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# The impacts of the number of prefreeze and postthaw blastomeres on embryo implantation potential

## A systematic analysis

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**Abstract**

To systematically analyze the potential of embryo implantation through comparison between the number of surviving blastomeres, the growth, and implantation rate.

Retrospective analysis on implantation rate and the growth of prefreeze-postthaw embryos with different blastomeres in 1487 frozen embryo transfer cycles.

In groups of postthaw embryos without damage, implantation rate and the average number of blastomere growth increased significantly with increasing number of blastomeres. The implantation rate and the number of blastomeres of embryos with 8-8c (the number of blastomeres in prefreeze embryo-the number of blastomeres in postthaw embryo) continued to grow at a significantly higher rate than that of 5-5c and 6-6c ($P < .05$). In groups of embryos with the same number of blastomeres before freezing and with partial damage after resuscitation, the implantation rates were lower and the average numbers of blastomere growth reduced as the number of damaged blastomeres increased. For embryos with good quality before freezing, 1 to 3 damaged blastomeres in postthawed embryos did not affect the development and implantation rate. Both implantation rate and growth rate of embryos with 8-6c were significantly higher than those of embryos with 6-6c ($P < .05$).

The number of surviving blastomeres and growth in frozen-thawed embryos could be important index to predict embryo development potential and clinical outcome of implantation. For embryos with good quality, a small amount of damaged blastomeres would not weaken embryo development potential and implantation rate after being thawed.

**Abbreviations:** FET = frozen embryo transfer, IVF = in vitro fertilization, PR = pregnancy rate.

**Keywords:** blastomere, frozen-thawed embryos, growth, implantation rate

## 1. Introduction

Because embryo cryopreservation was used in assisted reproductive technology, cumulative clinical pregnancy rate (PR) increased, whereas the number of embryos transplanted per cycle was reduced.[1] However, compared with fresh embryo transplantation, the clinical outcome of frozen-thawed embryo transfer was not good.[2] With recent development of biological freezing technologies, vitrification cryopreservation has significantly improved the rate of frozen-thawed embryos and clinical outcome of frozen embryo transfer.

Many factors, such as the age of the patient at time of cryopreservation, the cause of infertility, the grade of the transferred embryos, the damage of embryos after thawing, the level of estradiol, and the endometrial thickness on the day of transfer, may influence the implantation rate of freeze-thaw cycles.[3,4] Although transferring embryos with 50% intact blastomeres after thawing might lead to successful pregnancies, PR was higher when all the blastomeres survived.[5,6] Indeed, if the embryos survived the freeze-thaw process with all their blastomeres intact, PR was comparable to that of fresh in vitro fertilization (IVF) cycles. The most predictive parameter for blastocyst formation was time of morula formation.[7]

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It was reported that the growth rate of embryos and survival rate of blastomeres played an important role in improving embryo implantation rate.[8] The clinical pregnancy outcome may change if we select frozen-thawed embryos with different numbers of blastomeres.[9] In this study, we compared implantation rate, growth rate, and morula formation rate in 5, 6, 7, and 8c of frozen-thawed embryos, to systematically analyze the potential of embryo implantation and the effect of frozen-thawed embryos with different blastomeres on clinical outcome of frozen embryo transfer (FET).

## 2. Materials and methods

### 2.1. Embryo

This study was approved by Ethics Committee of Guangdong Women and Children Hospital and all patients provided informed consent. In this retrospective study, 1487 FET cycles were performed from August 2012 to December 2014 in the Department of Reproductive Medicine, Guangdong Women and Children Hospital. Based on the number of blastomeres in frozen-thawed embryos, embryos were divided into groups with 5c-5c, 6c-5c, 6c-6c, 7c-5c, 7c-6c, 7c-7c, 8c-5c, 8c-6c, 8c-7c, and 8c-8c. Among 1487 FET cycles, 24 FET cycles had only transplanted 1 embryo and 1 embryo was implanted; 635 FET cycles had transplanted 2 embryos and 2 embryos were both implanted. The remaining 828 FET cycles had transferred more than 2 embryos, and it was unclear which transferred embryos were implanted, and thus these cycles were excluded. Finally, a total of 1294 embryos ($24 \times 1 + 635 \times 2$) were frozen at day 3 in vitrification cryopreservation.

