Evaluation of Mouse Blastocyst Implantation Rate by Morphology Grading

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Abstract

The aim of our study is to observe the relationship between the blastocyst morphology and the implantation rate for mice. Mouse embryos obtained from the superovulated-ICR mice were cultured *in vitro* from 1-cell zygotes to blastocysts. Mouse blastocysts were then classified into 3 grades: grade I, small blastocysts; grade II, large blastocysts; grade III, hatching blastocysts. They were independently transferred into the uterus of recipient females mated with vasectomized male mice on 96 hours after the zygotes were cultured *in vitro*. The successful implantation was checked by injection of Chicago Sky Blue 6B on the second day after embryo transfer. Although there was no significant difference in the implantation rates between the grade III and grade II, grade I was significantly decreased, as compared with grade III. Grade I and grade II was also significantly decreased in both the diameter of blastocysts and cell number of inner cell mass (ICM) and trophectoderm (TE), as compared with grade III. These findings indicate that the expanded and hatching blastocyst selections for embryo transfer in *in vitro* fertilization were evaluated with the high implantation rate.

Key Words: blastocyst, implantation rate, inner cell mass (ICM), trophectoderm (TE)

Introduction

In human in-vitro fertilization (IVF) treatment programs, culture and transfer of blastocysts has been reported to generate high pregnancy and implantation rates (2,8,14). Many infertility centers use this treatment procedure for some or all of their IVF patients. Through the selection of smaller numbers of more competent embryos, blastocyst transfer may help reduce the frequency of multiple births resulting from IVF (6). To assess the blastocyst stage embryo characteristics that are indicative of viability will further develop the ability to distinguish those embryos more suitable for implantation.

It has been reported that blastocyst formation and hatching could not be used to assess subsequent developmental potential (12). Therefore, these findings constitute a paradox to our previous knowledge. Gardner and Schoolcraft (7) developed trichotomous qualitative assessments based on blastocyst expansion, inner cell mass (ICM) and trophectoderm (TE) to grade human blastocysts before transfer. However, they did not evaluate the number of cells in embryos. Blastocysts with relatively large and slightly oval

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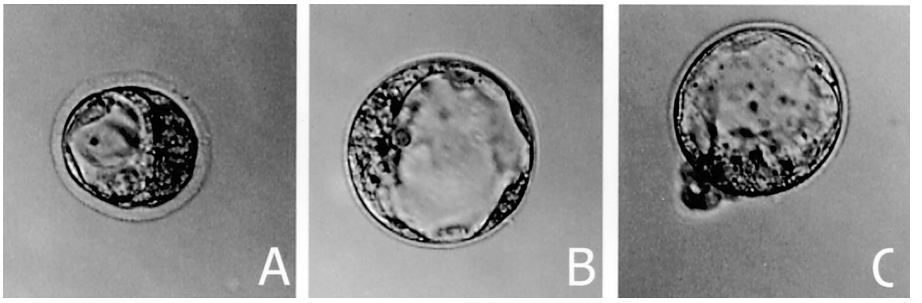

Fig. 1. The classification of three grades of blastocysts. (A) blastocyst from grade I. (B) blastocyst from gradeII. (C) blastocyst from grade III.

ICM were more likely to implant than other blastocysts (21). The number of TE and ICM cells allocated in normally fertilized human blastocysts appeared to be similar to that in mice (9). Quantitative measurements of the ICM were highly indicative of blastocyst implantation potential. It is important to analyze ICM and TE for the evaluation of blastocysts.

In this study, we propose an analysis method for the number of blastocyst cells and determine implantation potential using a mouse embryo test system. Mouse embryos have been studied extensively as models for mammalian embryogenesis. Because mice embryos can be grown in culture, they are also used as a routine "test system" for human *in vitro* fertilization (IVF) programs. We have studied the suitable quality for mouse blastocysts to uterus transfer from *in vitro* cultures. The aim of this research is to study the relationship between the morphology of blastocyst embryos and the implantation rate in mice.

