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# Euploidy Predictability of Human Blastocyst Inner Cell Mass and Trophectoderm Grading

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## Abstract

**Background:** The selection of viable, potentially normal, embryos is vital to successful embryo transfers. Subjective morphology grading of blastocysts has been the most accepted and reliable standard to judge embryo quality. However, good to excellent embryo development alone does not necessarily insure euploidy. Our objective was to determine if independent quality grades for blastocyst inner cell mass (ICM) and trophectoderm (TE) can predict early embryo euploidy. Secondly, we aimed to develop a comparison chart for preference of blastocyst quality grades utilizing both ICM and TE grades to improve the decision making process.

**Methods and Findings:** Elective TE biopsy patients (n=139) underwent pre -implantation genetic screening (PGS) for aneuploidy screening with subsequent embryo cryopreservation. The ICM/TE of day 5 and 6 blastocysts were independently graded (n>1200 blastocysts) from A to C: A=good to excellent, B=fair, and C=poor quality. All blastocysts achieved full blastocoele expansion prior to grading, laser-assisted blastocyst biopsying of the TE and then microSecure vitrification. Euploid embryos were subsequently selected for warming (>30 days post-PGS) and embryo transfer in 2014. Aneuploidy and pregnancy results were stratified by blastocyst grades. Combined quality grades determined an overall blastocyst grade and were further compared to evaluate preferences for euploidy. Chi-square analysis was used to determine significance.

**Results:** Independent grade "A" blastocysts resulted in higher ($p<0.05$) euploidy predictability (56-62%) compared to "B" quality blastocyst (37-43%). Overall blastocyst quality revealed the TE grade was more predictive of euploidy. Upon warming, 100% embryo recovery and survival was achieved applying vitrification. Cumulative implantation and ongoing pregnancy rates were high at 82% and 78% respectively, with an overall low risk of spontaneous abortion (SAB; 5%). When comparing all quality grades of transferred blastocysts, no significance was observed for implantation, ongoing pregnancy or SAB percentages.

**Conclusions:** Independent quality grades analysis indicated higher euploidy predictability given to better quality grades. Both ICM and TE grades show significance for euploidy predictability, with "A" quality TE being the preferred embryo selection criteria. Yet, the effectiveness of subjective morphological grading to optimize pregnancy outcomes is limited, as even fair to poor quality blastocysts may still be genetically normal. We have shown that high implantation and live birth rates are attainable, independent of the combined quality grade, once a PGS euploid blastocyst is transferred. Thus, it is unlikely that non-invasive morphology or morphokinetic measurements can ever match the accuracy of embryo biopsy, PGS euploid determinations for successful single embryo transfer.

**Keywords:** Blastocyst; Trophectoderm; Morphological grading; Euploidy; Predictability

# Introduction

In vitro fertilization success is highly dependent on proper embryo selection for transfer [1]. Embryo selection is imperative for improved pregnancy rates, but relies on variable technician and program dependent assessments [2]. Subjective morphology grading [1] has been the most accepted and reliable standard to judge embryo quality. Recently, morphokinetic studies utilizing time-lapse imaging have provided more accurate cleavage timing determinations with increased information to assess possible detrimental patterns of development [2]. Although morphokinetics analysis can lend itself to a ranking of predictability for potential euploidy, it does not diagnose aneuploidies [3]. Thus, the use of non-invasive time lapse image analysis is unlikely to discriminate aneuploid from euploid embryos and doubtfully approach the accuracy of preimplantation genetic screening (PGS) with array comparative genomic hybridization (aCGH) [4]. The need to more accurately choose embryos with a high probability for success proves difficult utilizing morphologic assessments alone [5].

Advancements in embryo culturing facilitated improved blastocyst development [1]. Blastocyst grading involves separate evaluation of: 1) the inner cell mass (ICM) and 2) the trophectoderm (TE) [6]. With two independent blastocyst grades, it has been debated whether preference should be given to the ICM or TE. Ahlstrom and coworkers [7] recently provided strong embryo transfer evidence that an "A" quality TE grade versus ICM grade is more predictive of live birth success based on more than a thousand frozen-thawed, non-PGS transfer cycles. When euploidy status is not determined, morphology and morphokinetics are the leading options available for embryo selection [3].

With an international movement toward single embryo transfers, the goal of this study was to determine if independent quality grades for blastocyst ICM and TE can predict early embryo euploidy. Utilizing the predictive ability of blastocyst morphology grades, we sought to develop a helpful comparison chart utilizing both ICM and TE quality to factor euploidy probabilities.

Furthermore, when transferring PGS screened blastocysts, we aimed to provide evidence that implantation and clinical pregnancy rates are comparable regardless of morphologic assessments and overall blastocyst quality grades.

