05$ ). Increasing the number of sponsoring embryos in the thawed batch did not significantly increase the multiple PR, which was 38% with two sponsoring embryos and 28.6% with three or more sponsoring embryos.

Although the pregnancy and implantation rates were lower for the older women, the effect of the sponsoring embryo showed the same trend in the two major age groups. The PR nearly doubled when a single sponsoring embryo was included in the thawed batch compared with ET cycles without sponsoring embryo: 18.4% versus 11.4% and 13.1% versus 7.3% in women aged ≤34 years and 35 to 39 years old, respectively. Because only four pregnancies were established in the ≥40-year-old age group (of 41 ET cycles), one cannot evaluate properly the effect of the sponsoring embryo in this group.

A similar effect of the sponsoring embryo was noticed when the data were analyzed according to the freezing protocol: using the propanediol protocol (268 ET cycles), the PR was 18.3% when a single sponsoring embryo was included in the thawed batch compared with 10.2% in ET cycles without it. The PR in embryos frozen by the DMSO protocol (172 ET cycles) was 17.3% in the single sponsoring embryo group compared with 8.9% in cycles without sponsoring embryos.

## DISCUSSION

The aim of the present study was to analyze the effect of a sponsoring embryo on the outcome of

thawing cycles when poor-quality embryos are cryopreserved. In addition, several other variables that could affect the PR were examined as well, namely: the patient's age, freezing protocol, and the endometrial timing-preparation method for ET.

Within 1 year after the cryopreservation of their supernumerary embryos, 85% of the patients who were ≥40 years of age returned for thawing cycle. Four pregnancies (9.8% per ET) were achieved in this age group compared with 72 pregnancies (18% per ET) in the group of <40 years of age ( $P < 0.05$ ). This effect of the women's age on the PR in IVF is well established (11) and needs no further confirmation. The interesting question of whether the presence of sponsoring embryos helps to achieve pregnancy in older patients cannot be answered at this stage, because of the small number of pregnancies in this age group. However, in the two younger age groups, namely 35 to 39 years, and ≤34 years old, the beneficial effect of the sponsoring embryo on the PR was clearly demonstrated when compared with PRs in ET cycles without sponsoring embryos.

As to the freezing protocol, we did not find a significant difference in PR per ET comparing the propanediol versus DMSO slow-cooling protocols (17.5% versus 16.8%) or in the effect of the sponsoring embryo on PR. These findings contradict the PR results of Van der Elst et al. (12), who found the DMSO to be superior to the propanediol protocol (PRs of 25% versus 6.5%, respectively). This discrepancy in the Van der Elst et al. (12) results when compared with our findings as well as with other propanediol protocol reports (6, 13) might be attributed to various differences in the freezing and thawing protocol, embryo's age at freezing, mean number of embryos transferred, time interval between thawing and ET, and luteal support in natural cycles.

The differences between the three methods of endometrial timing and preparation also were not

**Table 3** Multiple PR (per Clinical Pregnancy) Versus Number of Sponsoring Embryos in the Thawing Batch

No. of sponsoring embryos	Clinical pregnancy	Singleton	Twins	Triplets	Multiple pregnancies
					%
0	18	18	0	0	0
1	23	18	5	0	21.7*
2	21	13	7	1	38.0‡§
≥3	14	10	3	1	28.6§
Total	76	59	15	2	22.3

\*  $P < 0.05$  versus zero sponsoring embryos, Fisher's exact test.

† Not significant versus one sponsoring embryo, Fisher's exact test.

‡  $P < 0.005$  versus zero sponsoring embryos, Fisher's exact test.

§  $P < 0.05$  versus zero sponsoring embryos, Fisher's exact test.

|| Not significant versus one or two sponsoring embryos, Fisher's exact test.

found to be of statistical significance. These findings are in agreement with those of Queenan et al. (14) and Al-Shawaf et al. (15), who found that transferring cryopreserved-thawed embryos in a natural or programmed cycle yields similar pregnancy results.

The term "sponsoring embryo" was coined by Schalkoff et al. (6), who demonstrated that good quality frozen-thawed embryos are an important factor in achieving a consistently high PR. However, their data did not allow for the isolation of the effect of only one sponsoring embryo, but rather showed the beneficial effect of a group containing sponsoring embryos (one or more) compared with a group containing no sponsoring embryos. Our results confirm Schalkoff et al.'s (6) findings, but also clearly dictate the optimal guidelines for selecting embryo batches for freezing, based on the significant effect of one sponsoring embryo on the pregnancy outcome.

