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Efficacy of Coculture System in the Patients with Poor Prognoses on Human IVF-ET Program

Hye-Kyung Byun[1], Hye-Won Youm[1], Mi-Kyung Koong[2,3], Il-Pyo Son[2,3], Inn-Soo Kang[2,3] and Ho-Joon Lee[1]

[1]Infertility Research Lab., Cheil Medical Research Institute, [2]Infertility Clinic, Samsung Cheil Hospital & Women's Healthcare Center, [3]Department of Obstetics & Gynecology, SungKyunKwan University, Seoul, Korea

사람의 체외수정 시술시 저적응 예후를 보이는 환자에서 공동배양술의 효용성에 관한 연구

삼성제일병원 제일의학연구소 불임연구실[1], 불임크리닉[2],
성균관대학교 의과대학 산부인과학교실[3]

변혜경[1] · 염혜원[1] · 궁미경[2,3] · 손일표[2,3] · 강인수[2,3] · 이호준[1]

= 국문초록 =

본 연구는 *Vero* cell을 이용한 사람 배아의 공동배양술이 배아의 질을 향상시킬 수 있거나 또는 반복적 착상 실패를 극복하여 임신을 가능케 할 수 있는지 알아 보고자 시행되었다. 1996년 일년 동안, 반복적 착상 실패를 경험한 환자 (group I)와 이전 주기에서 배아의 질이 나빴던 환자 (group II)를 포함한 총 202례를 분석하여 대조군과 공동배양군 간의 배아의 질, 임신률, 임신유지율 및 착상률을 비교하였다.

Group I 93례 가운데 34례는 공동배양을, 나머지 59례는 기존의 체외수정을 시행하였다. Group II 109례에서는 공동배양 36례, 기존의 체외수정 73례를 시행하였다. Group I에서 공동배양군의 임신률, 임신유지율 및 착상률은 각각 14/34 (41.2%), 9/34 (26.5%), 16/81 (19.8%)로 대조군 (11/59 (18.6%), 8/59 (13.6%), 12/152 (7.9%))에 비하여 높았으며, 특히 임신률과 착상률은 유의한 차이를 나타내었다 (p=0.028, p=0.015). Group II에서는 공동배양군의 임신률과 임신유지율 및 착상률이 각각 8/36 (22.2%), 5/36 (13.9%), 8/87 (9.2%)로 대조군 (5/73 (6.8%), 3/73 (4.1%), 3/158 (1.9%))에 비하여 높았고, group I의 결과에서와 마찬가지로 임신률과 착상률의 유의한 차이를 나타내었다 (p=0.028, p=0.022).

이상에서 *Vero* cell을 이용한 공동배양술은 위의 두가지 주소를 가진 환자군에서 좋은 결과를 나타내었다. 또한 group II에서 3일-공동배양군의 임신률 역시 향상되어 (4/15 (26.7%)), 보조부화술을 겸비한 3일-공동배양이 이전 주기에서 배아의 질이 나빴던 환자군에 적용될 수 있음을 알 수 있었다.

결론적으로 *Vero* cell을 이용한 공동배양술은 반복적 착상 실패를 경험한 환자나 또는 이전 주기에서 배아의 질이 나빴던 환자에게 적용하여 임신률을 향상시킬 수 있을 것으로 사료된다.

INTRODUCTION

Fertilization in the human occurs in the ampullary region of Fallopian tube, and the fertilized oocyte cleaves in the tube. The embryo is transported into the uterus by the mechanism of ciliary action and muscular peristalsis 4 to 5 days after fertilization. Morula or blastocyst in the uterus expands to hatch and implants

into the musin layer in the endometrium. On the other hand, in human IVF-ET program, 4 to 8-cell stage embryos which are cultured *in vitro* are transferred into the uterus 2 or 3 days after oocyte retrieval; The transferred embryos have to cleave to blastocyst and wait until the timing of uterine receptivity for implantation in the uterus. Untimely embryo transfer may cause low pregnancy and take home baby rates. Attempts to prolong culture period have been tried in order to overcome these severe problems and to synchronize embryo with uterus.

Coculture systems with kinds of cell types have been proposed to obtain a high pregnancy rate in human IVF-ET program (Bongso *et al.*, 1989, 1992, 1994; Menezo *et al.*, 1990, 1992; Wiemer *et al.*, 1989a, 1989b). We followed our preliminary experimental method, replacing human oviductal epithelial cells with *Vero* cells (Lee *et al.*, 1994), in order to avoid the exposure of kinds of infection, and the transfer of diseases including AIDS by human oviductal cells.

