Preferable correlation to blastocyst development and pregnancy rates with a new embryo grading system specific for day 3 embryos

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Received: 8 August 2006 / Accepted: 7 November 2006 / Published online: 14 December 2006
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Abstract *Purpose:* To investigate the cleavage stage embryo quality by the correlation between the morphological features and blastocyst development rate to develop a new embryo grading system.

Methods: A retrospective analysis, including 216 cycles of cleavage stage embryo transfer and 251 cycles of blastocyst transfer. The correlation with blastocyst development of the embryo cleavage stage, fragmentation and uniformity of blastomeres was evaluated.

Results: There were significant differences in the blastocyst development rate between ≥ 7 cells and ≤ 6 cells (68.8% vs. 30.7%), $< 50\%$ fragmentation and $\geq 50\%$ fragmentation (51.9% vs. 25.7%), and evenly sized blastomeres and unevenly sized blastomeres (48.7% vs. 30.1%) on day 3. The new grading system defined by these 3 parameters showed a preferable correlation to the pregnancy rate.

Conclusions: The new grading system specific for day 3 embryos is useful for the selection of good quality embryos and may improve the pregnancy rate.

Keywords Blastocyst development · Embryo grading · Embryo morphology · *In vitro* fertilization · Pregnancy rate

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Introduction

One of the most important factors that can influence the outcome, such as the pregnancy rate, after *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) is the selection of embryos for transfer [1]. However, the increased probability of IVF pregnancy has come at the cost of multiple gestations. Therefore, fertility specialists and embryologists continue to search for methods to predict which embryos have the greatest potential for implantation, so that the least number of quality embryos can be transferred to maintain high pregnancy rates.

Originally, embryo scoring systems, which are based on the embryo morphology and/or cleavage stage, have been developed to assess the viability of human day 2 or day 3 preimplantation embryos for this purpose [2–4]. Recently, high pregnancy and implantation rates have been observed after blastocyst culture [5–7]. Blastocyst transfer has been suggested to result in the better selection of viable embryos [8, 9]. However, results have been contradictory among studies where the pregnancy rates between transfers performed at cleavage stage and blastocyst stage have been compared [7, 10]. One of the reasons for this controversy is that embryo scorings for cleavage stage embryos have been carried out in different ways [11–15]. For example, Veeck's criteria, one of the most frequently used conventional grading systems, consists of the form of blastomeres and the degree of fragmentation, and lacks the number of blastomeres [3, 16]. Furthermore, improved criteria, including the cleavage stage, vary in the period for evaluating embryos (day 2, day 3 or both) [12, 13, 17, 18]. Therefore, in the present study, we developed a new and simpler day 3-specific embryo scoring system, including the embryo morphology and cleavage state, according to the rate of blastocyst development. The aim of this study also included examining the correlation

between our new criteria and the pregnancy rate in comparison with the conventional criteria for embryo selection.

Materials and methods

Study population and data collection

All IVF/ICSI cycles at the assisted reproductive unit in the Clinic Mama, Ogaki, Japan between January, 2001 and December, 2005 were reviewed. Embryo transfers (ET) were routinely performed on the cleavage stage ($n = 216$, including 8 cycles with cancellation of ET) from January, 2001 to June, 2003 and on the blastocyst stage ($n = 251$, including 29 cycles with cancellation of ET) from July, 2003 to December, 2005. Information regarding IVF/ICSI cycles, including the fertilization rate, the number of embryos transferred and the cleavage or developmental state of each embryo transferred were collected, as well as demographic information, including age and the number of previous IVF/ICSI cycles. The IVF/ICSI outcome, including the implantation rate and pregnancy outcome, including spontaneous abortion and on-going pregnancy, were also collected.