Materials and Methods

Mouse Embryo Collection

Six- to eight-week-old virgin ICR mice were superovulated with an intraperitoneal injection of 5 IU pregnant mare serum gonadotropin (PMSG, Sigma), followed by 5 IU of human Chorionic Gonadotrophin (hCG; Serono, Rome, Italy) 48 hours later. After the hCG injection, each female mouse was placed in an individual cage overnight with a mature ICR male mouse with proven fertility. The next morning, successful mating was confirmed by the presence of a vaginal copulation plug. The appearance of a vaginal plug was designated day 1 of the pregnancy. Approximately 20 hours after hCG injection, mice were sacrificed and zygotes were obtained from the oviducts using needles. The zygote cumulus cells were removed by exposure to 80 unit/ml hyaluronidase (Sigma) for several minutes until all of them fell off. These zygotes were placed into wells with fresh modified human tubal fluid (Basal-HTF) medium (20) and incubated at 37(C and 5% CO_2 for about 4 hours. Two-pronucleus (2PN) embryos could be observed clearly selected under 40X phase-contrast microscopy. These 2PN embryos were transferred into dishes with pre-equilibrated Basal-HTF medium and incubated at 37°C and 5% CO_2 in air.

Blastocyst Grading

Six hundred and fifty zygotes were produced from 32 female mice. The pronucleus fertilization rate was 90.6% (589/650). The total number of blastocysts was 524. Blastocysts were classified based on the degree of expansion and hatching status, such as: grade I, a small blastocyst with a blastocoel equal to or less than half of the embryo volume; grade II, a large blastocyst with a blastocoel greater than half of the embryo volume or blastocyst with a blastocoel completely filling the embryo. Hatching embryos were designated as grade III (Figure I). The grade I percentage was 40.5% (212/524). Grade II was 38.5% (202/524) and 20.1% (110/524) of the blastocysts developed to the hatching stage.

Differential Staining

Some blastocysts were chosen from each grade randomly before embryo transfer and staining. Cells in the TE and ICM of the blastocysts were counted after differential nuclei staining using a modified method of Piekos et al (19). Embryos were submitted to zona removal using Tyrods' solution (pH2.2). The zona-free blastocysts were incubated at 5°C in M16 medium (Sigma) containing 10 mM trinitrobenzenesulphonic acid, 4.0 mg/ml polyvinylpyrrolidone and 0.015% Triton X-100 for 10 min. After washing in M2 medium (Sigma), the blastocysts were incubated in 0.1 mg/ml anti-dinitrophenol-BSA at 37°C for 15 min and washed again with M2 medium in triplicate. The blastocysts were then incubated in M2 medium containing a 1:10 dilution of guinea pig complement serum (Irvine, CA, USA) and 10.0 μg/ml

Table 1. Differential staining in the inner cell mass (ICM) and trophectoderm (TE) of the blastocysts from the three grades.

	Grade		
	I	II	III
No. of blastocysts measured	35	35	35
Diameter of blastocysts (μm)	89.3±1.2[a],*,†	110.7±1.5*	115.8±1.6
No. of blastomeres	39.3±1.3*,†	59.2±1.0*	77.4±2.6
No of cells in ICM	15.3±0.6*,†	26.7±0.5*	32.2±1.0
No of cells in TE	24.0±0.8*,†	32.5±0.6*	45.2±1.7
Ratio of ICM/TE cells (%)	64.1±1.9*,†	82.3±1.4*	73.0±2.1

[a]Mean ± SEM
*Compared with grade III by Student's *t*-test: $P<0.01$.
†Compared with grade II by Student's *t*-test: $P<0.01$.