We carried out this study to evaluate coculture system of human embryos with *Vero* cells, especially to evaluate whether the system can improve pregnancy rate in patients with two selected indications.

MATERIALS AND METHODS

Patients

From January to December 1996, 1158 IVF cycles were carried out at Infertility Clinic in Samsung Cheil Hospital, Seoul, Korea. Among them, coculture was performed in 70 cycles. Coculture was strictly performed in the two different categories of patient: (i) those with repetitive implantation failures and (ii) those with poor embryonic quality in their previous cycles. Conventional IVF of 132 cycles with the same indications and protocols of coculture group were selected and compared as a control.

Ovarian stimulation and In Vitro Fertilization

Controlled ovarian hyperstimulation was conducted using GnRH agonist (Superfact, Serono, USA) and FSH (Pergonal, Serono, USA) or FSH/hMG (Humegon, Organon, USA) (long and short protocols). Daily ultrasound monitoring for follicular measurement was started on the 3rd day of gonadotropin administration. Blood samples for measurement of serum E_2 concentration were drawn every 2 days. HCG (Pregnyl, Organon, USA) was administered when the diameter of dominant follicles was >17 mm. The oocytes were classificated by the cumulus spreading technique (Veeck, 1988). For 4 to 6 hours after oocyte retrieval, the oocytes were preincubated and inseminated. Eighteen to 20 hours later, the oocytes were exam-ined for the presence of two pronuclei.

Coculture of two pronuclear embryos with *Vero* cell monolayers

Frozen-thawed *Vero* cells were seeded into a multi-well dish under 5% CO_2 in air at 37°C on the day of oocyte retrieval. (About 5×10^4 cells/ml count were seeded for the wells of 1 and 2, and sequensely 2.5×10^4 cells/ml for 3 and 4 into a multi-well dish.) Ham's F10 culture medium (Irvine, USA) supplemented with 10% FBS (Gibco BRL, USA) was used. On day 1 after oocyte retrieval, cell proliferation for monolayer was confirmed and culture medium was changed with preequilibrated medium 1 to 2 hours before coculture. Two PN-embryos were transferred into 1 and 2 wells and cultured for 1 day. On day 2 after oocyte retrie-val, they were retransferred into 3 and 4 wells of the same dish. Freshly prepared monolayers as the same method were used during the coculture. Each monolayer was used for 1 day because the overgrowth of feeder cells may affect detrimental effect on embryos.

Embryo transfer on day 3 or 5

On day 3 after oocyte retrieval, cocultured embryos were observed for embryo transfer. Embryos which were thought to be unable to reach to blastocyst were transferred into the uterus on day 3 after assisted hatching (Cohen *et al.*, 1992; Mandelbaum, 1996; Wiemer *et al.*, 1996), whereas healthy embryos were continued coculture. On day 5, embryos that reached to blastocyst were selected for embryo transfer. Supernumerary blastocysts were cryopreserved with glycerol described by Menezo *et al.* (1995). Pregnancy was confirmed by RIA for β-hCG on 12 days post oocyte retrieval. Clinical pregnancy was defined as a visible gestational sac by ultrasoundsonography.

Statical analysis

Data was analysed by Fisher's exact test and Chi square test to determine statistical difference between groups. A statistically significant difference was defined as $p<0.05$.

RESULTS

A total of 591 two-pronuclear embryos from 70 cycles was cocultured with *Vero* cells. Of 509 cleaved embryos, 168 cleaved embryos were transferred into the uterus on day 3 or 5. There was significant difference in pregnancy rate between coculture and conventional IVF groups (Table I, II and III). In addition, there was an outstanding difference in implantation rate between two groups, whereas there was no significance in on-going rate. Table IV shows the low blastulation rate in the coculture group with the indication of poor embryonic quality.

DISCUSSION

Coculture systems have been used in human IVF program in order to prolong culture time and to improve the quality of embryo (Bongso *et al.*, 1990, 1991, 1995). Comparisons of feeder cells have shown that there is no cell-type specificity nor species-specificity in embryotrophic effect (Papaioannou and Ebert, 1986; Desai *et al.*, 1994). We have gotten a satisfactory result from the coculture system with *Vero* cells, which was the similar result with human oviductal epithelial cells (Lee *et al.*, 1994).

