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The Clinical Outcomes after Embryo Transfer (ET) on Day 2 and Day 5 or Subsequent ET on Day 2-5, 2-6, 2-7, 3-5 and 4-7 in *In Vitro* Fertilization-ET Cycles

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체외수정술에서 배아 이식 시기와 방법 (2일과 5일 및 2-5, 2-6, 2-7, 3-5, 4-7일 연속 이식)에 관한 연구

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연구목적: 일반적으로 IVF-ET에서 가장 높은 임신율을 얻는 방법은 5 day ET (배반포기 배아 이식)이지만 장기간 배양이 적절하지 못한 경우에는 2~4일째에 ET를 실시하고 나서 5~7일째에 배반포기에 도달한 배아를 재이식 (SET)하여, SET의 효용성에 대하여 조사하고자 실시하였다.

연구재료 및 방법: 48주기의 환자에서 회수한 난자와 수정란은 10%와 20% hFF가 첨가한 DMEM에서 이식 직전까지 각각 공배양하였다. 채란 2일 (group I, day 2 ET), 5일째 이식 (group II, day 5 ET) 또는 재이식 (group III, SET; 2-5, 2-6, 2-7, 3-5, 4-7일)을 실시하면서 수정률, 할구분할률 및 임신율을 각각 비교하였다. 결과에 대한 통계 분석을 SAS (version 6.2)를 이용한 Duncan's Multiple Range Test를 이용하여 p값이 0.05보다 작을 때 통계적으로 유의차가 있는 것으로 하였다.

결 과: 수정률은 group II (90.5%)가 다른 군에 비하여 높게 ($p<0.05$) 나타났다 (group I: 80.6%; group III: 82.9%). 할구분할률은 군간에 차이가 없었다 (수정란 당 93.3~99.1%). 임상적 임신율은 group II와 III (각각 58.3%)가 group I (33.3%) 보다 높게 나타났다. 그러나 처리군이 적어서 통계적인 차이는 없었다.

결 론: 배반포기 배아를 단독 이식하는 것이 임신율을 높일 수 있는 최선의 방법으로 나타났지만, 채란수가 적거나 수정률이 저조한 경우에는 2~4일째에 ET를 실시한 후 여분의 배아를 배반포기까지 배양한 다음 5~7일에 재이식 (SET)하면 blastocyst ET에서 나타날 수 있는 이식 자체의 실패를 방지할 수 있으면서 임신율을 높일 수 있는 이식 기법이 될 것이다.

Key Words: Day 2 ET, Day 5 ET, Subsequent ET (SET), Pregnancy rates

Transfer of human embryos to the uterus at the blastocyst stage has several advantages. There may be an improvement in success rates of pregnancy due to better synchronization of the uterine and embryonic development, self-selection of embryos for transfer along with the possibility of reducing the number of embryos transferred and the risk of multiple pregnancies without altering the overall

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pregnancy rate would be decreased. However, embryos are transferred to the uterus on day 2 or 3 after insemination before the blastocyst stage is reached in many cases. This may be a reflection of sub-optimal culture conditions although inherent abnormalities like chromosomal anomalies will also contribute to the loss.[1~8] Physiologically, the human endometrium prepares itself to its optimum approximately on days 5~7 after ovulation so as to receive a cavitated blastocyst from the fallopian tube for successful implantation.[1,9,10] However, conventionally, in most programs offering *in vitro* fertilization (IVF), 4~8 cell stage embryos are replaced to the uterus.[1,9,11] This asynchrony of embryonic stage and preparation of endometrium may be one major contributory cause of increased abortion and low take-home baby rates in infertility patients.[1,10~12]

Leaving all embryos in extended culture until they develop to the blastocyst stage might result in cancellation of the embryo transfer (ET) procedure if none of the embryos reach that stage. This could have a major adverse psychological impact on the patient, and also denies her the possibility of implantation of early embryos that did not develop to the blastocyst stage *in vitro* but might have done so *in vivo*.[2] To avoid such events while explore the feasibility of blastocyst transfer, subsequent ET (SET) of early embryos and blastocysts was applied on day 2~3 and 5.[2,6,7,13,14]

The current study was conducted to investigate the effectiveness of attempted SET of blastocysts on day 5~7 following initial multi-cell embryos transfer on day 2, 3 or 4 in IVF cycles.