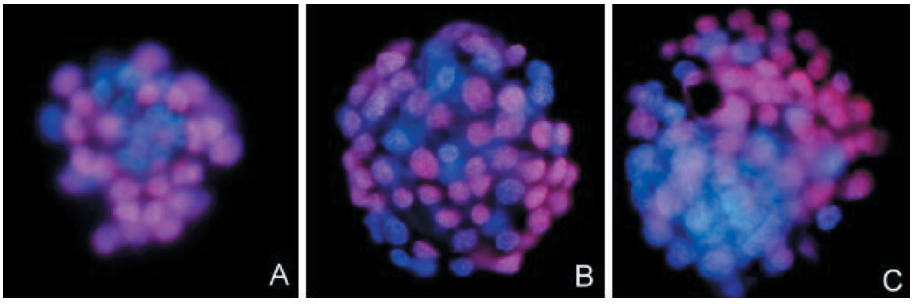

Fig. 2. Examples of differential staining of blastocysts.(A)blastocyst from grade I. (B) blastocyst from grade II. (C). blastocyst from grade III. Pink cells were trophectodermal blastomeres and blue cells were ICM.

propidium iodide (Sigma) at 37°C for 15 min and washed in Dulbecco's PBS (Gibco) in triplicate. After fixing in absolute ethanol containing 22.0 μg/ml bisbenzimide (Sigma) at 5°C overnight, individual blastocysts were mounted in glycerol on microscopic slides and compressed manually before visualizing by epi-fluorescence using Nikon filter to block UV-2A and G-2A. Blue nuclei were considered as originating from the inner cells and red-to- pink fluorescing nuclei as belonging to the outer cells.

Mouse Embryo Transfer

Recipient female mice (8-12 weeks old, ICR strain) were prepared by mating with vasectomized males of the same strain 4 days before embryo transfer. The embryo transfer procedures were performed according to Nagy *et al*. (16). Graded blastocysts were transferred to the top of the uterus by transfer pipettes. We transferred about 7 to 12 blastocysts to each recipient. The mice were sacrificed 2 days after embryo transfer. Successful implantation was verified and determined by injection of Chicago Sky Blue 6B (Sigma).

Statistical Analysis

The implantation rates were expressed as percentages. The numbers and diameters of cells were expressed as mean ± SEM and were statisticed using one way ANOVA followed by Student's *t* test for multiple comparisons. Differences between rates were determined using the chi-square test. Differences were considered significant if $P < 0.05$.

Results

Effects of Size and Differential Staining of Blastocyts

The diameter of blastocysts of grade I was significantly lower than grade II and III; the grade II was also significantly lower than grade III. We evaluated the quality of these three grades of blastocysts by differential stain (Fig. 2). The total ICM and trophectodermal cell numbers were calculated (Table 1). Thirty-five blastocysts were stained in grade I, the ICM number was 15.3±0.6 and the TE number was 24.0±0.8. Blastocysts had lower numbers of blastomeres in ICM or TE in grade I. Significant

Table 2. The implantation rate of the blastocysts from three grades.

	Grade		
	I	II	III
No. of recipients	6	6	6
No. of blastocysts transfer	60	60	66
Implantation rate (%)	56.7*	73.3	80.3

*Compared with grade III by X^2 test, $P<0.01$.

Table 3. Percent of hatching rate in Grade I and II Blastocyst cultures on days 6 and 7.

	Day 6	Day 7
Grade I (n=34)	43.3*	63.3
Grade II (n=32)	65.6	68.8

*Compared with grade II by X^2 test, $P<0.05$.

difference was found in the cell number of blastomeres, ICM and TE and ICM/TE ratio between grade I and grade II or III and between grade II and grade III.

Implantation Rate of Blastocysts

We assessed the implantation ability of blastocysts among these three groups by transferring embryos to uterus of foster-mothers. The implantation rates in grade I, II and III were 56.7% (34/60), 73.3% (44/61) and 80.3% (53/66), respectively (Table 2). The implantation rate in the grade III was significantly higher than that in the grade I.

