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Human Blastocysts : The Correlation Between Embryo Microscopical Assessments and Their Cell Number

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인간 배반포기 배의 현미경적 분류와 세포수의 상관관계에 관한 연구

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= 국문초록 =

본 연구는 인간의 체외수정 program으로 부터 생산된 여분의 배반포기 배를 이용하여 현미경에 의한 형태학적 판정과 differential labelling 기법을 이용한 세포 수의 상관관계를 조사하고자 실시하였다. 공시된 인간 배반포기 배는 체외수정 후 5일째에 36명의 환자로 부터 76개를 얻어 배반포강의 확대와 투명대 두께의 감소를 기준으로 early (ErB), early expanding (EEB), middle expanding (MEB) 및 expanded blastocyst (EdB)로 구분하였다. 분류된 배반포기 배의 크기와 투명대의 두께를 micrometer로 측정하였을 때, 그 크기는 각각 148.8-217.6μm, 1.2-14.4μm로 나타나 같은 배양조건에서 생산된 배아라도 그 차이는 크게 나타났다. Hoechst 염색을 이용하여 배반포기 배의 총 세포수를 조사하였을 때, 체외수정 후 5일째 생산된 배반포기 배는 ErB (39.1±3.6) 에서 EdB (89.6±3.3) 로 진행되는 동안 두배에서 세배정도 증가되는 양상을 나타내었다. 또한, differential labelling기법을 이용한 배반포기 배의 inner cell mass (ICM) 와 trophectoderm (TE) 세포수 조사 결과, 각각 11.9±1.8 - 22.2±4.3, 24.5±3.6 - 70.0±7.7을 나타내어 발달이 진행될수록 ICM과 TE세포수도 증가되는 것을 알 수 있었다. 특히, 형태학적으로 약한 ICM으로 판정된 EdB의 경우 differential labelling 후, 역시 적은 ICM 세포수를 나타내어 형태학적 판정과 세포수간에 밀접한 상관관계가 있음을 알 수 있었다. 따라서, ICM과 TE를 differential labelling하는 기법은 인간 배반포기 배의 quality를 평가하는데 매우 유용한 기법으로 형태학적인 구분과 병용된다면 인간 배반포기 배 이식 program의 임신율 증진을 위한 배아 선별의 중요한 자료로서 이용될 수 있다는 것을 시사한다.

INTRODUCTION

Production and transfer of blastocysts in the area of human IVF are now available as an ART program if it is ready for appropriate culture conditions. Transfer of human embryos to the uterus at the blastocyst stage would have several advantages as follows: i) synchronization between the embryonic development and uterine endometrium (Dokras *et al.*, 1993); ii) increase in implantation potential (Olivennes

et al., 1994) and iii) avoidance of multiple pregnancies (Bolton *et al.*, 1989). Especially, selection of better morphological blastocyst among the embryos in the same cycle is absolutely important for human blastocyst transfer program to obtain high pregnancy rates.

At present, selection methods of transferable embryo are mainly dependent on microscopic analysis of embryo itself and cleavage speeds (Dokras *et al.*, 1993). However, it is not clear which of the blastocyst grades would be most viable among the multiple variations in morphology and cleavage timings. Papaioannou and Ebert (1988) indicated that the cell number may be a valid indicator of the viability of preimplantation embryos although morphological criteria alone are poor indicators. Allocation to the ICM and TE cells of the blastocyst is of fundamental importance for later development. Recently, Handyside and Hunter (1984) established differential labeling of nuclei of the ICM and TE cells of mouse blastocysts *in situ* with two polynucleotide-specific fluorochromes. Differential labelling of ICM and TE nuclei *in situ* has two important advantages; first, the numbers of both these cell types can be determined for individual blastocysts, and second, spatial relationships are partially preserved so that regional interactions can be studied. Therefore, the results obtained from this study on cell number and the allocation of ICM and TE cells in later preimplantation stage can be used as an solid indicator of embryo quality (Conaghan *et al.*, 1993; Iwasaki *et al.*, 1994 a,b; Ray *et al.*, 1995). The objective of this study was to investigate correlation between the morphology by microscopic assessments of surplus blastocysts produced in human IVF program and their cell number obtained by differential labelling method.