All too often we are left with several spare poor-quality embryos after the fresh embryos are used for transfer. Here, one should first decide on the freezing policy: freeze only good-quality embryos, which, no doubt, provide a better chance for pregnancy (7, 16, 17), or freeze all the excess embryos except the totally degenerated ones. In the latter situation, a second decision should be made on how to group the embryos before freezing. A few strategies can be followed: random grouping or grouping the high-quality embryos separately from the low-quality ones. The latter strategy can be based theoretically on the assumption that low-quality embryos might interfere with the implantation rate of the high-quality ones. Our data suggest that there is, in fact, a third strategy, based on our assumption that the good-quality embryos may improve the chances of implantation of the low-quality embryos. The fact that both PR and implantation rate doubled when one sponsoring embryo was included in the transferred batch compared with ETs with low-quality embryos only, underscores the "added value" of this grouping strategy.

Given the study design we could not dissect reliably the potential beneficial effect of a sponsoring embryo on the implantation rate of a low-grade embryo. However, if we assume that the implantation

rate of a low-grade embryo is not affected by the presence of a sponsoring embryo, then we could estimate the implantation rate for the sponsoring embryos alone. Such speculative reasoning is given in Table 4, which, based on our data, suggests that the implantation rate for a single sponsoring embryo is 16%. We believe that a better estimate is approximately 10%, as given in Table 2, and found also by Mandelbaum et al. (18) who reported a 10% PR with the transfer of good-quality single frozen-thawed embryos. Hence, this difference can be taken to suggest that implantation rate for a low-grade embryo is improved in the presence of a sponsoring embryo. Further support of this hypothesis is the fact that all 18 pregnancies achieved with poor-quality embryos without any sponsoring embryos were singletons, whereas, in the presence of a single sponsoring embryo, there were 5 twin pregnancies (21.7%;  $P < 0.05$ ). Schalkoff et al. (6) reported similar findings: no multiple pregnancies in the nonsponsoring embryo group and 12% multiple PR in group containing one or more sponsoring embryos.

It therefore is tempting to speculate that the presence of a good-quality embryo may induce a favorable environment conducive for the development of pregnancy. The better morphology of the sponsoring embryo may reflect its potential to trigger the full scope of biochemical and molecular events (e.g., secretion of growth-enhancing factors) that are necessary for setting the stage for a successful pregnancy (19). Similarly, the disturbed morphology of the low-quality embryo may reflect its subsequent failure to induce the necessary events during the process of implantation. Consequently, the presence of the sponsoring embryo may take some of the "biochemical burden" off the low-quality embryo, paving its way to successful implantation. In support of this hypothesis, Moessner and Dodson (20) reported recently that culturing human embryos in groups enhances the quality of their growth by increasing the cleavage rates and embryo scores. This beneficial effect of grouped human embryo culture is consistent with the possible autocrine mechanism of enhanced growth in murine embryo model (19).

**Table 4** Estimation of the Implantation Rate of Sponsoring Embryos, if Low-Quality Embryos are not Affected by the Sponsoring Embryos

No. of sponsoring embryos	Grade 2 embryos	Grade $\geq 3$ embryos (sponsoring embryos)	Total no. of gestational sacs	Estimated no. of gestational sacs due to low-quality embryos*	Estimated no. of gestational sacs due to sponsoring embryo	Estimated implantation rate of sponsoring embryo
1	276	126	28	8.5	20	16 (20/126)
2	115	174	30	3.5	27	15.5 (27/174)
3	50	132	19	1.5	17.5	13.2 (17.5/132)

\* Based on a 3.1% implantation rate for low-quality embryos (see table 2).

In light of the fact that high-quality embryos survive the cryopreservation procedure better than low-quality ones, as well as our findings of the sponsoring embryo contribution to the implantation rate of poor-quality embryos, we suggest that the optimal strategy to ensure a reasonable and consistent PR would dictate the inclusion of at least one sponsoring embryo (when possible) in every batch of low-quality embryos. We emphasize that the results of this study apply to multicellular embryos and cannot be extrapolated to embryos frozen at the pronuclear stage.

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