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# Correlation between single embryo transfers (SET) on day five and day six, and clinical pregnancy rate in cases of intracytoplasmic sperm Injection (ICSI)

**Abdelrhman Saber Abdelnaby[1], Mohammed Ahmed Faris[2], Ahmed Kadry Gaber[3], Sayed Bakry Ahmed[4]**
[1]Queens Fertility Center-New Cairo, Egypt
[2] Department of Obstetrics and Gynecology - Faculty of Medicine, Ain Shams University, Cairo, Egypt
[3] International Islamic Center for Population Studies and Research- Al-Azhar University, Darassa, Cairo, Egypt
[4] Center for Genetic Engineering, Al-Azhar University, Nasr City, Cairo, Egypt

**Abstract**
**Objective (s):** one of the best tools to achieve high pregnancy rates is a successful embryo selection. The objective of this study is to assess the embryo grades on day five/six, and the potential of slow developing embryos.
**Rational:** morphological assessment of embryos is a routine practice in IVF lab. Embryo quality is mainly examined by microscope at certain times using morphological scoring systems, and it is still the best strategy of selection of embryos.
**Design:** prospective analysis of single embryo transfer (SET).
**Setting**: study was conducted in Queens Fertility Center, *Cairo, Egypt*.
**Subject:** women who had fresh single Embryo Transfer (ET) on day five/six, Age 20 – 35 years old were included. Embryo scoring was done using Gardner grading system, besides, 5 grades were added. Only non-compacting cells embryos were excluded.
**Intervention (s):** chromosomal analysis of each grade may add more prediction.
**Main Outcome Measure (s):** clinical pregnancy.
**Result (s):** analysis of single embryo transfer (n=326) revealed positive clinical pregnancy 138 (42.3%). Analysis of difference resulted single embryo with expansion or hatching (EH) stages or 1, 5, or 6 were significantly associated with higher clinical pregnancy rates.
**Conclusion:** This study concluded that, no a specific grade should be excluded except for arrested embryos, as no golden grade, however, prediction of pregnancy might be calculated according to our findings.

**Keywords:** embryo grading–pregnancy outcome–embryo quality–clinical pregnancy

## Introduction

The quality of embryo is the only parameter independently associated with ongoing pregnancy and mainly examined by microscope at certain times using morphological scoring systems, and it is still the best strategy for the selection of embryos **[1-2]**. Assessment of embryos using morphological and developmental criteria is the conventional method and inefficient indicator for pregnancy potential [3]. Combined factors was postulated to predict outcome as zygote quality, and in-vitro development because of embryo morphology alone isn't a predictive tool for IVF outcome [4]. However, both cytogenetic and molecular analysis could be performed, and the result may be used to score embryos. Since, chromosomal investigation of the embryo could provide embryologists with an additional marker for embryo quality which is partially independent of embryo morphology or using both morphological and chromosomal [5, 7].

Correlation between embryo morphology and pregnancy occurrence after IVF was assured, from 1993 a study of morphological assessment of embryos was directly correlated with implantation rate [8]. Morphological assessment leads to reduce the potential of multiple pregnancies through selecting fewer embryos at embryo at ET [9]. Top-quality embryos could be chosen by measurement of the cleavage rate and degree of fragmentation [10]. Early cleavage has shown to correlate with embryo quality, with the like hood to reach to blastocyst stage [11].

Five or six days post insemination, we have two choices for (ET), firstly; active, which means morphological and chromosomal assessment. Secondly; indirect by culturing the embryos as long as possible, in which unsuitable embryos will be arrested [5]. Many stages and grades of embryos were identified, dealing with this enormous numbers of forms make it difficult to deal with. There are many systems identified to make scoring easier. Embryo grading systems are useful in the prediction of embryo implantation [12]. Thus, the main aim of this study is to investigate the relationship between individual morphology parameters and clinical pregnancy outcome. In addition to adopt logistic regression model for prediction of the probability of which embryo could be transferred based on blastocyst morphology scoring.

