A new day 4 embryo evaluation criteria to predict the formation of blastocyst

Fangfang Dai
  Xingtai Infertility Specialist Hospital

Geng Yasong
  yasong2021@163.com
  Xingtai Infertility Specialist Hospital

Linlin Tao
  Xingtai Infertility Specialist Hospital

Guozhen Li
  Xingtai Infertility Specialist Hospital

Haoyang Dai
  Xingtai Infertility Specialist Hospital

Shusong Wang
  Hebei Research Institute of Family Planning Science and Technology

Bo zheng
  Xingtai Infertility Specialist Hospital

Research Article

Keywords: Embryo selection, Blastocyst formation, Morula, Day 4 embryo scoring

Posted Date: March 26th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4109442/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.
Abstract

Background

Currently, in vitro fertilization and embryo transfer (IVF-ET) typically involve transferring embryos on the third (D3) or fifth day (D5). However, a fresh cycle fourth day (D4) morula transfer offers a flexible and effective alternative. The compaction on the fourth day serves as a simple and reliable indicator to predict embryo implantation potential. The use of D4 transfer is gradually gaining popularity. Nevertheless, there is still a need for a comprehensive evaluation system for fourth day embryos. Thus, this study proposes to explore a day 4 embryo evaluation system based on the potential for the embryo to develop into a transferable blastocyst.

Methods

We observed the outcome of blastocyst culture for 1235 embryos from 199 patients. We considered the developmental stage, fragmentation, whether it was in a stage-specific cleavage pattern, and normal fertilization on the fourth day. The fourth day embryo evaluation system was proposed by comparing them with the effect on blastocyst formation rate. The rates of blastocyst formation with different embryo grades were compared. The receiver operative characteristics (ROC) curve was used to compare the predictive abilities of four criteria for day 4 evaluation to develop into transferable blastocysts and high-quality blastocysts.

Results

The developmental stage, fragmentation, normal fertilization and stage-specific cleavage pattern had the greatest impact on the formation of transferable blastocysts. There were significant differences in the formation rates of transferable blastocysts and high-quality blastocysts among different embryo grades. The areas under ROC curves of the day 4 embryo evaluation criteria constructed in this study was the highest in predicting formation of transferable or high-quality blastocyst, relative to the other three evaluation criteria. This study observed the presence of pseudo-compacted embryos similar to cleavage-stage embryos on the rate of blastocyst formation.

Conclusion

The Day 4 embryo evaluation criteria constructed in this study can effectively predict the ability to develop into a blastocyst.

Background

The embryos were usually transferred on the third or fifth day after fertilization. Compared with cleavage stage embryo, the blastocyst stage was beneficial for identifying the most viable embryo and could improve the synchronization of uterus and embryo and increase pregnancy rate[1, 2]. The blastocyst stage, which occurs on the fifth day, was favored for embryo transfer as it allows for the identification of the most potential embryos and improves the synchronization between the uterus and the embryo, leading to higher pregnancy rates[1, 2]. It has been reported that the embryonic genome was activated extensively on the fourth day, provides insights into the molecular mechanisms underlying embryo development and potential strategies for embryo evaluation[3]. Studies by Hsieh et al. had shown that the rate of euploidy was higher in morula and blastocyst transfer compared to cleavage transfer. In fresh
IVF-ET cycles, the clinical pregnancy rates of morula transfer were similar to that of blastocyst transfer, but the live birth rates were higher in the morula transfer[4, 5]. This provides flexibility in the timing of embryo transfer, allowing for individualized selection based on the patient's own culture conditions and the laboratory's culture conditions.