MATERIALS AND METHODS

1. Female patients

A total of 48 infertile couples was grouped by ET day and methods; group I: day 2 ET (n=12); group II: day 5 ET (n=24); group III: SET (n=12) [day 2-5 ET (n=6); 2-6 ET (n=1); 2-7 ET (n=1); 3-5 ET (n=2); 4-7 ET (n=2)]. Embryos (and/or blastocysts) were transferred on day 2, 5 or multi-day (SET). The mean age of the female patients in 3 study groups was 30.5±2.5, 30.2±3.4 and 30.5±2.0 (range=24~35) years respectively.

2. Follicular stimulation

Ovaries were stimulated by the gonadotropin-releasing hormone agonist (GnRHa)-menotropin regimen. Decapeptyl (Decapeptyl Inj 0.1 mg, Ferring, Germany) was administered starting at the mid-luteal phase. The hMG (IVF-M, LG, Korea) was started after down-regulation of the pituitary confirmed by serum estradiol. The subsequent dosage of hMG was adjusted according to the individual ovarian response in terms of daily follicle number and size. Ovulation was induced by a 10,000 IU injection of human chorionic gonadotropin (hCG, LG, Korea) when two or more follicles >17 mm in diameter were present. Oocyte retrieval was performed at 34~36 h after hCG injection. The oocytes were obtained by ultrasonography-guided transvaginal follicular aspiration.

3. Sperm preparation

Semen samples were obtained by masturbation and collected before oocyte retrieval. Semen parameters were evaluated according to WHO criteria.[15] After complete liquefaction, the sperm were washed twice with Ham's F-10 (81200-040, Gibco, USA) supplemented with 10% human follicular fluid (hFF) and antibiotics. The pellet was then resuspended to 1 ml of Ham's F-10 and centrifuged, and placed to swim up for 30 min to 1 h at 37℃ incubator. Sperm were collected in 5 ml tube (2003, Falcon, USA).

4. IVF and embryo culture

Retrieved oocytes were evaluated their maturity by simple method[8] and inseminated with approxi-

mately 2×10^5 sperm in 2 ml experimental media in 2-well dish.

Oocytes were considered fertilization if they exhibited clear 2-pronuclear (PN) at 16~18 h after insemination.

Fertilized oocytes (zygotes and embryos) were cultured on Vero cell monolayers until transferred.

5. Embryo co-culture on vero cell monolayers

The technical aspects of Vero cells maintenance have been described by Ouhibi *et al*[16]. Briefly, from the frozen cells, flasks were seeded with 2~3 $\times 10^6$ cells and reached confluence within 4 days (6~8 $\times 10^6$ cells/flask). After trypsinization with trypsin-EDTA (25300-054. Gibco, USA), the cell suspension was divided into three aliquots. One was used for seeding in a new flask, the other was frozen with a Cell Culture Freezing Medium (11101, Gibco, USA), and the third was used for seeding in culture dishes at 1×10^5 cells/well. Thus confluence was reached in 3 days. The media for Vero cell culture was TCM-199 (11150-059, Gibco, USA) containing 10% FBS and antibiotics. The media for oocyte and embryo culture was DMEM (119660-025, Gibco, USA) supplemented with hFF (10 and 20% respectively) on Vero cell monolayer.

6. Embryo and blastocyst grading

After cleavage of normally fertilized zygotes, the morphological grade of all embryos was assessed on day 2~3 and 5~7 post-oocyte retrieval as follows. Embryo and blastocyst grading was based on the system devised by Veeck[17], Dokras *et al*[11] and Park[8].