# Methods

## Patients selection and study design

TE biopsy was developed in our SCIRS laboratory as part of an IRB approved protocol. Consenting SCIRS patients electively selected TE biopsy with aCGH/PGS for aneuploidy determination, vitrification and subsequent transfer. All patients were fully informed of the potential risks of blastocyst biopsying and PGS procedures. Furthermore, they knowingly agreed that their data could be reviewed, summarized and published in a de-identified manner as required by professional/IRB standards. The data analyses were approved as IRB exempt research. Patient's ages ranged from 25-45 years old (mean age=35.7 ± 4.1) which included autologous (n=122) and donor (n=17) oocyte cycles. All vitrification transfer cycles in this study occurred between January 1, 2014 to 2015.

Retrospective analysis of over 1200 screened blastocysts were performed to compare independent (e.g., A) and combined (e.g., AB) morphologic quality grades. Day 5 and Day 6 blastocyst ICM and TE were independently graded from A to C quality, with A=excellent, B=good-fair and C=poor [1]. All blastocysts minimally achieved full blastocoele expansion prior to grading [8]. Grading was recorded for ICM first followed by TE. Combined overall blastocyst quality grades and euploidy outcomes were recorded. ICM and TE grades were then stratified and compared independently to pregnancy and genetic outcomes. Finally, combined grades were correlated to euploidy status and a chart compiled to indicate preference for euploidy predictability. Chi-square analysis was used to delineate significant differences.

Implantation determinations were assessed by transvaginal ultrasound visualization of gestational sac(s). Ongoing pregnancies were defined as all pregnancies that achieved a heartbeat and sustained their pregnancies without miscarriage to-date.

## Embryo culture, biopsy and PGS testing

Using MCO-5M mini Sanyo/Panasonic tri-gas incubators (5% 02/5.3-6.0% $CO_2$), we group cultured up to 8 embryos per 25 uL droplet of Global™ medium (LG; Life Global, Guilford, CT) supplemented with 7.5% synthetic protein supplement under Ovoil™ (Vitrolife, Englewood, CO) until blastocyst biopsy [8]. All oocytes retrieved were evaluated for maturity and had intracytoplasmic sperm injection (ICSI) performed 3-6 hours post egg retrieval [9]. Embryos were initially evaluated on day 3; laser zona dissection was performed using a 1410-nm diode laser (Zilos-tk™; Hamilton Thorne, Beverly, MA) and embryo incubation continued until day 5/6 evaluations [8]. The zona opening created on day 3 allowed TE to prematurely rupture through a 10-12-µm furrow in the zona. The same laser was used on day 5/6 for biopsying using a combination of laser pulses and mechanical separation to achieve 3-10 TE cells [4,8] used for aCGH and NextGen PGS (Genesis Genetics, Plymouth, MI).

## Vitrification (VTF) and embryo transfer

Fair to excellent quality blastocysts (≥3 BB grade) were vitrified on day 5 or day 6 using microSecure-VTF and hyaluronate-enriched, non-DMSO VTF solutions (Innovative Cryo Enterprises, Linden, NJ). Aseptic microSecure VTF was performed using: a 3-step dilution (5 min/5 min/1 min); individual blastocysts loaded into 300 µm ID flexipettes (Cook Medical, Spencer, IL; 3 µl volume); dried flexipettes inserted tip first into prelabeled 0.3 ml CBS™ embryo straws; the straw weld sealed; and plunged directly into LN2 [10]. The cooling rate was ≈1400°C/min, while rapid warming (≈6000°C/min) was achieved by direct placement of the vitrified flexipettes into a 37°C 0.5M sucrose bath [10]. Within 10 sec, each blastocyst was pipette directly from the flexipette into an open 200 µl droplet of 1.0M sucrose solution and then transferred to a 100 µl droplet under oil for 3 min. The embryo was then serially eluted in declining sucrose solutions (T2-T4, 3 min each), before isotonic equilibration in Hepes-LG medium. Warmed blastocysts were then cultured in LG medium + protein for 1-3 h prior to vitrified ET (VFET).

All VFET cycles were hormone replacement cycles using oral estradiol, estradiol patches or intramuscular (i.m) estradiol valerate followed by i.m. progesterone in oil. Progesterone in oil was started when endometrial thickness was >8 mm after documentation of serum progesterone level of <1.0 ng/ml. VFET was performed 5 days after progesterone was started. All transvaginal ultrasound guidance ET procedures were performed by a single physician. Pregnancies were initially tested 10 d post-ET and implantation subsequently assessed by transvaginal ultrasound beginning 4 weeks later.

## Results

Independent quality grades analysis indicated blastocysts with a grade "A" resulted in higher euploidy predictability (**Table 1**). When combining grades to access overall blastocyst quality, TE grade is more predictive (**Table 2**). Upon warming, 100% embryo recovery and survival was achieved using the microSecure VTF method. Cumulative implantation and ongoing pregnancy rates were high at 82% and 78% respectively, with an overall low risk of spontaneous abortion (SAB; 5%). When comparing all quality grades of transferred blastocysts, no significance was observed for implantation, ongoing pregnancy or SAB percentages (**Table 3**). In summary, if a TE is graded as A, that embryo consistently has a higher euploidy predictability and should always be chosen for transfer (**Table 4**), if available. The latter **Table 4** chart was created to be used as a tool for euploidy prediction in laboratories performing non-PGS SET procedures. Both ICM and TE grades show significance for euploidy predictability.