However, the lack of a standardized evaluation system for day 4 embryos is a major limitation for day 4 embryo transfer. Gemma Fabozzi et al. and Thomas Ebner et al. had developed evaluation criteria for day 4 embryos based on compaction and fragmentation rates respectively[6, 7]. The 2011 ESHRE Istanbul Consensus classified embryos in the compacted stage but did not specify grading for cleavage and early blastocyst stages[8]. Hong-Xing Li et al. categorized cleavage embryos based on blastomere number ratio (BNR, D4 blastomere number / D3 blastomere number), effectively predicting implantation rates (AUC, area under curve = 0.65) and live birth rates (AUC = 0.73)[9]. The embryo on the fourth day provides information not only on developmental speed and fragmentation rate. The other parameters should be incorporated into the embryo evaluation system. Compared to two-pronuclear (2PN) blastocyst transfer, monopronuclear (1PN) blastocysts transfer has higher abortion rates (AR) and lower live birth rates (LBR) while nonpronuclear (0PN) blastocyst transfer results in higher birth weight[10]. The presence of mononucleated blastomeres and the proportion of mononucleated blastomeres to total blastomeres may be positively correlated with embryo development potential and live births[11, 12]. The presence of vacuoles also affects the rates of aneuploidy and live births[13]. Theoretically, blastomeres produced by embryonic mitosis should be the same size. Embryos with uniform blastomeres have been shown to have lower rates of multinucleation and aneuploidy as well as significantly improved implantation rates[14]. However, the uniformity and size of blastomeres also depend on the regularity and specific stages of embryo development, known as stage-specific cleavage patterns. Therefore, considering more visual parameters will enhance the effectiveness of the embryo evaluation system. Combining embryo kinetic parameters based on time-lapse imaging techniques will greatly improve the accuracy of the embryo evaluation system, as even a small difference in the timing of dynamic embryonic development can lead to significant changes[15]. However, this will increase the difficulty of the evaluation system and make it more complex.

In this study, we propose a new set of evaluation criteria for day 4 embryos based on additional parameters beyond developmental stage and fragmentation. We re-evaluated embryos according to previously reported evaluation schemes and compared their accuracy and sensitivity in predicting the formation rate of transferable blastocysts.

**Materials and method**

**Study Population**

From January 2023 to August 2023, a total of 1235 embryos from 199 women undergoing their first IVF cycle at the Center for Reproductive Medicine in Xingtai Infertility Specialist Hospital (Xingtai, Hebei Province, China) were included in the analysis. The women's age was ≤ 35, with a range of 5–20
retrieved eggs, excluding patients with oocyte cryopreservation, in vitro maturation. Individuals with an endometrial history or significant chromosomal abnormalities including endometriosis, fibroids, intrauterine adhesion, endometritis and other chromosomal abnormalities were excluded. This study obtained approval from the Ethics Committee of the Xingtai Infertility Specialist Hospital (Approval Number: 2021-ER-06).

Treatment protocol

All patients used standard gonadotropin-releasing hormone (GnRH) agonist protocol or GnRH antagonist protocol. Patients used GnRH agonist protocol were injected intramuscularly with gonadotropin-releasing hormone analogue (GnRHa, Duferin, Ipsen Pharma Biotech, France) from the mid-luteal phase. After achieving the purpose of downregulation, recombinant FSH (Plecon, Meck Sharp&Dohme Limited, UK) was started to achieve the purpose of superpromoting ovulation. The GnRH antagonist protocol was started with recombinant FSH (Prilikon, Merck, the Netherlands) at 100 ~ 150U, the dose was adjusted according to the follicle growth measured by vaginal B ultrasound. HMG (Lizhu Pharmaceutical, China) was used to promote ovulation in the middle and late stage of follicles. Give the GnRH antagonist (Serono, Switzerland) 0.25mg daily when the dominant follicle reaches 12 mm in diameter. The administration of 5000 ~ 10000 IU human chorionic gonadotropin (hCG) (Lizhu Pharmaceutical, China) was used to trigger the ovary based on clinician's discretion as soon as one-third of all follicles reached a diameter of ≥ 18 mm. The retrieval of the oocyte was done using needle aspiration guided by transvaginal ultrasound, 36 to 37 hours after hCG injection. IVF/ICSI was carried out at 3 ~ 4 h after oocytes retrieval, and fertilization methods were performed depending on sperm parameters. IVF: It was performed by 30 to 50 ul micro droplets at 1 to 2 oocytes per drop with a concentration of 200 000 sperm per milliliter, and the granule cells surrounding the oocytes were removed from 4 to 6 h after fertilization.