Embryos were classified as follows; grade 1 (G1: blastomeres of equal size and no cytoplasmic fragments), G2 (blastomeres of equal size and minor cytoplasmic fragments or blebs), G3 (blastomeres of equal or unequal size and few cytoplasmic fragments or none), G4 (blastomeres of equal or unequal size and significant cytoplasmic fragmentation) or G5 (blastomeres of any size and severe or complete cytoplasmic fragmentation).

Blastocyst grades (BG) were classified as followed. BG1 demonstrated typical development with early cavitation occurring on day 5 or before day 6 of development resulted in an expanded single cavity lined with clear TE and a distinct ICM. BG2 had the same appearance as BG1 but their formation was delayed by 24~48 h after initial cavitation had begun. BG3 had dark degenerative area within the ICM and/or TE. An additional category of cavitating embryos was also identified. These were not considered to be blastocysts at all since they lacked a clear morula stage where the cells had undergone compaction and neither a distinct TE layer nor an ICM region was identifiable. These were often embryos of 6~8 cells that subsequently developed single or multiple cavities of different sizes and which were also unusual since they had well define, smooth edges.

7. ET and pregnancy monitoring

After assessment of embryo or blastocyst grade, embryos and/or blastocysts that showed the best morphological development were transferred to the uterine cavity on day 2, 3 or 4 and 5~7 post-oocyte retrieval. Catheter for ET was used Tom Cat Catheter (8890-793021, Sherwood, USA) but used Jansen-Anderson Bulb Tip Embryo Transfer Catheter Set (K-JET-3200, Cook, Australia) if Tom Cat Catheter was not inserted the uterine cavity.

Pregnancy was defined as serum ?-hCG level of >10 mIU/ml on day 10 after ET, and a clinical pregnancy was determined by the presence of gestational sac on vaginal ultrasound examination at 3 weeks after oocyte retrieval.

8. Statistical analysis

Data obtained from the experiment (fertilization, cleavage and clinical pregnancy rates) were analyzed using SAS (version 6.2). Values are the mean

Table 1. Clinical outcomes of transfer time and methods in IVF cycles

Variables	Groups							
	I	II	III					
	D2ET	D5ET	Subsequent ET					
			Total	2-5	2-6	2-7	3-5	4-7
No. of cycles included	12	24	12	6	1	1	2	2
Age of female patients								
Mean±SD	30.5±2.5	30.2±3.4	30.5±2.0	30.8	33	28	30	30
Range	25~34	24~35	28~34	29~34	33	28	30	28~32
No. of oocytes allocated	93	380	152	52	7	13	24	56
Mean±SD	7.8±1.9[a]	5.8±6.7[b]	12.7±10.4[ab]	8.7	7	13	12.0	28.0
Range	6~12	7~31	6~46	6~13	7	13	10~14	10~46
Fertilization rate (%)	75 (80.6)[ac]	344 (90.5)[b]	126 (82.9)[c]	39 (75.0)	6 (85.7)	9 (69.2)	24 (100)	48 (85.7)
Cleavage rate (per 2-PN, %)	70 (93.3)	341 (99.1)	122 (96.8)	38 (97.4)	5 (83.3)	8 (88.9)	23 (95.8)	48 (100)
No. of embryos transferred	50	88	45	25	4	5	7	6
Mean±SD	4.2±0.8	3.7±0.8	3.8±0.9	4.2	4	5	3.5	3.0
Range	2~5	2~5	3~5	3~5	4	5	3~4	3
Embryos / blastocysts	4.2/0	0/3.7	2.1/1.7	2.8/1.4	2/2	2/3	1.5/2	2/1
Clinical pregnancy rates (%)	4 (33.3)	14 (58.3)	7 (58.3)	3 (50.0)	0	1 (100)	2 (100)	1 (50)

[a,b,c]Means separation within a row by Duncan's Multiple Range Test, 5% level. Note: D2ET = Day 2 embryo transfer (ET); D5ET = Day 5 ET

±SD. Differences were analyzed by Duncan's Multiple Range Test. Results were considered statistically significant when p-values were less than 0.05.

