Can combination of Day 3 and Day 5 embryo morphology be useful to predict pregnancy in in-vitro fertilization cycles?

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Summary

Objective: To determine whether embryos having all top qualified both on Day 3 and Day 5 have higher pregnancy rates than the others. *Materials and Methods:* The study included 143 consecutive cycles were recruited in which Day 5 embryo transfer was available. Cleavage stage embryos were graded according to 1 to 4 scoring system, based on fragmentation, cell symmetry, and blastomere number. Among cleavage stage embryos, Grade 1 and Grade 2a/2b were further stratified as 'top quality' embryos to be transferred, others were defined as control group. Blastocyst stage embryos were graded from 1 to 6 according to intracellular mass (ICM) and trophectoderm (TE). Day 5 fresh embryo transfer was performed in all cases using soft catheter. Positive pregnancy test was accepted when serum beta-human chorionic gonadotrophin (ß-hCG) exceeded 20 mIU/ml. *Results:* On the cleavage stage, top quality embryo was available in 47 of 143(32.9%) cases. Of the 47 embryos, the number of cases reaching any Grade 4, 3 quality, and early blastocyst on Day 5 were 22 (46.8%), 15 (31.9%), and 10 (21.3%). The respective figures on the control group (n=96) were 33 (34.4%), 37 (38.5%), and 26 (27.1%) ($p > 0.05$). The pregnancy rates were also similar. *Conclusion:* All top qualified embryos both on Day 3 and 5 did not reveal higher pregnancy rate than the others.

Key words: Embryo morphology; Blastocyst; In vitro fertilization.

Introduction

Since the first infant was achieved via in-vitro fertilization (IVF) in 1978 [1], numerous researches have been made for better understanding the development of human embryo. In clinical practice, regarding the day of transfer, either cleavage-stage embryo or blastocyst might be preferred according to patient characteristics, number of available embryos, and local legislation. However, irrespective from the day of transfer, assigning the one to be transferred within the available embryos mainly depends on cell number and morphology.

Nowadays, single embryo transfer revealing singleton pregnancy and a healthy infant is the most desired outcome in IVF cycles. Therefore, selecting the single embryo having the highest chance of implantation is crucial to prevent multiple pregnancies while keeping the success rate at a reasonable level. Although there is enough evidence addressing improved pregnancy rate with blastocyst transfer compared with cleavage stage [2], there is paucity of data whether the former quality on Day 3 affects the morphology and implantation potential of an embryo on Day 5. Nevertheless, one may assume that, the implantation potential might present diversity within blastocysts having similar scores for morphology on Day 5 but having different scores on Day 3.

In the current study, primarily, the authors sought to determine whether embryos having all top qualified according to morphological assessment both on Day 3 and Day 5 have higher pregnancy rates than the remaining. Secondly, the authors aimed to observe the natural way of cleavage-stage embryos according to blastocyst morphology on Day 5.

Materials and Methods

Between March 2010 and September 2012, a total of 143 consecutive cycles were recruited retrospectively in which Day 5 embryo transfer was available. Frozen-thawed cycles and women that were transferred on Day 3 were excluded from the final analysis.

Controlled ovarian hyperstimulation

All patients underwent controlled ovarian hyperstimulation consisting of either luteal long gonadotrophin-releasing hormone (GnRH) agonist or fixed GnRH antagonist protocols with gonadotrophin. The starting dose of gonadotrophin was determined based on female age, antral follicle count at baseline transvaginal ultrasonography, body mass index (BMI), and previous ovarian response, if available. Ovarian response was monitored with frequent serum estradiol measurements and transvaginal ultrasonography, as described previously [3]. The criterion for human chorionic gonadotrophin (hCG) administration was the presence of two or more follicles exceeding 17 mm in diameter. Oocyte retrieval was carried out under local anesthesia using vaginal ultrasound-guided puncture of follicles 36 hours after hCG administration. Embryo transfer was performed using soft catheter under transabdominal ultrasound guidance. The luteal phase was supported by daily vaginal progesterone suppositories starting one day after oocyte pick-up.

Embryo and blastocyst grading

All oocytes are fertilized using intracytoplasmic sperm injection (ICSI). For the procedure of ICSI, the most morphologically normal motile spermatozoa were identified. Where all spermatozoa

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had morphological defects, spermatozoa with fully developed tails and grossly normal heads were injected. Vitality testing of immotile spermatozoa was not performed. The presence of fertilization was evaluated by examining oocytes 12–17 hours after injection for the presence of distinct two pronuclei and two polar bodies.

Cleavage stage embryos were graded according to a 1 to 4 scoring system, which was based on fragmentation, cell symmetry, and number of blastomere as referred by Hardarson *et al.* [4] The scoring system was as follows: Grade 1 embryo: no fragmentation with equal size homogenous blastomeres, Grade 2a embryo: <10% fragmentation with equal size homogenous blastomeres, Grade 2b embryo: unequal size blastomeres with no fragmentation, Grade 2ab embryo: unequal size blastomeres with <20% fragmentation, Grade 3 embryo: equal or unequal size blastomeres with 30-50% fragmentation, Grade 4 embryo: >50% fragmentation. Among cleavage stage embryos, whereas Grade 1 and Grade 2a / 2b were further stratified as 'top quality' embryos to be transferred, others were defined as control group.