Embryo morphology assessment

The evidence of fertilization was evaluated 16–18 hours after insemination, and zygotes with two pronuclei indicated that fertilization was normal. Multinucleation and mononucleation were observed at 44 ± 1 hours after fertilization, while the number and stage-specific cleavage pattern of blastomeres, as well as fragmentation, were observed at 68 ± 1 hours after fertilization. The degree of compaction and fragmentation are observed at 92 ± 2 hours after fertilization. Vacuoles were recorded during each observation. All embryos were classified as follows: A - Normal fertilization with early blastocysts that follow a stage-specific cleavage pattern; B - Normal fertilization with early blastocysts that do not follow a stage-specific cleavage pattern or have a compaction ratio ≥ 50% and fragmentation ≤ 10%; C - Abnormal fertilization with early blastocysts, fusion ratio ≥ 50% and fragmentation between 10–50%, or compaction ratio < 50% and fragmentation ≤ 10%; D - Compaction ratio < 50% and fragmentation between 10–50% or BNR (blastomere nuclear ratio) ≥ 1.2 and fragmentation < 10%; E - Fragmentation > 50%, BNR < 1.2, or BNR ≥ 1.2 and fragmentation > 10% (Fig. 2). The embryos were further re-graded according to previously reported evaluation systems, as detailed in the reference[6–8, 16]. The blastocysts were scored at 116 ± 2 hours and 140 ± 2 hours after fertilization, using the Gardner scoring
system[16]. The transferable blastocysts were at graded as 3–6 (i.e., full blastocysts onward) with an inner cell mass grade of B or higher and a trophectoderm grade of C or higher. High-quality blastocysts have both the inner cell mass and trophectoderm graded as A or B.

**Statistical analysis**

The statistical analysis was performed using IBM SPSS 25.0 software (IBM Corporation, NY, USA). Continuous variables were expressed as median (25–75 percentile), and comparisons were performed using the Kruskal-Wallis H test. Count data were indicated as a percentage (%), and comparisons were performed using the \( \chi^2 \) test. Binary logistic regression analysis, presented as unadjusted odds ratio (crude odds ratio (OR)) or adjusted odds ratio (aOR) with a 95% confidence interval (CI), was used to determine the relationship between different morphologic parameters and the formation rate of transferable blastocysts. The predictive effect of embryo grading on transferable blastocysts and high-quality blastocysts was evaluated through ROC curves. Statistical significance was accepted at \( p < 0.05 \).

**Results**

**Basic demographic and clinical characteristics of patients**

During the study period, a total of 199 women who met the inclusion criteria obtained 2567 oocytes. Among them, 513 oocytes were immature, 291 zygotes were \( \geq \) three pronuclears, 122 oocytes were unfertilized, and 406 embryos were not cultured until the fifth day. The remaining 1235 embryos were available for observational research (Fig. 1). 94 cases (47.24%) were primary infertility, and 52 cases (26.13%) underwent ICSI. The average number of retrieved oocytes was 13 (10–16), with a blastocyst formation rate of 41.46% (512/1235) and a high-quality blastocyst formation rate of 13.36% (165/1235) (Table 1).

**The effect of morphological parameters on the formation rate of transferable blastocysts**

Binary logistic regression analysis revealed that developmental stage at 92 ± 2 hours, fragmentation ratio, stage-specific cleavage pattern, and non-2PN origin significantly influenced the formation rate of transferable blastocysts (\( p < 0.05 \), while multinucleation and vacuolization had no effect on blastocyst formation (\( p > 0.05 \)). In this study, 50 pseudo-compacted embryos were observed (Fig. 2G), of which 13 formed transferable blastocysts (26%), and none formed high-quality blastocysts. These embryos were similar to cleavage-stage embryos and were combined for analysis as cleavage-stage embryo. The formation rate of transferable blastocysts significantly increased with closer developmental stage to early blastocysts, smaller fragmentation ratio, stage-specific cleavage pattern, and 2PN origin. Among them, the effect weight of early blastocysts vs BNR < 1.2 was the highest (OR = 139.887, \( p < 0.001 \), followed by fragmentation < 10% vs \( \geq \) 50% (OR = 8.511, \( p < 0.001 \)); non-2PN origin significantly reduced the formation rate of transferable blastocysts, Non-2PN vs 2PN (OR = 0.339, \( p = 0.010 \)), followed by non-stage-specific cleavage pattern, Non-stage-specific vs stage-specific (OR = 0.545, \( p = 0.002 \)) (Table 2).
Comparison of blastocyst formation rate among different grading embryos