RESULTS

The purpose of this study was to investigate the effects of transfer time (day 2~7) and methods (day 2, 5, or SET) on the clinical outcomes in IVF cycles.

A total of 48 couples (group I: n=12; II: n=24; III: n=12) was included in this study. A total of 625 oocytes was used to each groups (group I: n=93; II: n=380; III: n=152). The results regarding fertilization, cleavage and pregnancy rate are summarized in Table 1.

Fertilization rates were significantly higher ($p<0.05$) in group II (90.5%, 344/380) than in I (80.6%, 75/93) and III (82.9%, 126/152). No differences was found in the cleavage rate (93.3~99.1%, per fertilized oocytes). Mean percents of embryo development (per fertilized oocytes) in group I, II and III were 93.3 (70/75), 99.1 (341/344) and 96.8% (122/126) respectively. No differences were found in cleavage rate among three groups. Clinical pregnancy rates were higher in group II (58.3%, 14/24) and III (58.3%, 7/12) than that in group I (33.3%, 4/12), however due to the small number of cycles involved in ET, the differences were not statistically significant. Rates of implantation, multiple pregnancy, abortion, on-going pregnancy and take home baby were not shown in this results.

In day 2-5, 2-6, 2-7, 3-5 and 4-7 ET of SET group, fertilization (75.0, 85.7, 69.2, 100 and 85.7%), cleavage (97.4, 83.3, 88.9, 95.8 and 100%) and pregnancy rates (50.0, 0, 100, 100 and 50.0%)

indicated that SET is effective method in terms of pregnancy rate.

DISCUSSION

The rates of failure to develop to the blastocyst stage are between 0~37%, depending on the patient selection criteria.[2,18~27] Ashkenazi *et al*[2] reported that 12.8% of patients who were designed for a double transfer did not have a blastocyst available for the second transfer. They reported that the double (consecutive) transfer of early embryos (on day 2 or 3) and blastocyst (s) (on day 5) does not improve either pregnancy (36.8~41.4%) or implantation (14.6~19.8%) rates compared with the traditional early transfer of high-quality cleaving embryos.[2] Jung *et al*[14] concluded that the second transfer (on day 3 and 5) did not have a significant effect on the pregnancy rate. The most important factor for the pregnancy seems to be the quality of the embryos transferred on day 3 following oocyte retrieval.[14] Another possible explanation is an adverse effect of the second transfer on the implantation process. A second insertion of a catheter through the cervix might cause a trauma to the uterine lining or stimulate secretion of prostaglandins that could produce uterine contractions. It might also introduce more mucus or additional microbial contamination to the uterine cavity.[28] However, Rijuders and Jansen[29] have shown that the predictive value of embryo morphology for subsequent blastocyst formation is limited before the full activation of the embryonic genome. In their study, 49% of the embryos preselected on day 3 were not transferred on day 5, partly because there were better embryos present on day 5 that had not been preselected, but mostly because these embryos showed an arrested development or degradation, despite their good quality on day 3.[29] Ben-Schlomo *et al*[30] concluded that double transfer of 48~72 h embryos and late blastocysts was not detrimental treatment cycles. Their results show that pregnancy rates per ET were 52.0% for double ET, 34.5% for single ET ($p<0.01$). Beauchamp *et al*[13] reported that double ET replacing embryos in two attempts resulted in a 14% increase in pregnancy rate. Multiple pregnancy rate was 25.0%. The increased ET fluid volume had no deleterious effect in pregnancy and implantation rates (15.0%) or spontaneous abortion rate (15.0%) or ectopic pregnancies (0%). A good second ET was associated with higher pregnancy rates. Routine double ET under sonogram guidance increase overall ET efficiency and pregnancy rates.[13]

The results of this study showed that subsequent ET (SET) (on 2-5, 2-6, 2-7, 3-5, 4-7 day ET) improve pregnancy rates compared with the ET on 2 day, but similar to the rate by blastocyst ET on day 5, and SET is effective method in term of pregnancy rate in an unselected population of patients.

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