**Table 1** Euploidy occurrence by individual (a) ICM and (b) TE quality grades.

| (a) ICM Grade | A | B |
|---|---|---|
| # Aneuploid | 398<br>44% | 206<br>57% |
| # Euploid | 501<br>56%* | 206<br>43%* |
| (b) TE GRADE: | A | B |
| # Aneuploid | 286<br>38% | 189<br>63% |
| # Euploid | 466<br>62%* | 318<br>37%* |

* Table 1 a/b row values are different between columns ($p<0.01$).

**Table 2** Euploidy occurrence by combined blastocyst quality grade.

| Total Blastocyst grade | AA | AB | BA | BB |
|---|---|---|---|---|
| % Euploid | 62% | 41% | 62% | 33% |

**Table 3** pregnancy outcomes by blastocyst grades.

| | % Implantation | % Ongoing pregnancy | %SAB | p Value |
|---|---|---|---|---|
| AA (n=123) | 82.90% | 77.10% | 6.80% | N/S |
| BA (n=9) | 87.50% | 87.50% | 0% | N/S |
| AB (n=16) | 77.80% | 77.80% | 0% | N/S |
| BB (n=13) | 76.90% | 76.90% | 0% | N/S |

## Discussion

In the process of successfully applying blastocyst biopsy with array based PGS testing, the need to correlate morphology to euploidy status has proven difficult. Blastocyst quality grades are subjectively applied between technicians and programs, resulting in a variety of grading protocols and preference for embryo selection. This study clearly shows in our program that good to high quality blastocysts have an increased potential for euploidy, while fair and poor quality blastocysts are more likely aneuploid. Interestingly, this study provides data to support the concept that a high quality TE is the best predictor for selecting a single blastocyst for ET to optimize pregnancy outcomes [7].

It is noted that many fair morphologically graded blastocysts prove to be euploid and that implantation rates between top quality and lower quality blastocysts showed no significance. Our data supports the assessments of Kramer and coworkers that blastocyst morphology is greatly inaccurate in diagnosing euploidy [4]. The commonality that good quality embryos are better predictors of success is not a new discovery. However, the relatively high euploidy rate of fair quality blastocysts yields to the notion that morphology is not definitive. Although blastocyst grades are predictive of potential euploidy, the morphologicial quality assessment is not absolute and some poor morphological graded blastocysts may be euploid. With high pregnancy rates attained transferring fair quality euploid embryos, it appears that genetic health is of higher importance for success than subjective embryo grading. With relevant inconsistencies between morphology and euploidy, single embryo transfer success is still reliant on PGS testing in our ART program.

As this was not a randomized clinical trial, we have identified some limitations to our study. By electively choosing to biopsy and test only blastocysts with a discernible ICM (i.e., "A" or "B" quality), the question arises whether blastocysts with top quality TE and poor/no ICM have the same predictability for euploidy and relative success? Although the degree of ICM growth in vivo from transfer to implantation is unknown, it is assumed that embryos with "C" quality ICM are at high risk for miscarriages. A better understanding of ICM development could assist programs in deciding between transfer, cryopreservation or discarding blastocysts with a poor to nonexistent grade "C" ICM. In addition, since the transfer arm of this study was not randomized and the best quality embryo available for transfer was chosen, our data is bias toward top quality embryos. It remains unclear whether blastocyst quality grades would show significance for implantation predictability if transfer selection was randomized.

When aneuploidy screening is unavailable, morphology and morphokinetics are the leading technologies available for accurate embryo transfer selection. This study has generated a blastocyst quality comparison chart to assist technicians with embryo selection based on their euploidy predictability. It is the goal of this chart to help clinics accurately "choose" embryos with an increased potential for euploidy by emphasizing TE health in the selection process. It is clear that genetic health cannot be diagnosed without invasive technologies. Finally, it appears that PGS testing of blastocysts is the ideal method to accurately

**Table 4** Combined grade comparison chart, reflecting euploidy predictability and embryo selection preferences made by row vs. column.

| Quality Grades | AA | AB | BA | BB |
|---|---|---|---|---|
| **AA** | Neutral | *S- Choose **AA** | N/S- Choose either | *S- Choose **AA** |
| **AB** | *S-Choose **AA** | Neutral | *S- Choose **BA** | N/S- Choose Either |
| **BA** | N/S- Choose either | *S-Choose **BA** | Neutral | *S-Choose **BA** |
| **BB** | *S-Choose **AA** | N/S- Choose either | *S- Choose **BA** | Neutral |

diagnose euploidy and maximize the success of single embryo transfers.

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