### 2.2. Controlled ovarian hyperstimulation

Controlled ovarian stimulation was performed with 2 approaches: the standard long protocol and antagonist protocol. Patients in the former group were treated with GnRHa (1.0–1.875 mg Diphereline) during the previous menstruation mid-luteal phase, and stimulated with Gn (recombinant human follicle stimulating hormone (FSH)) 2 weeks later. Patients in the latter group were directly stimulated with Gn from the second to fifth days of menstruation, when the ovary was during the basal follicular phase. Gn doses were adjusted according to ultrasound monitoring and serum E2, luteinizing hormone (LH), and FSH levels. In addition, antagonist (Ghani Rick 0.25 or 0.125 mg) was added when LH value was high or the follicle diameter reached 10 to 12 mm. Oocyte maturation was induced by administering human chorionic gonadotropin (5000–10,000 IU) when the majority of follicles reached 16 to 18 mm. Transvaginal follicular aspiration was carried out 34 to 36 hours later. Regular IVF/intracytoplasmic sperm injection (ICSI) was performed according to what was required for the patients.

### 2.3. Embryo scoring and selection

All the embryos included in this study were transferred at 16 hours after thawing. Cleavage stage embryos were graded according to the (Society for Assisted Reproductive Technology) scoring system of embryos.[10] Our selection criteria were as follows: the number of cells was $>5$, and the fragmentation rate was $<25\%$.

### 2.4. Freezing and thawing

The embryos were transferred from culture into basal medium (BM) containing HEPES (Quinn's SAGE, Knardrupvej, Denmark) with 20% v/v human serum albumin (Vitrolife, Sweden), and then transferred into vitrification solution 1 (BM solution containing 7.5% v/v DMSO and 7.5% v/v ethylene glycol) for 2 to 7 minutes. When the shrinkage of embryos expanded to 80%, the embryos were transferred into vitrification solution 2 (BM solution containing 15% v/v DMSO, 15% v/v ethylene glycol, and 10% v/v sucrose) for 30 seconds. Within 5 to 10 seconds, the embryos were collected in a minimal volume and put into the cryodevice, which were then immediately put into liquid nitrogen to cryopreserve at −196°C.

For thawing, the embryos were transferred from cryodevice into warming solution 1 (BM solution containing 1 M sucrose) for 1 minute, and then transferred into warming solution 2 (BM solution containing 0.5 M sucrose) for 3 minutes. Last, the embryos were transferred into the BM and remained in the solution for 5 minutes. Next the embryos were rinsed in G2 solution several times and cultured at 37°C in 6% $CO_2$ incubator overnight.

### 2.5. Choice of transplanting time

The urinary LH of patients with natural ovulation was monitored with ultrasound. The freeze-thawed embryos were transplanted on the third day after ovulation, on which day the thickness of endometrium was $\leq 7$ mm. Hormone replacement therapy was adopted in patients without natural ovulation or with irregular menstrual cycle. Estradiol valerate with 2 to 4 mg/day was prescribed from the third day of the menstrual cycle, progesterone was injected from 8 to 10 days with 80 mg/day, embryos were transplanted on 11 to 15 days under the guiding of ultrasound.

### 2.6. Judgments

Implantation rate: After 4 weeks of the embryo transplantation, the percentage of the total number of gestational sac to the total number of embryos transferred. Growth data: At the time of transplantation of the number of blastomere minus the number of surviving blastomeres after thawing (the same culture time). Morula formation rate: Morula accounted for the proportion of the total of embryos transplantation.

### 2.7. Statistical analysis

All the data were shown as mean ± standard error. Analysis of variance (1 way) was applied to calculate statistical significance followed by post hoc Dunnett test. Chi square analysis was used to compare implantation rate between groups. All statistical analyses were performed using SPSS 19.0 software (SPSS, Chicago, IL) and $P < .05$ indicated statistically significant differences.