MATERIALS AND METHODS

1. Human preimplantation embryos

Human day 5 surplus embryos were obtained with permission from patients undergoing *in vitro* fertilization. After pituitary-gonadal suppression with a luteinizing hormone releasing hormone (LHRH) agonist (Buserelin, Hoechst), patients were superovulated with gonadotrophin (FSH/hMG or hMG alone). 10,000 i.u. human chorionic gonadotrophin (hCG; Humegon, Organon) was given 34-36 hr before egg collection. Oocytes were collected, preincubated, inseminated (day 0) and checked for pronuclei on the following day (18 hr after insemination). Fertilized embryos were then cocultured with cumulus cells in culture drop (1×10^4 cumulus cells/10μl) covered with mineral oil in 5% CO_2 in air at 37℃ incubator. On day 5 (118-120 hr after IVF), embryos were transferred to the patient's uterus and then some of the remaining surplus embryos were allowed to investigate their quality under patient's consent. For these experiments, 76 surplus blastocysts were obtained from 36 patients.

2. Blastocyst classification

Blastocysts were classified to ErB, EEB, MEB, EdB according to their blastocoel expansion and zona thickness as follows: i) ErB (blastocoel is smaller than 2/3 of the whole embryonic cell); ii) EEB (blastocoel is larger than 2/3 of the whole embryonic cell); iii) MEB (blastocoel is larger than 2/3 of the whole embryonic cell and thinning zona) and iv) EdB (typical blastocyst; blastocoel expansion is maximum and zona pellucida is almost not seen) (Fig. 1). Also, ovum size and zona thickness were measured by eyepiece micrometer (Olympus) fitted on inverted microscope (Olympus). Observation has mainly done from two – to six hundred magnification.

3. Total cell count

To count total number of blastomeres, the classified blastocysts were fixed with 2% formalin solution for 2-3 min. prior to bis-

benzimide (No. 33342, 2.5μg/ml, Sigma) treatment. Fixed blastocysts were washed, placed on a slide glass. And then 50μl of bisbenzimide solution was dropped beside the coverslip, filled the square under coverglass with the solution and sealed the edges with fingernail polish. Observation was carried out under fluorescent microscope one day after sample preparation.

4. Differential labelling of ICM and TE nuclei

Differential cell counts were carried out by the method of Hardy *et al.* (1989) with some modifications. Briefly, embryo zona was removed in 0.5% pronase (Sigma) solution and allowed to recover for 10 min. in TL – Hepes. Embryos were placed on ice for 15 min. in 15 mM trinitrobenzene sulfonic acid (TNBS; Sigma) to label cell surface proteins with covalently bound trinitrophenol (TNP) groups. This allowed the use of an antiserum against dinitrophenol (DNP) which crossreacts with TNP – labelled proteins. Embryos were then washed completely and incubated in 0.1mg/ml anti – DNP – BSA (ICN Immunological.) in TL – Hepes for 10 min. at 39℃. Embryos were again washed sufficiently in TL – Hepes and then incubated in 0.01mg/ml propidium iodide (PI) and 15% (v/v) guinea pig complement (Sigma) in TL – Hepes for 20 – 30 min. at 39℃. This step resulted in the lysis of the TE cells and the red staining of TE nuclei. Immediately after this step, embryos were placed in absolute alcohol containing the fluorochrome bisbenzimide (0.05mM, Sigma). Following overnight storage at 4℃, the embryos were washed in absolute alcohol for at least 1 hr, and mounted in glycerol under a coverslip on a slide glass. Labelled nuclei were observed under fluorescent microscope and TE nuclei labelled with PI and bisbenzimide appeared pink or red, ICM nuclei labelled with bisbenzimide appeared blue or unlabelled.

5. Statistical analysis

Difference in number of cells between development groups was compared using the Student's t – test.