Based on the developmental stage of embryos at 92 ± 2 hours post-fertilization, proportion of fragmentation, whether they originated from 2PN, and whether they exhibited stage-specific cleavage pattern, embryos were divided into five grades: A, B, C, D, and E. The transferable blastocysts rate of grade A embryos significantly increased (93.23% vs. 68.51%, 51.89%, 32.17% and 9.75%), while the other grades decreased sequentially, and there were significant differences between different grades (p < 0.05) (Table 3). The formation rate of high-quality blastocysts in grade A significantly increased, while the rates of the other grades decreased sequentially, and there were significant differences between different grades (p < 0.05) (Table 4).

The rate of transferable blastocyst formation gradually decreased in grade B (adjusted odds ratio, aOR:0.412, P < 0.001), grade C (aOR: 0.152, P < 0.001), grade D (aOR: 0.095, P < 0.001) and grade E (aOR: 0.034, P < 0.001) relative to grade A (Table 3), and also decreased for high-quality blastocyst formation (Table 4). This trend remains when embryos are classified into IVF and ICSI (Fig. 3).

Comparison of predictive abilities of four different evaluation systems for blastocyst formation rate

A total of 1235 embryos that met the inclusion criteria were re-evaluated using four grading systems, and the proportions of embryos covered by different grading systems were calculated. The coverage rates of the embryo evaluation system proposed in this study and Feil 2008 were 100% (1235/1235), while ESHRE 2011 and Gemma 2015 had a coverage rate of 88.02% (1087/1235) due to lack of classification for early blastocysts (Table 5). The four embryo evaluation systems had significant predictive values for transferable and high-quality blastocysts (P < 0.05). The evaluation system proposed in this study had the highest area under the curve (AUC = 0.817, P < 0.001) for predicting the formation rate of transferable blastocysts, followed by Feil 2008 (AUC = 0.716, P < 0.001), ESHRE 2011 (AUC = 0.643, P < 0.001), and Gemma 2015 (AUC = 0.739, P < 0.001). The evaluation system proposed in this study also had the highest area under the curve (AUC = 0.780, P < 0.001) for predicting the formation rate of high-quality blastocysts (Fig. 4), followed by Feil 2008 (AUC = 0.726, P < 0.001), ESHRE 2011 (AUC = 0.667, P < 0.001), and Gemma 2015 (AUC = 0.705, P < 0.001) (Fig. 5).

Discussion

This study demonstrated that the developmental stage of the embryo on the fourth day after fertilization, the proportion of fragmentation, the presence of stage-specific cleavage patterns, and whether the
embryo is derived from a 2PN source all have an impact on the formation of transferable blastocysts. The developmental stage of the embryo on the fourth day after fertilization has the greatest impact on the formation of transferable blastocysts, followed by subgroup analysis based on other morphological parameters. The ROC results showed that the evaluation criterion for fourth-day embryos established in this study can effectively predict the formation of transferable and high-quality blastocysts.