## Patients and Methods

### Design of Study

A prospective analysis, observational study of Single Embryo Transfer (SET) was conducted in a Queens Fertility Center *Cairo, Egypt*. Inclusion criteria were women who had fresh (SET) on day 5/6, Age 20 – 35 years old. While Cases of well-known poor endometrial receptivity, uterine anomalies, and who had systemic conditions that may predispose to miscarriage were excluded. Regarding data documentation all demographic and initial characteristics of women included, type of the protocol used for ovarian stimulation, number of oocytes retrieved, number of embryos transferred, and their grading were documented daily during work. Women included were contacted directly in the center to record either positive or negative pregnancy.

**Controlled Ovarian Stimulation (COH)**

Hormonal profile including estradiol (E2), luteinizing hormone (LH), prolactin (PRL), and follicular stimulating hormone (FSH) were monitored. Doses of initial gonadotropin used for ovarian stimulation is 75-300 IU/ml adjusted according to patient's age, Body Mass Index (BMI), previous response to stimulation and FSH concentrations on day 2 or 3 of menstruation. Patients underwent ovarian stimulation by rFSH (Fostimon; Serono Laboratories Inc, USA) with gonadotrophin-releasing hormone (GnRH) agonist pituitary down-regulation by menotrophen (Merional, IBSA International) or GnRH antagonist by cetrorelix acetate (Cetrotide, EMD Serono, USA), and trigger human chorionic gonadotrophin (HCG) (Choriomon, IBSA International). It was given when two or more follicles reach a diameter of ≥18 mm. Egg retrieval was done by trans-vaginal ultrasound 36 h after HCG administration. After (ET) patients were asked to administer 80 mg/day of progesterone (Prontogest, Marcyrl, Egypt) till clinical pregnancy.

**Follicular Aspiration and Semen Preparation**

Follicles were retrieved from the follicular fluid and poured in a Falcon dish **(**Falcon, USA) with 3 ml of HEPES-MOP'S buffer medium (Multipurpose Handling Medium, MHM® with Gentamicin, Irvin Scientific). Once all oocytes were retrieved, they were washed in 3 ml of same buffer. And semen was prepared discontinuous density gradients (Isolate, Irvin Scientific), and simple washing by (Sperm wash, Irvin Scientific).

**Intra Cytoplasmic Sperm Injection and Culture System**

Cumulus oocytes were denuded after one hour of retrieval by two alternative steps; Biochemical in which buffered Hyaluronidase Solution 80 IU (90101, Irvin Scientific) in 100 µl for 30-45 seconds. Secondly; mechanical in which flexible pipettes used for denuding by gently multiple movements with appropriate diameter 170 µl (Cook® Flexipet® Pipette, Cook® medical). Upon completion, oocytes were graded for maturity then placed into the incubator (37°C) for 1–2 h until ICSI. 38-39h post HCG ICSI was started, the first polar body either at the 6 o'clock or 12 o'clock position. A single spermatozoon was aspirated into the cytoplasm and then back into the needle, the spermatozoon and aspirated cytoplasm were expelled to the oocyte. The oocytes were incubated in single step media (Global® TOTAL, life global group) at 37°C in 6.9% $CO_2$, 5.0% $O_2$, 98% humidity, PH 7.24 to 7.28 in (Forma water jacket incubator, Thermo fisher scientific, USA).

**Pronucleus Check, and Embryo Grading**

Zygots were evaluated for pronucleus (PN) 16–18 hours after injection. Fertilization was considered normal when only two (PN) were seen, abnormal were excluded.

Assessment two days post-insemination, the evaluation considered the number of blastomeres, degree of fragmentation, multi-nucleation, and symmetry of blastomeres. On day five, grading was conducted according to Gardner grading system/ Gardner – Schoolcraft grading system [13]**.** Fourteen days later, biochemical test for β-HCG level was measured. After 7 weeks female was asked for ultrasound to record clinical pregnancy.