RESULTS

A total of 76 surplus human blastocysts used in this experiment were obtained from 36

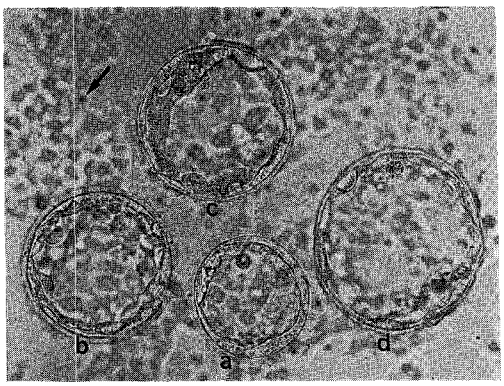

Fig. 1. Morphology of day 5 human blastocysts produced *in vitro* by coculture with homologous cumulus cell monolayer. Arrow indicates cumulus cell monolayer. a) Early blastocyst, b) Eary expanding blastocyst, c) Middle expanding blastocyst, d) Expanded blastocyst. x200 magnification.

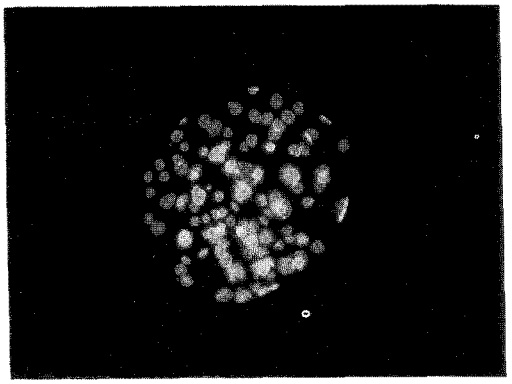

Fig. 2. Fluorescence micrographs of nuclei stained with bisbenzimide (hoechst staining). Total cell number (TCN) of human expanded blastocyst produced at 5 day after IVF (TCN; 102). x300 magnification.

Table 1. Description of the surplus blastocyst

Blastocyst no.	Rate (%) of classified blastocysts			
	ErB	EEB	MEB	EdB
76	27 (35.5)	13 (17.1)	20 (26.3)	16 (21.1)

Table 2. Ovum size size and zona thickness according to blastocyst classification

Development stage	No.	Ovum size (μm)	Zona thickness (μm)
ErB	27	*160.2±1.8 (148.8-172.8)[a]	12.6±0.4 (9.6-14.4)[a]
EEB	13	171.2±2.8 (163.2-177.6)[b]	10.3±1.6 (6.4-14.4)[b]
MEB	20	184.9±2.2 (169.6-192.0)[c]	6.0±0.6 (2.4-9.6)[c]
EdB	16	199.1±3.7 (176.0-217.6)[d]	2.9±0.3 (1.2-4.8)[d]

*Values are means±standard errors
[a,b,c,d] Means in the same column without common superscripts are significantly different (P <0.05).

patients (mean 2.1 per patient; range 1 – 4) on day 5 after IVF. These embryos were classified to ErB, EEB, MEB, EdB according to their blastocoel expansion and zona thickness and their classified rates were 35.5, 17.1, 26.3 and 21.1%, respectively (Table 1).

When the ovum size and zona thickness of classified blastocysts were measured using micrometer, ovum sizes of ErB, EEB, MEB and EdB were 160.2±1.8, 171.2±2.8, 184.9±2.2 and 199.1±3.7μm, respectively (Table 2), and their zona thickness were 12.6±0.4, 10.3±1.6, 6.0±0.6 and 2.9±0.3μm, respectively. It proved that expansion of blastocoel resulted in increased ovum size and concomitantly thinned zona pellucida.

Table 3. Total cell number of human IVF blastocysts using hoechst staining

Development stage	No.	Cell number
ErB	16	* 39.1±3.6 (24-64)[a]
EEB	5	47.1±5.2 (34-76)[a]
MEB	8	70.4±5.4 (40-96)[b]
EdB	6	89.6±3.3 (68-120)[c]

* Values are means±standard errors
[a,b,c,d] Means in the column without common superscripts are significantly different (P <0.01).