Blastocyst stage embryos were graded from 1 to 6 according to intracellular mass (ICM) and trophectoderm (TE), as reported by Gardner and Schoolcraft [5]. In this grading system, grades refer to: Grade 1: early blastocyst (blastocoel being less than half the volume of embryo), Grade 2: blastocyst (blastocoel being greater than half the volume of embryo), Grade 3: full blastocyst (blastocoel completely fills the embryo), Grade 4: expanded blastocyst (blastocoel volume is larger than that of early embryo and zona is thinner). ICM is graded as A, B, and C. Whereas A refers to tightly packed and many cells, B describes loosely grouped and several cells. C refers to very few cells. TE is also graded as A, B, and C. While A refers to many cells forming cohesive epithelium, B signifies few cells forming loose epithelium. C includes TE with very few large cells. Women were further categorized according to the quality of transferred embryos on Day 5 as 4AA/4AB; 4BB/4BA; 3AA/3AB; 3BB/3CC, and early blastocyst. Positive pregnancy test was accepted when serum b-hCG exceeded 20 mIU/ml. Ethical approval was available from Hacettepe University.

Statistical analysis

Statistical analysis of the results was performed using statistical package for the social sciences software (SPSS version 16.0). Clinical characteristics are compared using independent samples t test. The χ^2 test and Fisher's exact test were used to analyse variables in the form of frequency tables. A p value < 0.05 was considered statistically significant.

Results

On the cleavage stage, top quality embryo was available in 47 of 143 (32.9%) cases. The mean female age, BMI, duration of infertility, and number of oocytes retrieved were comparable among patients having top quality embryo on Day 3 and controls (Table 1).

Table 1. — *Characteristics of the patients according to embryo quality on Day 3.*

	Top quality on Day 3 (n=47)	Controls (n=96)
Female age (years)	28.3 ± 3.3	28.9 ± 3.8
BMI (kg/m^2)	24.3 ± 3.7	25.3 ± 4.3
Duration of infertility (months)	67.6 ± 40.5	58.6 ± 43.3
No. of oocytes retrieved	13.2 ± 4.5	12.2 ± 6.0
Number of cells of day 3 embryos	7.8 ± 0.7	7.7 ± 1.1
Fertilization rate (%)	83	82

Data given as mean ± standard deviation, unless stated otherwise.
All comparisons are non-significant.

Of the 47 embryos, the number of cases reaching any Grade 4, 3 quality, and early blastocyst on Day 5 were 22 (46.8%), 15 (31.9%), and 10 (21.3%). The respective figures in the control group was 33 (34.4%), 37 (38.5%), and 26 (27.1%) ($p > 0.05$). On Day 5, the pregnancy rate per embryo transfer was highest when 4AA/4AB was available when compared with 3AA/3AB and early blastocyst arms (Table 2). However, success rate did not present significance when matched according to the former quality of embryos on Day 3 (Table 2).

To investigate the natural course of embryo morphology, the scores on Days 3 and 5 are given in Table 2. However pregnancy rates were similar when embryos were categorized according to the quality both on Days 3 and 5.

Discussion

To the best of the authors' knowledge, there is no study that has examined the pregnancy outcome by combining Days 3 and 5 embryo morphology. Therefore the aim of this study was to search whether the pregnancy rate could be increased by selecting the best embryo to be transferred by combining scores on both days. However, the authors failed to present any advantage of combination embryo grades in means of pregnancy rate. Of note, a top qualified embryo on Day 3 did not reveal a clear course for the quality on the stage of blastocyst. Nevertheless, only 22 of 47 top qualified embryos (46.8%) reached a Grade 4 embryo on Day 5. Similarly, of the 35 blastocyst assigned to Grade 4, 17 of them (48.6) had been scored top qualified on Day 3 and pregnancy rate did not differ; either they were all top qualified or not (Table 2). Therefore, once an embryo

Table 2. — *Pregnancy rates according to morphology on Days 3 and 5.*

	Day 5 embryo quality (n, %)					
	4AA/ 4AB	4BB / 4BA	3AA / 3AB	3BB / 3CC	Early blastocyst	Total
Top quality on Day 3 (n=47)	10/17 (58.8)	2/5 (40.0)	4/10 (40.0)	2/5 (40.0)	4/10 (40.0)	22/47 (46.8)
Controls (n=96)	12/18 (66.7)	8/15 (53.3)	4/20 (20.0)	9/17 (52.9)	8/26 (30.8)	41/96 (42.7)
Total	22/35 (62.9)[a, b]	10/20 (50.0)	8/30 (40.0)[a]	11/22 (50.0)	12/36 (33.3)[b]	63/143 (44.1)

[a]$p = 0.006$ and [b]$p = 0.018$.

reached blastocyst stage, its preceding quality on Day 3 seemed to have no impact on the pregnancy rate.