Stimulation protocol and IVF/ICSI procedure

Controlled ovarian stimulation for all patients consisted of down-regulation with gonadotropin-releasing hormone agonist (Nasanyl; Yamanouchi Pharmaceutical, Tokyo, Japan), followed by stimulation with human menopausal gonadotropin (HMG “Nikken”; Nikken Chemicals, Tokyo, Japan or Pergogreen; Serono Japan, Tokyo, Japan). Human chorionic gonadotropin (Gestron; Nikken Chemicals) was administered when dominant follicles reached a mean diameter of 18 mm. Oocyte retrievals were carried out 35.5 h later using transvaginal ultrasound imaging. The procedures of sperm and oocyte preparation, IVF and ICSI have been described previously [19]. Fertilization was assessed by the presence of two pronuclei 18–19 h after insemination or ICSI.

Embryo culture, evaluation, transfer and luteal support

Embryos were cultured in Human Tubal Fluid (HTF; Irvine Scientific, Santa Ana, CA, USA) with 6% Synthetic Serum Substitute (SSS; Irvine Scientific) from January, 2001 to June, 2003. From July, 2003 to December, 2005, oocytes were cultured in Universal IVF Medium (Medi-Cult, Denmark) until being assessed for fertilization. Then, the embryos were transferred to Medium 1 and Medium 2 of Blast Assist System (Medi-Cult) on day 1 and day 3, respectively.

The cleavage embryos were observed on day 2 (44–46 h after insemination or ICSI) and/or on day 3 (66–71 h after insemination or ICSI) considering the number of blastomeres, the degree of fragmentation and the uniformity of blastomeres. The degree of fragmentation was expressed as a percentage of the perivitelline space occupied by anucleate cytoplasmic fragments. Then, each embryo was assessed by conventional criteria as described by Veeck [16] as follows; Grade 1: blastomeres of equal size and no fragmentation, Grade 2: blastomeres of equal size with minor fragmentation, Grade 3: blastomeres of distinctly unequal size with little to no fragmentation, Grade 4: blastomeres of equal or unequal size with significant fragmentation, Grade 5: few blastomeres of any size and severe or complete fragmentation. Day 5 (114–119 h after insemination or ICSI) embryos were scored according to the expansion of the blastocoe cavity and the number and integrity of both the inner cell mass and trophectoderm cells, as described previously [9]. All observations were performed with an inverted microscope (Olympus IX70; Olympus Electric Industry Co., Tokyo, Japan) using Hoffman modulation contrast optics at $\times 400$ magnification. Embryo transfer was performed on either day 2 or day 3 for the cleavage stage embryo or either day 5 or day 6 for the blastocyst stage. A maximum of 3 embryos in the cleavage stage or 2 embryos in the blastocyst stage were transferred, except for a few cases with very poor quality embryos. The luteal phase was supported by either human chorionic gonadotropin (Gestron) or progesterone (Oophormin Luteumdepot; Aska Pharmaceutical Co., Ltd., Tokyo, Japan). Clinical pregnancy was confirmed by presence of a gestational sac using transvaginal ultrasound imaging.

Statistical analysis

Data were analyzed with StatView 4.0 (SAS Institute Inc., Cary, NC, USA) and SigmaStat (SPSS Inc., Chicago, IL, USA). Student's *t*-test, the Mann-Whitney *U* test and the χ^2 -test were applied where appropriate. Odds ratios and 95% confidence intervals (CI) were estimated with the univariate logistic regression model. The statistical significance was defined as $p < 0.05$.