The count of total blastomere number by hoechst staining was presented in Table 3 and Fig. 2. As shown in Table 3, total cell numbers of ErB, EEB, MEB and EdB were 39.1±3.6, 47.1±5.2, 70.4±5.4 and 89.6±3.3, respectively. It showed that total blastomere number was increased by two to three fold during the transition period from ErB to EdB.

ICM and TE cell number by differential labelling with immunosurgery and polynucleotide – specific fluorochreome is summarized in Table 4 and Fig. 3. As shown in Fig. 3 B and D, the TE nuclei labelled with PI and bisbenzimide appeared red or pink and the ICM nuclei labelled with bisbenzimide appeared blue. In Table 4, ICM cell numbers of ErB,

Table 4. Numbers of ICM and TE cells of human IVF blastocysts using differential labelling

Development stage	No.	ICM cell number	TE cell number	Total cell number
ErB	11	* 11.9±1.8 (2-18)	24.5±3.6 (18-40)[a]	35.9±2.9 (24-48)[c]
EEB	8	12.2±1.8 (3-18)	28.7±5.2 (16-66)[a]	40.9±6.3 (23-78)[c]
MEB	12	22.2±4.3 (12-35)	49.8±7.2 (34-72)[b]	76.0±6.0 (58-96)[d]
EdB	10	19.7±2.9 (12-36)	70.0±7.7 (40-82)[b]	85.3±3.5 (68-98)[d]

*Values are means±standard errors
Means in the same column without common superscripts are significantly different: [ab]P<0.05; [c,d]P<0.01
*6 out of embryous showed morphologically abnormal characteristics of poor ICM.

EEB, MEB and EdB were 11.9±1.8, 12.2±1.8, 22.2±4.3 and 19.7±2.9, respectively and their TE cell numbers were 24.5±3.6, 28.7±5.2, 49.8±7.2 and 70.0±7.7, respectively. It also showed the increased pattern of combined cell numbers of ICM and TE with advance in developmental level. But ICM cell number of EdB was smaller than that of MEB. This result should be obtained by added up data from some abnormal EdB (Fig. 3 C and D).

DISCUSSION

Embryo morphology has long been used to assess viability and has been the basis of scoring systems to quantify the extent of development (Shea, 1981; Cummins *et al.*, 1986). Mohr *et al.* (1983) observed that at all stages of development, human embryos which resulted in a pregnancy were the most rapidly di-