**Statistics**

Our results were statistically analyzed using Statistical Package for Social Science (SPSS®) software (Statistical Package for Social Science, version 23, Illinois, USA). Results were expressed as means ±SD. Analysis of difference using Mann-Whitney's U-Test and Chi-Squared Test. Differences were statistically significant when $p < 0.05$.

**Results & Discussion**

**Results**

Included range of female 20 – 35 years with Mean ± SD (28.57 ± 4.08). The major of included indications was male factor (44.8%), while the minor indication was gender selection. Table (1) shows the characteristics of inclusion.

Cycles stimulated using GnRHa Protocol represented (87%), and GnRHant Protocol were (12.8%). Wide range of number of retrieved oocytes (1-40) median 10 (6-14) as shown in table (2).

(SET) analysis (n=326) revealed positive clinical pregnancy 138 (42.3%). Analysis of difference using Mann-Whitney's U-Test and Chi-Squared Test resulted that single embryo with expansion or hatching (EH) stages or 1, 5, or 6 were significantly associated with higher clinical pregnancy rates. On the contrary, transfer of a single embryo with stages of morula (20%), compacting cells (30.9%), and cavitating morula (25%). There was no significant association between clinical pregnancy outcome and inner cell mass (ICM) (A) grade (63.6%) P=0.372 (NS), (B) grade (40%) P=0.134 (NS). Otherwise, trophoectoderm (TE) grade (A) showed significantly positive clinical pregnancy (67.7%) P=0.027 (S). Table (3) shows the difference between women who had positive clinical pregnancy outcome and those who did not regarding EH Stage.

**Table 1:** initial characteristics.

| **Age (years)** | |
|---|---|
| Range | 20 – 35 |
| Mean ± SD | 28.57 ± 4.08 |
| Duration of Infertility (years) | |
| Range | 2 – 21 |
| Median (IQR) | 7.95 ± 3.86 |
| Indication for ICSI | |
| Male Factor | 146 (44.8%) |
| Tubal Factor | 30 (9.3%) |
| Endometriosis | 31 (9.6%) |
| Anovulation | 26 (8.4%) |
| Unexplained Infertility | 41 (12.6%) |
| Combined Factors | 36 (11.1%) |
| Gender Selection | 14 (4.2%) |

SD standard deviation –IQR interquartile range - IVF *in vitro* fertilization - ICSI intracytoplasmic sperm injection

Data presented as range, mean ± SD; or number (percentage)

**Table 2:** ICSI Cycle Characteristics

| Protocol of COH | |
|---|---|
| GnRHa Protocol | 284 (87.2%) |
| GnRHant Protocol | 42 (12.8%) |
| Source of Sperm Harvesting | |
| Fresh Ejaculate | 262 (80.4%) |
| Frozen Ejaculate | 15 (4.5%) |
| Fresh TESE | 6 (2%) |
| Frozen TESE | 30 (9.2%) |
| Fresh FNA | 9 (2.7%) |
| Frozen FNA | 4 (1.3%) |
| No. of Retrieved Oocytes | |
| Range | 1 – 49 |
| Median (IQR) | 10 (6 – 14) |

ICSI intracytoplasmic sperm injection – COH controlled ovarian hyperstimulation GnRHa gonadotropin releasing hormone agonist GnRHant gonadotropin releasing hormone antagonist TESE testicular sperm extraction – FNA fine needle aspiration Data presented as number (percentage); or range, median (IQR)