Currently, in vitro fertilization and embryo transfer (IVF-ET) typically involve transferring embryos on the third (D3) or fifth day (D5). However, a fresh cycle fourth day (D4) morula transfer offers a flexible and effective alternative. The compaction on the fourth day serves as a simple and reliable indicator to predict embryo implantation potential. The use of D4 transfer is gradually gaining popularity. In recent years, there has been an increasing number of studies on the evaluation of fourth-day embryos[9, 17, 18]. Among them, ESHRE 2011 and Gemma 2015 did not classify early blastocysts, resulting in a coverage rate of only 88.02% (1087/1235) in this study[7, 8]. Feil 2008 mainly referred to developmental stage and fragmentation ratio as morphological indicators[6]. When comparing the clinical outcomes between good morula embryo transfer (MET) on day 4 and good blastocyst embryo transfer (BET) on day 5, the results were consistent[4]. There were no significant differences in implantation rate (48.8% vs 41.1%, p = 0.335), clinical pregnancy rate (55.0% vs 53.2%, p = 0.867), and live birth rate (47.5% vs 47.1%, p = 1.000). The term birth rate in the MET group on day 4 was also higher than that in the BET group on day 5 (100% vs 78.3%, p = 0.025). The degree and pattern of fragmentation significantly affected pregnancy and implantation, and the application of microsurgical fragmentation removal before ET improved the implantation rate of embryos[19]. However, whether stage-specific cleavage patterns, normal fertilization, multinucleation, and vacuoles also affect the euploidy rate and live birth rate of embryos. Compared to 2PN, 1PN had higher AR and lower LBR, while 0PN resulted in a higher birth weight[10]. Therefore, 2PN blastocyst transfer should be prioritized after 2PN fertilization. The closer the developmental stage was to the early blastocyst, the smaller the fragmentation ratio, the presence of stage-specific cleavage patterns, and the evidence of normal fertilization, all significantly increase the formation rate of transferable blastocysts. Among them, the influence weight of early blastocysts vs BNR<1.2 was the highest (OR = 139.887), followed by fragmentation <10% vs ≥ 50% (OR = 8.511). Non-2PN significantly decreased the formation rate of transferable blastocysts compared to 2PN (OR = 0.339), followed by non-stage-specific cleavage patterns compared to stage-specific (OR = 0.545). The presence of mononucleation in embryos and the proportion of mononucleated embryos to total embryos may be positively correlated with embryonic development potential and live birth rate[11, 12]. The presence of vacuoles also affects the rate of aneuploidy and live birth of embryos[13]. But, multinucleation and vacuoles were not observed to affect the formation of transferable blastocysts in this study, possibly because the frequency of multinucleation and vacuoles was too low to be prioritized for transplantation. Therefore, it is recommended to designate multinucleation and vacuoles as special events and not prioritize their application in transplantation.

This study observed the presence of pseudo-compacted embryos (Fig. 2G), and it was reported for the first time. These embryos displayed characteristics similar to cleavage-stage embryos. This phenomenon could possibly be attributed to the physical compression caused by an increase in
blastomeres during embryonic cleavage, leading to the disappearance of cell boundaries. It suggests that the compaction observed is not a result of an increase in intercellular connections. In this study we combined pseudo-compacted embryos with cleavage-stage embryos for analysis.

Therefore, this study established an evaluation system for fourth-day embryos based on the developmental stage, fragmentation ratio, stage-specific cleavage patterns, and normal fertilization in embryos on fourth day, dividing embryos into five grades: A, B, C, D, and E. The transferable blastocysts rate of grade A embryos significantly increased (93.23% vs. 68.51%, 51.89%, 32.17% and 9.75%), while the other grades decreased sequentially, and there were significant differences between different grades (p < 0.05). In addition to the transferable blastocysts rate, the formation rate of high-quality blastocysts was also analyzed. It was found that the formation rate of high-quality blastocysts was significantly higher in grade A embryos compared to the rates of other grades. Similar to the transferable blastocysts rate, the rates of high-quality blastocyst formation decreased progressively with each grade, indicating a decrease in overall embryo quality. The differences between different grades were also statistically significant, emphasizing the impact of embryo grading on the development of high-quality blastocysts. Berger DS, et al. compared two groups, Group A and Group B, which received different types of sperm for intracytoplasmic sperm injection (ICSI)[20]. Group A received teratozoospermic sperm (0–2% normal), while Group B received dysmorphic sperm (5–13% normal). It was found that by day 4, a higher percentage of embryos in Group B had compacted compared to Group A. This suggests that the use of dysmorphic sperm may have a positive impact on the early embryonic development. Therefore, we adjusted the mode of fertilization and the fourth day 4 scoring system had similar predictive value for transferable blastocyst formation and high quality blastocyst formation. The rate of transferable blastocyst formation gradually decreased in grade B (adjusted odds ratio, aOR:0.412, P < 0.001), grade C(aOR: 0.152, P < 0.001), grade D(aOR: 0.095, P < 0.001) and grade E(aOR: 0.034, P < 0.001) relative to grade A (Table 3), and also decreased for high-quality blastocyst formation(Table 4). This trend remains when embryos are classified into IVF and ICSI (Fig. 3).