Results

In all, 467 cycles were performed during the study period. Table 1 presents the clinical characteristics, treatment data and IVF/ICSI outcomes. There were no significant differences in age or the numbers of previous IVF/ICSI cycles between the Day 2–3 ET group and the Day 5–6 blastocyst transfer (BT) group. No significant difference existed in the

Table 1 Patient characteristics and IVF/ICSI outcomes for day 2–3 Embryo Transfer (ET) vs. day 5–6 Blastocyst Transfer (BT)

Characteristics and outcomes	Day 2–3 ET	Day 5–6 BT	<i>p</i> value
No. of cycles (IVF/ICSI)	216 (107/109)	251 (113/138)	NS
Age (y)	33.4 ± 4.6	32.6 ± 3.9	NS
No. of previous IVF/ICSI cycles	0.9 ± 1.4	0.9 ± 1.3	NS
No. of oocytes retrieved	12.5 ± 6.8	14.3 ± 7.7	< 0.01
Ampoules of 75 IU hMG	53.2 ± 23.9	47.5 ± 21.2	< 0.01
No. of embryos transferred	3.4 ± 1.4	1.8 ± 0.9	< 0.001
Fertilization rate [%; (n/N)]	54.2 (1468/2706)	56.2 (2019/3591)	NS
Implantation rate [%; (n/N)]	16.8 (125/742)	35.0 (162/463)	< 0.001
Blastocyst formation rate [%; (n/N)]	–	43.7 (882/2019)	–
Clinical pregnancy rate per retrieval [%; (n/N)]	38.0 (82/216)	47.4 (119/251)	< 0.05
On-going pregnancy rate per retrieval [%; (n/N)]	31.5 (68/216)	38.6 (97/251)	NS
Multiple pregnancy rate per pregnancy [%; (n/N)]	42.7 (35/82)	31.9 (38/119)	NS
ET cancellation rate [%; (n/N)]	3.7 (8/216)	11.6 (29/251)	< 0.05

Note. Values are expressed as mean ± SD. NS, not significant; hMG, human menopausal gonadotropin.

fertilization rate, either. On the other hand, we found significantly increased rates of implantation and clinical pregnancy in the Day 5–6 BT group. We also found that a significant difference existed in the ET cancellation rate.

First, we analyzed the correlation between the rates of blastocyst development and the numbers of blastomeres, the degree of fragmentation or the form of blastomeres in the Day 5–6 BT group to explore a new embryo scoring system specific for day 3 embryos. When the numbers of blastomeres were divided into four groups, the blastocyst development rates were 4.4% (≤ 3 cells), 39.0% (4–6 cells), 71.6% (7–8 cells) and 54.1% (≥ 9 cells), respectively (Fig. 1A). There were significant differences among all groups (at least $p < 0.01$). Furthermore, we found that the blastocyst development rates decreased with increasing fragmentation. The blastocyst development rates were 55.3% ($< 5\%$ fragmentation), 54.8% (5–24% fragmentation), 48.3% (25–49% fragmentation) and 25.7% ($\geq 50\%$ fragmentation), respectively (Fig. 1B). The group of embryos with $\geq 50\%$ fragmentation was significantly lower in the blastocyst development rate than the other groups ($p < 0.001$). We also found that embryos with evenly sized blastomeres showed a higher blastocyst development rate (48.7%) than those with unevenly sized blastomeres (30.1%, $p < 0.001$, Fig. 1C). Table 2 shows the predictive analyses for the blastocyst development rate, using univariate logistic regression models. The cell numbers seemed to be the most important factor contributing to an increased blastocyst development rate, and the odds ratio was 4.98 (95% CI 4.08–6.08), although the degree of fragmentation and the form of blastomeres were also involved in blastocyst development.

Next, we developed our new grading system for day 3 embryos based on the present data (Table 3), mainly using the cell numbers and assessing the rate of blastocyst development, as compared with the conventional Veeck's criteria

that were formerly used (Fig. 2). The rates of development to the blastocyst of conventional grade 1 to 5 embryos were 55.1%, 60.9%, 36.9%, 47.9% and 25.8%, respectively. On the other hand, our new grading system specific for day-3 embryos showed a more strict correlation with the blastocyst development rate (grade 1 to 5; 76.6%, 50.0%, 40.9%, 25.9% and 17.5%).