**Table 3:** Difference between Women who had Positive Clinical Pregnancy Outcome and Those who did Not among Women who had Single Embryo Transfer regarding EH Stage, ICM and TE Grades

| Single Embryo Transfer (n=326) | Positive Clinical Pregnancy (n=138) | Negative Clinical Pregnancy (n=188) | RR (95% CI) | P |
|---|---|---|---|---|
| EH Stage | | | | |
| 1 (n=28) | 18 (64.3%) | 10 (35.7%) | 1.6 (1.17 to 2.17) | 0.014 (S) |
| 2 (n=28) | 16 (57.1%) | 12 (42.9%) | 1.4 (0.99 to 1.98) | 0.097 (NS) |
| 3 (n=16) | 10 (62.5%) | 6 (37.5%) | 1.51 (1.01 to 2.26) | 0.094 (NS) |
| 4 (n=20) | 6 (30%) | 14 (70%) | 0.7 (0.35 to 1.38) | 0.249 (NS) |
| 5 (n=40) | 28 (70%) | 12 (30%) | 1.82 (1.42 to 2.34) | <0.001 (HS) |
| 6 (n=4) | 4 (100%) | 0 (0%) | 2.4 (2.11 to 2.73) | 0.019 (S) |
| Contracted (n=6) | 4 (66.7%) | 2 (33.3%) | 1.59 (0.89 to 2.84) | 0.223 (NS) |
| Cavitating Morula (n=32) | 8 (25%) | 24 (75%) | 0.57 (0.31 to 1.04) | 0.037 (S) |
| Abnormal Cavity (n=2) | 2 (100%) | 0 (0%) | 2.38 (2.1 to 2.71) | 0.098 (NS) |
| Morula (n=40) | 8 (20%) | 32 (80%) | 0.44 (0.23 to 0.83) | 0.002 (S) |
| Compacted cells (n=110) | 34 (30.9%) | 76 (69.1%) | 0.64 (0.47 to 0.88) | 0.003 (S) |
| ICM Grade | | | | |
| A (n=66) | 42 (63.6%) | 24 (36.4%) | 1.27 (0.7 to 2.31) | 0.372 (NS) |
| B (n=10) | 4 (40%) | 6 (60%) | 0.62 (0.28 to 1.35) | 0.134 (NS) |
| C (n=2) | 2 (100%) | 0 (0%) | 1.65 (1.38 to 1.98) | 0.257 (NS) |
| TE Grade | | | | |
| A (n=62) | 42 (67.7%) | 20 (32.3%) | 1.81 (0.94 to 3.48) | 0.027 (S) |
| B (n=6) | 2 (33.3%) | 4 (66.7%) | 0.52 (0.17 to 1.64) | 0.139 (NS) |
| C (n=10) | 4 (40%) | 6 (60%) | 0.62 (0.28 to 1.35) | 0.134 (NS) |

EH expansion or hatching – ICM inner cell mass – TE trophoectoderm Data presented as number (percentage) RR risk ratio and its 95% confidence interval
1 Analysis of Difference using Mann-Whitney's U-Test
2 Analysis of Difference using Chi-Squared Test
S significant – HS highly significant – NS non-significant

## Discussion

Three independent factors in blastocyst may affect outcome. EH, TE, and ICM were independently measured if any has influence. Assessment of shape and size of embryo may provide more insight to predict outcome. A lot of scoring method s have been developed. Gardner's is widely using in IVF laboratories. This system, which has been shown to provide higher implantation rates and better selection than other systems [14].

### Gardener's Included Stages

Previous results of day five embryos recorded high implantation rate of expanded and Hatched blastocysts they achieved (64%) and (63%) respectively [15]. In 2013, a retrospective analysis on 1860 blastocyts, EH 5 showed the highest pregnancy rate among other grades (41.8%) [16]. But it was thawed embryos which may explain low pregnancy rate, however, the highest between all other EH grades. Other study recommended that the most potential parameter in blastocyst is EH stage, and EH 5 of SET showed (50%) clinical pregnancy [17].