Due to the inherent maternal and neonatal risks of multiple gestations, there is a trend towards transfer of a single blastocyst on day 5 in the field of assisted reproductive technology (ART) in order to minimize multiple pregnancies. According to current legislation in Turkey after March 2010, it became obligatory to transfer one embryo in the first two cycles of IVF in patients <35 years of age and only two embryos can be transferred at maximum after the age of 35, whatever the cause of infertility is. When transferring a single embryo, finding the best embryo having the highest potential of implantation is crucial. On the cleavage stage, morphologic assessment based on cell symmetry and fragmentation is widely preferred [6] since non-morphologic tools called omics technologies (genomics, transcriptomics, proteomics, and metabolomics) has been a challenging but promising task. Despite the developments in omics, the knowledge of the protein secretome of preimplantation embryos remains limited. The combined effects of limited template, low protein expression, and lack of sensitivity of current proteomics platforms remain main hurdles [7]. To date there is no non-invasive platform, including non-invasive proteomics that has proven to be of true clinical predictive value or been examined in prospective randomized control trials to be better than current morphology based selections methods. As for metabolomics, several studies have suggested the presence of metabolic differences between embryos with different reproductive potential [8]. However the application of these technologies to a clinical setting has remained limited for a variety of reasons. Many of these technologies are expensive, require dedicated equipment and technical staff, and frequently do not produce results quick enough to allow information to be used clinically in the limited window of time acceptable for embryo transfer. Moreover, none of these technologies has ever been validated using culture media evaluated in a blinded fashion and shown to correlate with the implantation potential of embryos that have been transferred. There appears to be a clear relationship between morphologic grading of cleavage stage embryos and their implantation potential [9]. Excellent and good quality embryos (grade 1 and 2) have a higher chance of implantation compared to fair and bad quality embryos (Grades 3 and 4). With regards to blastocyst embryos, grading is based on morphology of ICM and TE as well as expansion of the blastocyst cavity [10]. Blastocyst quality has been shown to be clearly associated with implantation and pregnancy [11]; with good quality blastocysts yielding very high implantation rates and hatching blastocysts having the highest. The present data also show that highest quality of embryos at blastocyst stage have the highest pregnancy rates.

The relationship between cleavage-stage embryo quality and blastocyst quality was studied by Rjinders and Jansen [12]. They concluded that the predictive value of day 3 embryo morphology regarding subsequent blastocyst formation is limited because only 51% of the embryos that would have been preselected for transfer on day 3 could reach blastocysts on day 5. Although 47% of the good quality embryos reached the blastocyst stage, only 21% of the poor quality embryos did so on day 5 [11].

Prolonged in vitro culture and transfer at the blastocyst stage of poor quality cleavage-stage embryos appears to increase implantation rates. Balaban *et al.* reported that despite fewer embryos being available for transfer on day 5, pregnancy rates are not compromised when compared with day 3 transfers [11]. But at the time this study was conducted, multiple embryo transfers were allowed. Their mean number of embryos transferred was 5.2 at day 3 and 2.4 at blastocyst stage. The present authors transferred single embryo and found pregnancy rates of blastocyst arising from both good and poor quality embryos to be same.

In a recent study, Mackenna *et al.* searched sibling embryo blastocyst development as a prognostic factor for the outcome of day 3 embryo transfer [13]. They stated that development of sibling embryos to blastocyst is a prognostic factor for the outcome of the cycle in which transfer is performed at day 3 and provides valuable information about the prognosis of the subsequent cycle. Although they graded the embryos transferred at day 3, no data has been given about the quality of blastocysts developed from sibling embryos. According to Mackenna *et al.* sibling embryo blastocyst development is important whereas the present authors only included embryos reaching blastocyst stage in this study, and according to these results, once an embryo reaches blastocyst stage, its respective day 3 quality possibly has no importance.

Overall, analysis of embryo morphology and using various grading systems may aid in the selection of embryos that may have the highest potential for implantation. However, they remain insufficient to predict the successive pregnancy. Non-invasive methods that assess the embryo culture media like proteomics and metabolomics may be combined with the embryo grading systems in the near future to further select the best embryo.

Limitations of the present study are: firstly it is not a prospective randomized study that is several biases of a retrospective study might have been seen. Secondly, the authors did not search for the rate at which poor or good quality embryos reached blastocyst stage as would have been done in a prospective study, since it was previously reported that embryos reaching blastocyst stage might have increased pregnancy rates. Instead the authors evaluated their respective day 3 embryo qualities of embryos reaching blastocyst stage. This would not be applicable in patients having few number of embryos which would be transferred at day 3, whether being poor or good quality due to the risk of halting embryo development before reaching day 5. Thirdly, the authors did not have any Grade 2 blastocysts transferred that might have possible effects on the results.

In conclusion, embryos on Day 3 present no clear course regarding the morphology at the blastocyst stage. All top qualified embryos both on Day 3 and 5 do not reveal higher pregnancy rates than the others. Those findings once again suggest that morphological-only assessment is not competence enough for the prediction of pregnancy in IVF.

The question of which embryo to be selected in the situation where multiple embryos of the same patient reach the same grade of blastocyst stage may be subject of future studies.

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