## 3. Results

### 3.1. Patient characteristics

The basal parameters such as age and endometrium thickness of each patient showed no significant difference (Table 1).

### 3.2. Comparison between no blastomere damage groups

The implantation rates of frozen-thawed embryos with 5-5c, 6-6c, 7-7c, and 8-8c were 6.09%, 14.94%, 36.1%, and 45.1%, respectively (Table 2). There were significant differences between

**Table 1**

**Patient characteristics for frozen embryo transfer cycles.**

| Frozen-thawed embryo | The number of embryos | Age | Thickness of endometrium (cm) |
|---|---|---|---|
| 8c-8c | 814 | 31.83±5.44 | 9.73±1.96 |
| 8c-7c | 54 | 32.03±5.18 | 9.71±2.05 |
| 8c-6c | 28 | 31.97±4.61 | 9.66±2.19 |
| 8c-5c | 19 | 31.92±4.72 | 9.86±2.24 |
| 7c-7c | 158 | 31.95±5.66 | 9.79±2.16 |
| 7c-6c | 19 | 31.98±5.28 | 9.83±1.97 |
| 7c-5c | 15 | 31.94±5.23 | 9.76±2.33 |
| 6c-6c | 87 | 31.55±4.84 | 9.92±2.05 |
| 6c-5c | 18 | 31.25±4.64 | 9.74±2.31 |
| 5c-5c | 82 | 31.84±4.72 | 9.81±2.76 |
| *P* value | | >.05 | >.05 |

Differences between groups were not significant ($P > .05$).

the 4 groups. The average numbers of blastomere growth of each group were 1.56±0.141, 1.88±0.169, 2.32±0.163, and 3.24±0.087, respectively. Blastomere growth was significantly reduced with the decline in total number of blastomeres ($P < .05$) (Fig. 1). The implantation rate and embryo growth rate were higher in post-thawed embryos with higher survival rate of blastomeres.

### 3.3. Comparison between blastomere damage groups

The implantation rates of frozen thawed embryos with 8-8c, 8-7c, 8-6c, and 8-5c were 45.1%, 40.7%, 32.1%, and 21.4%, respectively, showing a significant downward trend (Table 2). The average numbers of blastomeres growth in each group were 24±0.087, 2.90±0.321, 2.80±0.381, and 1.91±0.392, respectively. The number of blastomere growth in the group of embryos with 8-8c was significantly higher than that of embryos with 8-5c ($P < .05$) (Fig. 2A). The implantation rates of 7-7c, 7-6c, and 7-5c were 36.1%, 26.3%, and 13.3%, respectively, with a significant decreasing trend ($P < .05$) (Table 1). The average numbers of blastomere growth were 2.32±0.163, 2.07±0.370, and 1.71±0.266, respectively. There was no significant difference between embryos with 6, 7 cell prefreeze groups (Fig. 2B and C). The implantation rate of embryos with less damaged blastomeres was significantly higher than that of embryos with more damaged blastomeres, whereas the growth of the blastomere was decreased with the increase of damaged blastomeres.

**Table 2**

**Implantation rates classified according to the number of blastomeres in the prefreezing embryos and the number of blastomeres in post-thawing embryos.**

| | Post-thaw 8c | Post-thaw 7c | Post-thaw 6c | Post-thaw 5c | *P* |
|---|---|---|---|---|---|
| Prefreeze 8c | 45.1% (367/814) | 40.7% (22/54) | 32.1% (9/28) | 21.4% (4/19) | <.05 |
| Prefreeze 7c | | 36.1% (57/158) | 26.3% (5/19) | 13.3% (2/15) | <.05 |
| Prefreeze 6c | | | 14.9% (13/87) | 11.1% (2/18) | >.05 |
| Prefreeze 5c | | | | 6.09% (5/82) | |
| *P* value | | >.05 | <.05 | <.05 | <.05 |
| Total | 45.1% (367/814) | 37.3% (79/212) | 20.1% (27/134) | 9.6% (13/134) | <.05 |