The four embryo evaluation systems have predictive significance for the formation of transferable and high-quality blastocysts (P < 0.05). The evaluation system proposed in this study had the highest area under the curve (AUC = 0.817, P < 0.001) for predicting the formation rate of transferable blastocysts, followed by Feil 2008 (AUC = 0.716, P < 0.001), ESHRE 2011 (AUC = 0.643, P < 0.001), and Gemma 2015 (AUC = 0.739, P < 0.001). The evaluation system proposed in this study had the highest AUC (AUC = 0.780, P < 0.001) for predicting the formation rate of high-quality blastocysts, followed by Feil 2008 (AUC = 0.726, P < 0.001), ESHRE 2011 (AUC = 0.667, P < 0.001), and Gemma 2015 (AUC = 0.705, P < 0.001). With the development of imaging technology, time-lapse imaging technology allows for tracing the developmental parameters of embryos, which can capture the development of fourth-day embryos well[18, 21–23]. This study comprehensively considered the impact and weight of various parameters on blastocyst formation through regression analysis, taking into account the effects of stage-specific cleavage patterns and normal fertilization. Those may be the main reasons for the more accurate prediction of embryonic developmental potential using the fourth day embryo evaluation system in this study.
However, it is important to note that when transferring two embryos, the grading of the two embryos may differ. As a result of selectively transferring a high-quality embryo, there was a lack of clinical pregnancy data for both general and poor-quality embryos. Additionally, the quality of the endometrium plays a significant role in determining live births. To obtain a more precise assessment of the developmental potential of embryos, this study primarily focuses on blastocyst formation. However, it is important to acknowledge that without additional clinical pregnancy and live birth data, further observation is necessary to determine the impact of the Day 4 evaluation system on these outcomes. Considering the simplicity and easy generalizability of the evaluation system, the necessity of embryo kinetic parameters based on time-lapse imaging technology is not emphasized in this study's embryo evaluation system, although data tracing was conducted using time-lapse imaging technology.

**Conclusion**

This study observed the presence of pseudo-compacted embryos, and it was reported for the first time. The evaluation system developed in this study, which incorporates multiple morphological parameters, had demonstrated high predictive accuracy for the formation of transferable blastocysts and high-quality blastocysts. These findings have the promise to contribute to the advancement of IVF-ET techniques and improve the selection of embryos with higher developmental potential.

**Abbreviations**

- **IVF-ET**  In vitro fertilization and embryo transfer
- **ROC**  The receiver operative characteristics
- **BNR**  The blastomere number ratio (D4 blastomere number / D3 blastomere number)
- **AUC**  Area under curve
- **2PN**  Two-pronuclear
- **1PN**  Monopronuclear
- **0PN**  Nonpronuclear
- **AR**  Abortion rates
- **LBR**  Live birth rates
- **GnRH**  Gonadotropin-releasing hormone
- **hCG**  Human chorionic gonadotropin
Declarations

Acknowledgments

We would like to express our gratitude to all those who helped us during the writing of this manuscript. Thanks to all the peer reviewers for their opinions and suggestions.

Author Contributions

BZ, FD and SW contributed to conception and design of the study. FD, HD and YG organized the database. HD, LT and GL performed the statistical analysis. YG and FD wrote the first draft of the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Funding Acquisition: The work was supported by the Health Commission of Hebei Province (Grant No. 20240234)

Data availability

The datasets generated and analyzed during the current study are not publicly available, since the dataset will be used for other retrospective analyses. The data are available from the corresponding author upon reasonable request.

Conflicts of interest: All authors declare no conflict of interest.

Ethics approval and consent to participate

All procedures used in this study were conducted in accordance with the principles of conducting experiments with human participants as outlined in the Declaration of Helsinki. This study adopted the consent of the Ethics Committee of Xingtai Infertility Specialized Hospital (Approval Number: 2021-ER-06). All process in IVF-ET were obtained by written informed consent.

Consent for publication

Not applicable.

Competing interests
The authors declare no competing interests.

** Conflict of interests 

All authors declare no conflict of interest.