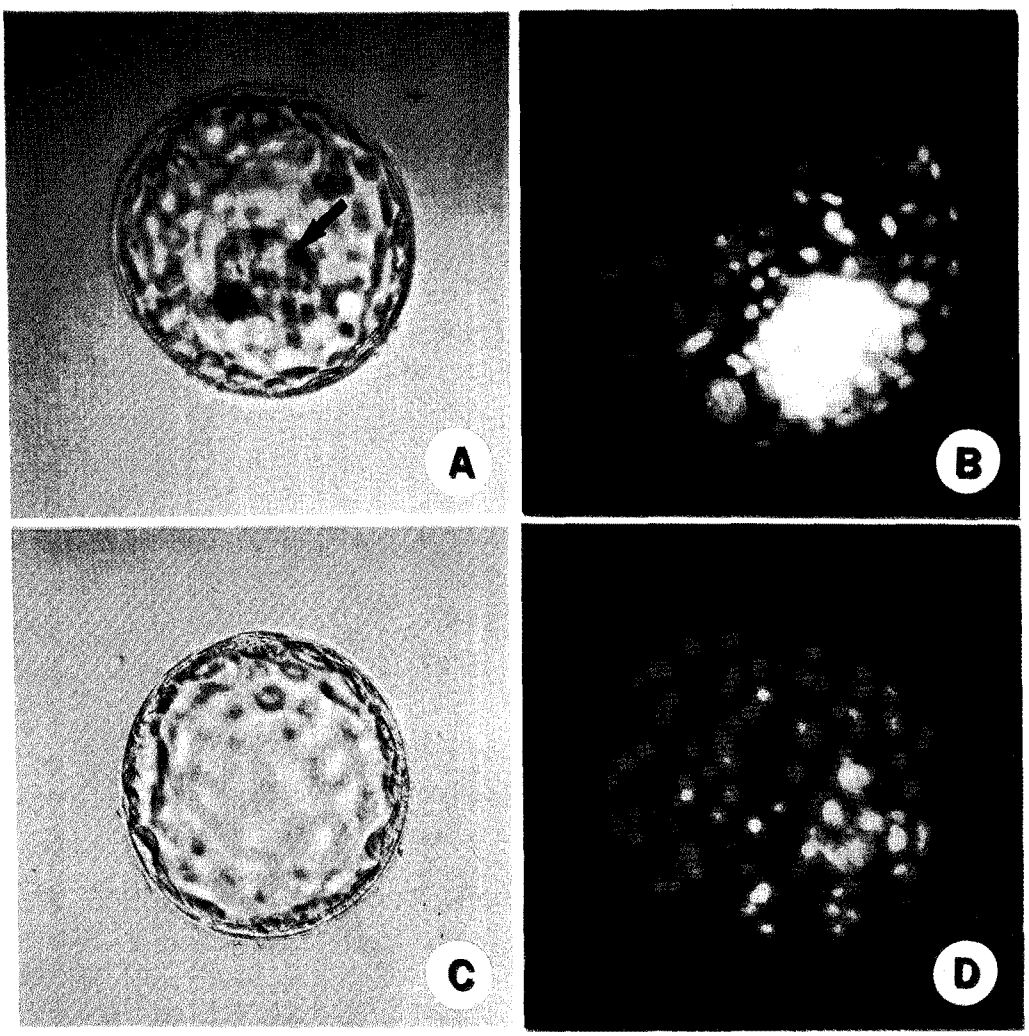

Fig. 3. Fluorescence micrographs (FM) of nuclei stained differentially with bisbenzimide and proidium iodide (differential labelling) in expanded blastocyst on day 5 after IVF. (B and D) FM, ×300 magnification; (A and C) corresponding phase micrographs, ×200 magnification; (A and B) Morphologically excellent embtyo(Ich; 30, TE; 68). (C and D) Morphologically abnormal embryo of poor ICM (ICM; 12, TE; 72). Blue ICM nuclei and pink TE nuclei are easily distinguished. Arrow indicates healthy ICM cells.

viding ones. On the other hand, Lopata *et al.* (1985) indicated that even those embryos that were slowest to divide could also be viable and that the speed of cleavage alone could not be used as an indication of embryo viability. However, selection based on morphological data is useful, but it is of poor clinical value to predict the viability of a specific embryo (Roux *et al.*, 1995). In our results, although the embryos were included in the same classification, their ovum size and zona thickness varied significantly. And also, there are some embryos in the EdB group of which ova sizes are smaller than those in MEB group although their blastocoels are fully expanded. This variation may be caused by the size differences of ova from different donors.

Total blastomere count by hoechst staining was carried out to compare with the cell number counts of ICM and TE by differential labelling. In blastomere count using hoechst staining, embryos in EdB group had two or three fold more cells than those in ErB group (Table 3). Also, mean cell number (89.6 ± 3.3) of EdB produced in our culture system was very high when compared with the mean cell number (58.3 ± 8.1) of day 5 blastocysts obtained *in vitro* by Hardy *et al.* (1989). This difference may be caused by different culture system between coculture and medium only culture. Especially, our results showed that total cell number of each embryos harvested from the same donor was remarkably similar, whereas there was significant difference in cell number when source of the embryos is different (data not shown). This tendancy suggests that all the embryos from the same donor have the same timings of fertilization and cleavage. On the other hand, there are correlation between cell number and morphological assessment. Papaioannou and Ebert (1988) also indicated that there was a good correlation between cell number and morphological development although cell number within each morphological category varied widely and there was considerable overlap between categories. And those large variation within a category may be caused by a variation in the quality of embryos (Iwasaki *et al.*, 1990).