Potential Hatching Rate of Blastocysts

The ability of potential hatching in blastocysts was evaluated. The partial embryos in grades I and II were cultured on day 6 and day 7 after the zygotes were chosen. The hatching rate in grade I was 43.3% (13/34) and grade II was 65.6%(21/32) on day 6 (Table 3). The potential ability of embryo hatching in grade II was higher than that in grade I on day 6. However, there was no significant difference between both grades on day 7.

Discussion

Selecting good embryos with high implantation potential is one of the most important factors in assisted reproduction. The benefits of blastocyst transfer include suitable synchronization between the endometrium and the embryo and selection of embryos with a higher implantation potential (2). Scoring blastocysts is more complex than scoring embryos in the cleavage stage for increased embryo transfer selection. Blastocyst culture and transfer have previously been shown to be effective in decreasing multiple gestations and thereby avoid the complications associated with such pregnancies (6, 14). Human blastocyst grading can be quantitated using a three-part scoring system that selects blastocysts for transfer (7). In our study, the quality of mouse blastocysts assessment also affected implantation. Our data indicated that crucial factors in successful implantation may include blastocoel and hatching formation as shown in our results (Table 2). Similar results were reported by Balaban *et al* (1). They reported that transfer of at least one good quality blastocyst or one hatching blastocyst into women undergoing the IVF cycle was associated with very high implantation and pregnancy rates.

Microscopic observations frequently do not allow the proportion of ICM and TE cells to be determined in a precise manner. This can be achieved by differential ICM and TE blastocyst staining during immunosurgery, using two different fluorescent dyes. The total number of blastocysts was regulated using different conditions, such as half embryo, isolated blastomeres and growth environment (17, 22, 23). The total cell number of blastocysts and the ICM ratio in our study were similar to that in a previous study (11). An acceptable ICM to total cell development ratio is thirty percent. The three grades in our study showed good blastocyst development. In conclusion, the morphology of blastocysts was good indicators for embryos transferred in the mouse model.

It was reported that there was a significant positive correlation between number of blastocyst cells and number of ICM cells and subsequent fetal development, but morphology as assessed by blastocyst formation and hatching was not correlated with subsequent fetal development (12). However, they didn't re-classified quality of blastocysts in different treatments before transferring to pseudopregnant recipients. The detail variation of blastocysts in each treatment afterwards may disregard in previous peports. We propose to assess fetus development from blastoctst before blastocyst transfer, as shown in this study. The role of hatching for implantation was more important than the cell number or blastocyst volume in this study.

The potential of embroyos' being developed into blastocysts and being implanted may relate to factors in the embryos themselves, such as their gene expression in the nucleus or cytoplasm. One major view was that the disruption of the zona pellucida in an estrogen-sensitized uterus was accomplished through the action of a uterine protease or pronase (10). Previous studies documented the induction of blastocyst hatching by TGF-α (3) and HB-EGF (4) or

interferon (INF-τ) (13). When embryos were grown *in vitro*, successful hatching was dependent on blastocyst expansion and was based on a minium number of embryonic cells. The blastocysts continued their growth and, after having reached a certain threshold in the mean number of embryonic cells, underwent blastocyst in CB6F1 mice (15). Our data showed that successful hatching *in vitro* also depends on a sufficiently high number of embryonic cells, as shown in our previous study.

More than thirty percent of the blastocysts in grade I and II could not achieve the hatching stage. Some of the embryos achieved full to expand blastocysts but failed to hatch (Table 3). Embryos that fail to hatch by day 6 may have a lower implantation potential. Embryo transfer can be delayed to day 6 after oocyte insemination, at which time a small percentage of embryos will hatch, and delayed blastocyst growth or hatching did not improve implantation (5). Embryo and uterus cross-reaction is needed in a successful implantation process (18). Therefore, we suggested that predominately hatching or large volume blastocysts may be used for embryo transfer to produce high implantation rates.

In conclusion, we were able to achieve high implantation rates using hatching and expanded blastocysts. Which genes express failure or delay at the implantation stage is yet unknown. The blastocyst genes and hatching environment will be important topics for future research.

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