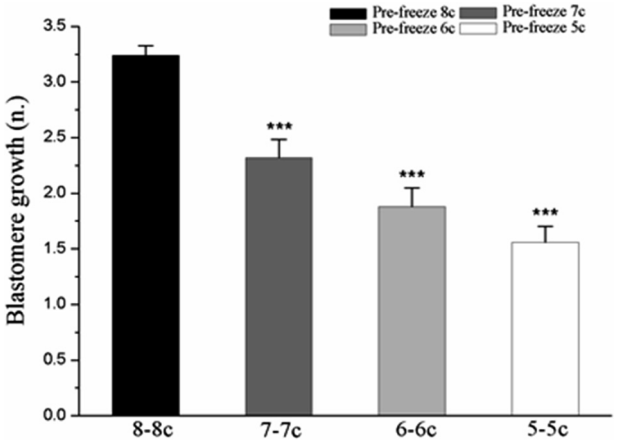


**Figure 1.** The average number of blastomere growth of no blastomere damage groups after 16 hours of thawing. Blastomere growth significantly reduced with the decline in total number of blastomeres (***$P < .001$).

### 3.4. Comparison of the same number of blastomere groups

Overall, the implantation rate decreased as the number of blastomeres decreased significantly after resuscitation ($P < .05$). The implantation rates of frozen-thawed embryos with 5-5c, 6-5c, 7-5c, and 8-5c were 6.09%, 11.1%, 13.3%, and 21.4%, respectively, showing significant differences between each 2 groups (Table 2). The average numbers of blastomeres growth were 1.56±0.141, 1.63±0.287, 1.71±0.266, and 1.91±0.392, respectively (Fig. 3A). The growth of embryos with 8-5c was significantly higher than that of embryos with 5-5c ($P < .05$). The implantation rates of frozen-thawed embryos with 6-6c, 7-6c, and 8-6c were 14.94%, 26.3%, and 32.1%, respectively. There were significant differences between each 2 groups (Table 2). The number of blastomere growth in embryos with 8-6c was significantly higher than that in embryos with 6-6c ($P < .05$) (Fig. 3B). The implantation rate of embryos with more blastomeres before freezing was significantly higher than embryos with less blastomeres. There was no significant difference between embryos with 8-7c and embryos with 7-7c ($P > .05$) (Fig. 3C).

### 3.5. Comparison of morula formation rate

Because embryos in part of morulas were transplanted in this study, we compared the morula formation rate. The morula formation rate of 8-8c, 8-7c, 8-6c, and 8-5c were 46.68%, 33.33%, 28.57%, and 15.79%, respectively, showing significant differences (Table 3).

## 4. Discussion

The selection of frozen-thawed embryos is critical in the FET cycle. Survival rate of post-thawed embryo blastomere was evaluated as embryo damage index.[6] It was reported that the injury of blastomeres would weaken the potential of embryo implantation. Damaged embryos reduced the number of blastomeres and decreased growth rate, which may have a "toxic effect." The toxic effect caused damage to the internal structure of embryos, and affected development, hatching, and