The allocation of cells to the ICM and TE of the blastocyst is of fundamental importance for later development. In the mouse, allocation to the two lineages occurs during the fourth (Handyside, 1981) and fifth cleavage divisions (Pederson, 1986; Fleming, 1987) at the morula stage. Following implantation, the TE only gives rise to the placenta and extraembryonic membranes whereas the ICM forms all three germ layers of the fetus as well as complementary contributions to the extra embryonic membranes (Gardner and Papaioannou, 1975). Also, it is well known that allocation and differentiation to the ICM and TE lineage in preimplantation embryo development are affected by experimental treatment of cleaved embryos (Handyside *et al.*, 1984). The morphological characteristics of blastocysts vary, with some having distinct ICM and well laid down TE while others do not. In our results, by using differential labelling, ICM cell numbers between ErB and EdB were ranged from 11.9 ± 1.8 to 22.2 ± 4.3, but these cell numbers were not significantly different among the classified groups (Table 4). However, TE cell numbers (24.5 ± 3.6 to 70.0 ± 7.7) were significantly different between ErB and EdB, but there were not signifiantly different between ErB and EEB or between MEB and EdB. Also, ICM number (19.7 ± 2.9) of day 5 EdB is very similar when compared with the ICM number (20.4 ± 4.0) of data of Hardy *et al.* (1989). Although total cell number of EdB produced on day 5 is high (Table 3), ICM cell number of EdB is smaller than that of MEB. This result should be obtained by added up data from some abnormal EdB morphologically (Fig. 3 C and D). However, it

showed the increased pattern of cell number according to the developmental level.

The proportion of ICM cells in blastocysts is crucial. Iwasaki *et al.* (1990) presented that the proportions of ICM cells in blastocysts from *in vitro* fertilization decreased gradually with advance in development, although those from the *in vivo* fertilization were identical in the early and hatched stages. Also, they demonstrated that the cell – cell contacts of ICM cells in blastocysts derived from *in vivo* fertilization were tighter than those from in vitro fertilization and the reduced cell proliferation of ICM cells may be the cause of the low pregnancy rates of blastocysts derived from in vitro fertilization. In this viewpoint, selection of better morphological blastocysts among the embryos in same cycle is absolutely important for human blastocyst transfer program to obtain high pregnancy rates. Therefore, the differential labelling of ICM and TE nuclei in situ is a very useful technique to evaluate the quality of human blastocyst and can be used as an important criterion, if it is accompanied with morphological assessments, in selecting the better embryo for improving the pregnancy rates in human blastocyst transfer program.

SUMMARY

The objective of this study was to investigate correlation between the morphology by microscopic assessments of surplus blastocysts produced in human IVF program and their cell number obtained by differential labelling method. For these experiments, 76 surplus human blastocysts were obtained from 36 patients on day 5 after IVF, the embryos were classified to early (ErB), early expanding (EEB), middle expanding (MEB), expanded blastocyst (EdB) according to their blastocoel expansion and zona thickness. When the ovum size and zona thickness of the classified blastocysts were measured using micrometer, although the embryos were produced in the same culture condition, there were significant variances in ovum size (148.8 – 217.6μm) and zona thickness (1.2 – 14.4μm). Total blastomere cell number counted after hoechst staining was increased by two to three fold during the transition period from ErB (39.1±3.6) to EdB (89.6±3.3) stage on day 5 after IVF. ICM (11.9±1.8 – 22.2±4.3) and TE (24.5± 3.6 – 70.0±7.7) cell numbers using differential labelling were also showed the increased pattern according to the developmental level. Especially, EdB which showed poor ICM morphologically also indicated the low ICM cell number after differential labelling. This demonstrated that there is good correlation between the morphological assessment and the cell number. The count of ICM and TE nuclei using differential labelling can be used as an important criterion, if it is accompanied with morphological assessments, in selecting the better embryos for improving the pregnancy rates in human blastocyst transfer program.

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