However, a frozen embryo transfer FET research revealed lowest pregnancy of EH 5, then 6 among included grades [18]. And one more recent showed poor outcome of EH 5 also, it achieved (33.3%) compared to EH 4 (57.8%) [19]. In this study, it's noted that only 3 embryos were included in EH 5, moreover, probability of damage after thawing because of grading was done before vitrification. Early blastocyst is the smallest EH between all blastocysts. It was more likely to achieve low pregnancy compared to EH 5. It was the lowest pregnancy [20], and [17]. Other studies reported higher results [15]. EH2 achieved higher in euploid embryos [19].

It has proven from current analysis and [15, 17, 21, 22] that EH grade correlates with pregnancy rate. EH 5 is the best for transfer, EH 6 and 1 have high clinical pregnancy. On contrary, some papers postulated that EH grade didn't affect pregnancy [18, 19, 20]. In our findings, women who received single embryo transfer of EH 5 and 6 were the highest but EH 6 was only 6 embryos.

Morphology of TE may be the most important parameter when selecting a single blastocyst for transfer, it is the strongest morphological predictor for outcome A grade was the highest then B and C [18, 15] In contrary [19, 21] didn't find any relation. TE morphology may affect the rates of ongoing pregnancy. In our findings, TE with grade A was significantly the highest clinical pregnancy. This may be due to high numbers of cells and compaction of grade A which help in implantation. The ICM, The higher ongoing pregnancy embryos those had a grade compared to C [18, 15]. But it wasn't significantly linked with pregnancy [15]. Among poor quality or average quality embryos, ICM morphology priority should be chosen with better grade because it is a better predictor of pregnancy outcomes than other blastocyst components [19]. The relation between ICM and predicting pregnancy has been linked [17, 21] also. On the other hand, studies found that no significant association between ICM grade and clinical pregnancy outcome [15, 18, 20]. In current study, ICM wasn't important parameter when selecting SET. It's found that a grade associated (not significantly) with clinical pregnancy.

### Gardener's not Included Stages

Morula four days post insemination is normal according to the healthy developing embryo [23]. Therefore, when single elective morula was transferred on day 4, it was an alternative to single elective day 5 blastocyst [24]. Low pregnancy (15.8% for morula, and 21.1% for Cavitating morula) was recorded on day 5 [23] Cavitating morula, morula and unexpanded early blastocyst in one group achieved 30% implantation rate [25]. According to our results, transfer of a single embryo Gardner not included stages; cavitating morula, morula, or compacting was significantly associated with lower clinical pregnancy rates.

In a simplified grading system morula and Cavitating morula reported implantation, but live birth rate was higher in cavitating embryos [26]. But included cavitating and morula groups were limited as mentioned, morula or cavitating weren't significantly different from they poor blastocyst also. A similar result was reached, about 36% of morula implanted successfully, but higher miscarriage rate occurred [15]. No pregnancies were observed following transfers of 9 embryos non-cavitating on day five [27]. But only 3 transfers were considered. Clinical pregnancy of cavitating morula was slightly higher than morula of included embryos in the previous study. In comparison of transferring of fresh embryos and culturing for one more day, higher blastulation rate of Cavitating morula was recorded than morula, however, low pregnancy achieved after cryopreservation [23]. Many studies added compacting embryos to morula or compacting morula [25, 27] but it was separated here to assess their potential. Slow developing embryos might be by the action of chromosomal abnormality, it was stated that auto-correction of late developed embryos was done, the progression of normal cells exceeded from (12.5%) on day six to (47.8%) on day twelve [28]. Low clinical pregnancy was achieved, there were transferred early day five. Their potential was low, however, they should be considered after patients counseling about low outcome instead of excluding if they were the only available grades. And Non-compact Cells embryos on day 5 were excluded from current study. It was considered as arrested embryos [25].

## Conclusion

EH and TE were the most blastocyst variables may influence outcome, no specific grade should be excluded except for arrest embryos, as no golden grade, however, Otherwise, ICM grade was not significant. The correlation between morphological grading of day five and/or day six embryos and clinical pregnancy rate might be calculated according to previous finding. We suggest a chromosomal analysis of each grade.

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