Finally, we re-evaluated the clinical pregnancy rate in the Day 2–3 ET group according to our new grading system. Cycles were classified into 3 groups: Group A, transfer with only good embryos (G1 and G2); Group B, transfer with mixed good and poor embryos (G3, G4 and G5); Group C, transfer with only poor embryos. Then numbers of embryos transferred were similar among all groups (data not shown). The pregnancy rates re-evaluated by a new grading system were significantly higher in Group A (65.0%) and Group B (54.3%) as compared with Group C (18.2%, $p < 0.05$). A significant difference was not recognized among the groups based on the conventional grading system, although there were trends that Group A (60.0%) and Group B (58.6%) were higher in their pregnancy rates as compared with Group C (29.4%, Fig. 3).

Discussion

Multiple gestations remain one of the major problems associated with human assisted conception [20]. Therefore, recent guidelines published in September, 2004 by the American Society for Reproductive Medicine (ASRM) recommend that the number of embryos transferred be limited to two or less in women under 35, and only one embryo in women with the “best prognosis” [21]. In addition, single embryo transfer has been demonstrated to decrease multiple pregnancy rates in IVF/ICSI [22]. There is therefore a pressing

Fig. 1 Correlation between the blastocyst development rates and the cell numbers on day 3 (**A**), the degree of fragmentation on day 3 (**B**) or the form of blastomeres on day 3 (**C**). Each parameter is predictive of blastocyst development. * $p < 0.01$ vs. all other groups, ** $p < 0.001$ vs. all other groups, *** $p < 0.001$ vs. the other group

need to identify those embryos with the highest developmental potential in order to increase the chance of implantation, thereby reducing the number of embryos transferred in order to decrease multiple pregnancies.

Blastocyst transfer has been suggested to result in the better selection of viable embryos, and therefore, to be a promising method available for attaining consistently high implantation and pregnancy rates [5–9]. In addition, prolonged culture has been suggested to allow the selection of chromosomally competent embryos, because embryos failing to develop more often show multiple aneuploidy [23, 24]. However, the possible influences of an extended embryo culture to the blastocyst stage on the incidence of monozygotic multiples and the cancellation of embryo transfer may be of concern [25–27]. Furthermore, recent meta-analysis showed that there is no evidence for a difference in live birth or pregnancy outcomes between Day 2 to 3 and Day 5 to 6 transfer embryos [28]. How does this discrepancy occur? One of the problems seems to be that the criteria for choosing optimal embryos remain controversial. Several investigators suggest that the cleavage state is a superior predictor for embryo implantation [4, 29] and that embryo morphology analysis adds little to the prediction of implantation; whereas, morphology, based on fragmentation and blastomere size, has also been considered to be an important property of embryo development because embryo fragmentation is theorized to occur as a result of apoptosis with the subsequent loss of regulatory proteins [30]. In addition, the time of evaluation for cleavage embryos has not been consistent. Therefore, in the present study, we re-evaluated the cleavage stage and the cell morphology, including the degree of fragmentation and the uniformity of blastomere size on day 3, not day 2, from the standpoint of the development to blastocyst, which showed higher implantation and pregnancy rates in its transfer than cleavage stage embryo transfer. We found that each incidence has an influence on the rate of blastocyst development. Although the cell numbers seemed to be the most important factor contributing to an increased blastocyst development rate, the degree of fragmentation and the form of blastomeres also showed increased odds ratio of more than two. Based on these data, we defined a new grading system for day 3 embryos, which is easily used. Compared with conventional evaluation with Veeck's criteria on day 2–3, our new grading system showed a more preferable correlation to the rates of blastocyst development and pregnancy. This increased predictability of new criteria might be involved in including the cleavage stage and specification for day-3 evaluation. Embryo transfer on day 3 allows for the additional assessment of embryonic development and improvement in the selection of morphological criteria.