**References**


Tables

Table 1 Demographic and clinical characteristics
### Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Whole cohort (N=199)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age of women</strong> (years)</td>
<td>31 (28-33)</td>
</tr>
<tr>
<td><strong>Body mass index</strong> (kg/m²)</td>
<td>24.2 (21.6-27.3)</td>
</tr>
<tr>
<td><strong>Primary infertility rate (%)</strong></td>
<td>94 (47.24%)</td>
</tr>
<tr>
<td><strong>Duration of infertility</strong> (years)</td>
<td>4 (2-5)</td>
</tr>
<tr>
<td><strong>Basic follicle stimulating hormone</strong> (IU/L)</td>
<td>6.18 (5.15-7.58)</td>
</tr>
<tr>
<td><strong>Basic luteinizing hormone</strong> (IU/L)</td>
<td>3.94 (2.67-5.41)</td>
</tr>
<tr>
<td><strong>Anti-mullerian hormone</strong> (ng/ml)</td>
<td>3.33 (2.24-4.68)</td>
</tr>
<tr>
<td><strong>Total gonadotropin dose</strong> (IU)</td>
<td>2700 (2250-3300)</td>
</tr>
<tr>
<td><strong>Duration of Gn stimulation</strong> (days)</td>
<td>11 (10-12)</td>
</tr>
<tr>
<td><strong>Estradiol on the trigger day</strong> (ng/ml)</td>
<td>2000 (1579-2810)</td>
</tr>
<tr>
<td><strong>Progestin on the trigger day</strong> (ng/ml)</td>
<td>0.72 (0.56-0.88)</td>
</tr>
<tr>
<td><strong>Rate of intracytoplasmic sperm injection (%)</strong></td>
<td>52 (26.13%)</td>
</tr>
<tr>
<td><strong>Number of oocytes retrieved</strong></td>
<td>13 (10-16)</td>
</tr>
<tr>
<td><strong>Transferable blastocyst formation rate (%)</strong></td>
<td>512/1235 (41.46%)</td>
</tr>
<tr>
<td><strong>High-quality blastocyst formation rate (%)</strong></td>
<td>165/1235 (13.36%)</td>
</tr>
</tbody>
</table>

Continuous data are expressed as median (25–75th percentile), while categorical data are expressed as N(%)

---

**Table 2** Comparison of transplantable blastolyst formation rates among different morphologic parameters
<table>
<thead>
<tr>
<th>Morphologic parameters</th>
<th>Transferable blastocyst formation rate (%)</th>
<th>Odds ratio</th>
<th>Confidence interval (95%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>The stage of development</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BNR≥1.2</td>
<td>17/263(6.46%)</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Early blastocysts vs BNR≤1.2</td>
<td>135/148(91.22%)</td>
<td>139.887</td>
<td>65.894-296.968</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>≥50% compaction vs BNR≤1.2</td>
<td>160/255(62.75%)</td>
<td>22.687</td>
<td>13.034-39.490</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>&lt;50% compaction vs BNR≤1.2</td>
<td>133/287(46.34%)</td>
<td>11.634</td>
<td>6.749-20.055</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>BNR≥1.2 vs BNR≤1.2</td>
<td>67/299(22.41%)</td>
<td>3.890</td>
<td>2.216-6.829</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fragmentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥50%</td>
<td>10/90(11.11%)</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>≤10% vs ≥50%</td>
<td>383/743(51.55%)</td>
<td>8.511</td>
<td>4.342-16.682</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10%-25% vs ≥50%</td>
<td>81/229(35.37%)</td>
<td>4.378</td>
<td>2.150-8.915</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>25%-50% vs ≥50%</td>
<td>38/173(21.97%)</td>
<td>2.252</td>
<td>1.064-4.765</td>
<td>0.034</td>
</tr>
<tr>
<td>Stage-specific versus non-stage-specific cleavage patterns</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage-specific</td>
<td>485/1141(42.51%)</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Non-stage-specific</td>
<td>27/94(28.72%)</td>
<td>0.545</td>
<td>0.343-0.865</td>
<td>0.010</td>
</tr>
<tr>
<td>Multinuclear or vacuolization</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>465/1119(41.55%)</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yes vs No</td>
<td>47/116(40.52%)</td>
<td>0.958</td>
<td>0.649-1.414</td>
<td>0.829</td>
</tr>
<tr>
<td>Normal fertilization</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2PN</td>
<td>501/1180(42.46%)</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Non-2PN vs 2PN</td>
<td>11/55(20%)</td>
<td>0.339</td>
<td>0.173-0.663</td>
<td>0.002</td>
</tr>
</tbody>
</table>