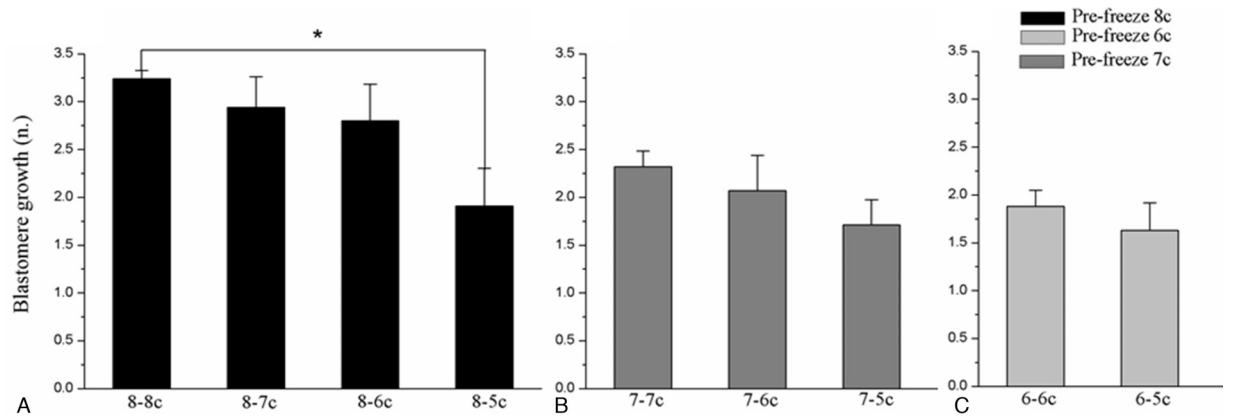


**Figure 2.** The average number of blastomere growth in different groups after 16 hours of thawing. The number of blastomere growth in the group of embryos with 8-8c was significantly higher than that of embryos with 8-5c ($^{*}P < .05$).

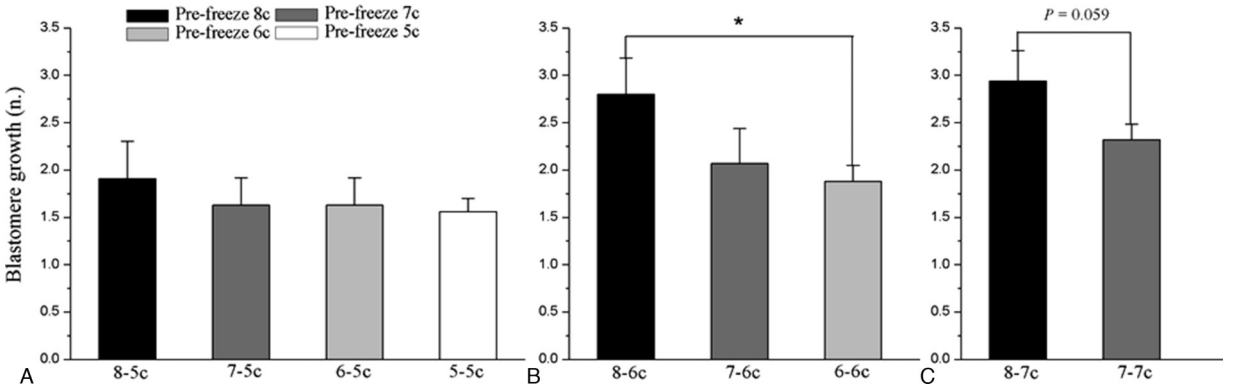


**Figure 3.** The average number of blastomere growth of the same number of blastomere groups post-thaw after 16 hours of thawing. The number of blastomere growth in embryos with 8-6c was significantly higher than that in embryos with 6-6c ($^{*}P < .05$) There was no significant difference between embryos with 8-7c and embryos with 7-7c ($P > .05$).

implantation.[11,12] The presence of 1 to 2 lysed blastomeres in the thawed day 3 embryos did not appear to have a negative influence on the development of sibling intact cells.[13] Therefore, using the blastomeres survival rate of embryos to predict subsequent developmental potential and clinical outcomes needs further study.

During the freezing and thawing process, the embryos undergo a series of changes in physical, chemical, and osmotic pressure. These changes affect pellucid zone at different degrees, and clinical PR may significantly decrease if we transfer embryos with abnormal pellucid zone.[14] Compared with no blastomere damage embryos, transplantation of partially damaged embryos

**Table 3**

**Morula formation rate classified according to the number of blastomeres in the prefreezing embryos and the number of blastomeres in post-thawing embryos.**

| | Post-thaw 8c | Post-thaw 7c | Post-thaw 6c | Post-thaw 5c | *P* |
|---|---|---|---|---|---|
| Prefreeze 8c | 46.68% (380/814) | 33.33% (22/54) | 28.57% (8/28) | 15.79% (3/19) | <.05 |
| Prefreeze 7c | | 30.38% (48/158) | 26.32% (5/19) | 13.3% (2/15) | >.05 |
| Prefreeze 6c | | | 22.99% (20/87) | 11.1% (2/18) | >.05 |
| Prefreeze 5c | | | | 7.32% (5/82) | |
| *P* value | | >.05 | >.05 | >.05 | <.05 |
| Total | 46.68% (380/814) | 33.02% (70/212) | 24.63% (33/134) | 8.96% (12/134) | <.05 |