Table 2 Univariate logistic regression for Blastocyst development

Factor	Blastocyst development	Odds ratio	95% confidence intervals	<i>p</i> value
Cell number (≥ 7 cell vs. ≤ 6 cell)	68.8% vs. 30.7%	4.98	4.08–6.08	<0.001
Fragmentation ($< 50\%$ vs. $\geq 50\%$)	51.9% vs. 25.7%	3.12	2.54–3.84	<0.001
Form of blastomere (even vs. uneven)	48.7% vs. 30.1%	2.21	1.79–2.72	<0.001

Table 3 New grading system specific for day 3 embryos based mainly on the cell number

Grade 1 :	cell number ≥ 7 cells with $< 50\%$ fragmentation and even sized blastomeres
Grade 2 :	cell number ≥ 7 cells with $\geq 50\%$ fragmentation or uneven sized blastomeres
Grade 3 :	cell number ≥ 7 cells with $\geq 50\%$ fragmentation and uneven sized blastomeres
Grade 4 :	cell number ≤ 6 cells with $< 50\%$ fragmentation and even sized blastomeres
Grade 5 :	cell number ≤ 6 cells with $\geq 50\%$ fragmentation or uneven sized blastomeres

Fig. 2 Correlation between the blastocyst development rates and the new grading system specific for day 3 embryos (white bars) or the conventional grading system (black bars). The new grading system showed a better correlation to the rate of blastocyst development than the conventional grading system. * $p < 0.001$ vs. all other grades in the new grading system

A scoring system specific for day 3 embryos has not been generally observed, although various scoring systems, including day 3 evaluation have been proposed. A study on day 3 embryos suggests that the pattern of fragmentation on day 3 may in fact give a better measure of the implantation potential than the degree of fragmentation, which has always been the predominant feature in day-2 embryo scoring systems [30]. In another study by Alikani et al., embryos with < 7 cells or > 9 cells formed developing blastocysts signif-

Fig. 3 Clinical pregnancy rates by the new grading system specific for day 3 embryos (white bars) and the conventional grading system (black bars). Group A: transfer only good embryos (G1, G2), Group B: transfer both good and poor embryos (G3, G4, G5), Group C: transfer only poor embryos. The new grading system showed a better correlation to the pregnancy rate than the conventional grading system. * $p < 0.05$ vs. Group C in new grading system

icantly less frequently than those with 7–9 cells [31]. It has been reported that extremely fast developing embryos may exhibit high levels of chromosomal aberration [32], which may account for our results that embryos with ≥ 9 cells on day 3 showed reduced rates of blastocyst formation, similarly to the results of Alikani et al. They also demonstrated that normal blastocyst formation rate of embryos with 0–15% fragmentation was significantly higher than that of embryos with more than 15% fragmentation. Our results showed that embryo viability was preserved on those with less than 50% fragmentation, which seems more generous than any other studies. Recently, Dennis et al. reported that the average morphological grade, which consists of the degree of fragmentation and uniformity of blastomeres on postconception day 3, was highly predictive of the implantation and live birth rate, and that the embryo cell number on postconception day 3 was not predictive of the pregnancy outcome [33]. In their study, the majority of embryos transferred was > 6 cells; therefore, there were no statistical differences among < 6 cells, ≥ 6 , < 8 cells and ≥ 8 cells in implantation rate or live birth rate. Considering this, it could not be concluded that the embryo cell number on day 3 is not predictive for pregnancy outcome, if there were few embryos with < 6 cells transferred.

In the present study, we developed a new embryo grading system, including three basic features of embryo viability: cleavage stage, fragmentation and form of blastomeres, which showed a better correlation to the rates of blastocyst development and pregnancy than the conventional criteria. In addition, this new grading system can be easily applied because of the criteria specific for day 3 embryos. Although further studies, including prospective randomized studies, will be required to define the usefulness of this new grading system, our results suggested that using this new grading system might achieve the comparable implantation and pregnancy rates and decrease multiple pregnancy rates with blastocyst transfer on day 5–6, avoiding complicated extended culture using sequential media and concern about the cancellation of transfer.

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