**Table 3** Different grades of day 4 embryo and their ability to develop transferable blastocysts
### Table 4 Different grades of day 4 embryo and their ability to develop High-quality blastocysts

<table>
<thead>
<tr>
<th>Grade</th>
<th>Transferable blastocyst formation rate</th>
<th>Crude</th>
<th>Adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Odds ratio</td>
<td>Confidence interval (95%)</td>
</tr>
<tr>
<td>A</td>
<td>124/133 (93.23%)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>B vs. A</td>
<td>124/181 (68.51%)</td>
<td>0.158</td>
<td>0.075-0.3329</td>
</tr>
<tr>
<td>C vs. A</td>
<td>151/291 (51.89%)</td>
<td>0.078</td>
<td>0.038-0.160</td>
</tr>
<tr>
<td>D vs. A</td>
<td>74/230 (32.17%)</td>
<td>0.034</td>
<td>0.017-0.072</td>
</tr>
<tr>
<td>E vs. A</td>
<td>39/400 (9.75%)</td>
<td>0.008</td>
<td>0.004-0.017</td>
</tr>
</tbody>
</table>

Adjusted: ICSI

### Table 5 The coverage rates of day 4 embryos by different evaluation systems
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>The coverage rate</td>
<td>100%</td>
<td>100%</td>
<td>88.02%</td>
<td>89.30%</td>
</tr>
<tr>
<td></td>
<td>(1235/1235)</td>
<td>(1235/1235)</td>
<td>(1087/1235)</td>
<td>(1235/1383)</td>
</tr>
</tbody>
</table>

**Figures**

**Fig. 1 Flow of selection of the study population**

The 199 couples who met the inclusion and exclusion criteria received 2,567 oocytes

1332 oocytes were excluded
- Not MII n=513
- >3PN n=291
- Unfertilized oocytes n=122
- Culture was not continued until the fifth day n=406

1235 embryos were included for analysis

**Figure 1**

See image above for figure legend
Fig. 2 Grading of embryos on day 4. (A) Early blastocyst (Grade A). (B) \( > 50\% \) compaction and fragmentation \( < 10\% \). (C) \( > 50\% \) compaction and fragmentation 10-50\%. (D) 10-50\% compaction and fragmentation \( < 10\% \). (E) 10-50\% compaction and fragmentation 10\%-50\%. (F) No compaction. (G) False compaction. (H) Fragmentation \( > 50\% \).

Figure 2

See image above for figure legend

Fig. 3 Blastocyst formation rates of different embryo grades on day 4. (A) Embryos were obtained from the conventional in vitro fertilization, *** p<0.001. (B) Embryos were obtained from the intracytoplasmic sperm injection, *** p<0.001.

Figure 3

See image above for figure legend
### Table 1

<table>
<thead>
<tr>
<th>Evaluation system</th>
<th>Area under the curve (AUC) (95% CI)</th>
<th>P value[^a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>XT2023</td>
<td>0.817 (0.793–0.840)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Feil2008</td>
<td>0.776 (0.75–0.802)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ESHRE2011</td>
<td>0.643 (0.607–0.679)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gemma2015</td>
<td>0.739 (0.708–0.771)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

[^a]: Compared with null hypothesis (diagonal line, i.e. AUC of 0.5). *p*<0.05 (statistically significant)

---

**Figure 4**

Receiver operating characteristic (ROC) curve of different evaluation systems and transferable blastocyst.

See image above for figure legend
### Evaluation System

<table>
<thead>
<tr>
<th>Evaluation System</th>
<th>Area under the curve (AUC) (95% CI)</th>
<th>P value (^\wedge)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XT2023</td>
<td>0.780 (0.742–0.817)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Feil2008</td>
<td>0.726 (0.686–0.765)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ESHRE2011</td>
<td>0.667 (0.608–0.726)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gemma2015</td>
<td>0.705 (0.650–0.759)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\(^\wedge\)Compared with null hypothesis (diagonal line, i.e. AUC of 0.5). *p<0.05 (statistically significant)

**Figure 4** Receiver operating characteristic (ROC) curve of different evaluation systems and high-quality blastocyst.

**Figure 5**

See image above for figure legend.