would significantly reduce the implantation rate and clinical PR. The reason is that the damage of blastomere could weaken the developmental potential of surviving blastomeres and reduce the rate of blastocyst formation.[5,15] The cell debris of post-thawed embryos cannot be completely absorbed by the embryos. Previous studies have demonstrated that the removal of fragments in mouse embryos could significantly improve the growth and implantation rate of embryos.[16–18]

Clinical pregnancy outcome was positively related to the number of surviving blastomeres in FET cycles.[6] Transplantation of embryos with <6 blastomeres could significantly reduce clinical PR and increase abortion rate. Transplantation of embryos with >9 blastomeres could increase aneuploidy rate.[19,20] However, one study reported no significant difference in clinical pregnancy outcome between transplantation of embryos with 1 to 2 cells impaired or damaged and transplantation of embryos with intact cells.[13] Other studies showed that few blastomere debris in the cleavage stage embryos did not affect embryo development, and embryos could remove the debris during embryonic development.[16,21] The developmental stage and the speed of development could predict the developmental potential of mouse embryos.[22] Moreover, embryos with fast growth speed before being frozen showed higher recovery rate, cleavage rate, and implantation rate than embryos with slow growth speed.[8] As one of the optimizing conditions, the growth rate of embryos is widely used in human embryo transfer.[23,24] Our results showed that the implantation rate of frozen-thawed embryos without cleavage damage increased dramatically with cleavage increasing, which was consistent with most studies. The implantation rate reduced dramatically as the number of damaged blastomeres increased. In group of thawed embryos with same number of live blastomeres, the more the number of blastomeres before freezing, the higher the implantation rate. Although there were 1 to 3 impaired blastomeres, the implantation rate of embryos with 8c was not reduced. The implantation rate of thawed embryos with 8-6c was significantly higher than that of embryos with 6-6c, and that of embryos with 8-7c was significantly higher than that of embryos with 7-7c. The implantation rate of embryos with 7 or 8 blastomeres before freezing was better,[20,25] perhaps due to the function of self-clearing debris. According to the implantation outcome, few damaged blastomeres could not affect the developmental potential of embryos.[16,26]

Furthermore, we confirmed that the number of blastomeres was correlated with ongoing growth. In group of frozen-thawed embryos without impaired blastomeres, the growth index increased dramatically as the total number of blastomeres increased before freezing. In group of frozen-thawed embryos without damaged blastomeres, the growth increased significantly with the increase of the total number. In group of embryos with damaged blastomeres and with the same number of blastomeres before freezing, the less damaged blastomeres post-thawed, the larger the number of growth. In group of embryos with different number of blastomeres before freezing, if the numbers of blastomeres after thawing were the same, the more blastomere before frozen, the more growth. The number of blastomere growth in group of 8-6c was significantly higher than that in group of 6-6c. 8c fresh embryos even with 1-3 damaged blastomeres post-thawed did not affect the continuous growth. In this study, transplantation of embryos included the cleavage stage and morula. We analyzed the blastomere growth of cleavage stage embryos, and calculated the morulas formation rate. The results showed the same trend. Therefore, the number of frozen-thawed embryo blastomere survival could effectively predict the potential of developmental and clinical outcome of implanting. For the same number of embryonic blastomeres after resuscitation, selecting the better growth of blastomeres with good quality before freezing might have a better implantation rate. Our study provides an important reference basis for the selection of embryos in FET cycles.

## Author contributions

**Conceptualization:** Feng-hua Liu.
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**Methodology:** Wen-juan Liu.
**Resources:** Yu-jiang Wang.
**Software:** Zi-tao Li, Yu-qiang Huang.
**Supervision:** Dun Liu, Chuang-qi Chen.
**Validation:** Zi-tao Li, Chuang-qi Chen.

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