Table I. Results of patients with repetitive implantation failures who were underwent coculture compared to control

Group	No. of cycles	Mean age of patients	Mean No. of IVF attempts	No. of oocytes (mean ± SE)	No. of embryos (mean ± SE)
Control	59	33.8 ± 0.4	3.8 ± 0.2	849 (14.4 ± 0.4)	451 (8 ± 0.0)
Implantation failures	34	27.1 ± 0.7	4.4 ± 0.2	516 (15.2 ± 0.5)	254 (7.5 ± 0.2)
Total	93	33.7 ± 0.4	4.0 ± 0.0	1365 (14.7 ± 0.3)	705 (7.6 ± 0.2)

Table II. Results of patients with poor embryonic quality who were underwent coculture compared to control

Group	No. of cycles	Mean age of patients	Mean No. of IVF attempts	No. of oocytes (mean ± SE)	No. of embryos (Mean ± SE)
Control	73	33.4 ± 0.1	1.6 ± 0.1	687 (9.4 ± 0.7)	225 (3.1 ± 0.5)
Poor embryonic quality	36	32.6 ± 0.7	4.3 ± 0.0	571 (15.9 ± 0.7)	255 (7.1 ± 0.5)
Total	109	33.1 ± 0.1	1.9 ± 0.0	1258 (11.5 ± 0.9)	480 (4.4 ± 0.3)

Table III. Pregnancy rate in the patients underwent conventional IVF or coculture

Group	No. of cycles transferred	No. of embryos transferred	No. of preg (%)	No. of on-going (%)	No. of implantation per transferred embryos
Control	59	152	11 (18.6)	8 (13.6)	12 (7.9)
Implantation failures	34	81	14* (41.2)	9 (26.5)	16** (19.8)
Control	73	158	5 (6.8)	3 (4.1)	3 (1.9)
Poor embryonic quality	36	87	8*** (22.2)	5 (13.9)	8**** (9.2)

* P values is 0.0280, ** P values is 0.0147
*** P values is 0.0282, **** P values is 0.0219.

Table IV. Developmental rate to blastocyst in those with two prognoses who were underwent coculture

Group	No. of 2PN	No. of embryos	No. of blastocyst (%)	No. of blastocyst transferred
Implantation failures	217	197	86 (43.7)	62
Poor embryonic quality	178	162	55 (34.0)	46

Although so strict indications, embryonic development in two coculture subgroups was enhanced which was compared with the previous cycles. Higher pregnancy and implantation rates in the coculture group regardless of the two indications show the positive effect of coculture. Furthermore, coculture underwent in the patients with poor embryonic quality improved higher pregnancy and implantation compared with conventional IVF in the patients with the same indication. For these patients, 3 day-coculture system with assisted hatching is recommended in order to confirm the ability of the embryos to develop and to avoid the cancellation of embryo transfer.

This study shows that there is no difference in blastulation between long and short protocols (data was not shown), which agrees to the result of Menezo *et al.* (1992). Blastulation rate in coculture group is significantly higher than control; Interestingly, of coculture group, the subgroup with the indication of poor embryonic quality shows the improved blastulation rate compared to control. This suggests, coculture with *Vero* cells has a positive effect on the embryonic development like other feeder cells.

Of our data with implantation rate per transferred embryo, there is a significant difference between coculture and control groups. This agrees to the results of Menezo *et al.* (1992) and Wiemer *et al.* (1996). On the other hand, the use of coculture system in the groups with non-selected patients failed to show a significant improvement of pregnancy rate (Guerin *et al.*, 1991; Janny *et al.*, 1993). This suggests that coculture system can be used for selected indications in order to get successful pregnancy rate. Higher implantation rate in the subgroup with the indication of poor embryonic quality can make it possible to propose coculture system for the patients with the indication.

Even though coculture system has advantages in human IVF program, it is a time-consuming method and has risks of cross-contamination of diseases and of bacterial and viral infection.

The use of animal cells recovered from the slaughterhouse or human heterologous cells should be avoided. Constant quality control of *Vero* cells should be performed for the prevention of those risks. The use of human autologous cells could be recommended, but further study should be conducted whether those cells can improve development and blastulation of human embryos.

In conclusion, coculture system with *Vero* cells improves pregnancy and implantation rates in the patients with the problems of repetitive implantation failures or those with the poor embryonic quality in their previous cycles. In the patients with the poor embryonic quality, 3 day-coculture with assisted hatching is recommended in order to avoid the cancellation of embryo transfer and to get higher pregnancy rate. Further study has been conducted for the determination of embryotrophic factors secreted from the feeder cells which are used in human IVF program. Synthetic culture medium which provides human embryos with optimal culture condition to blastocyst stage would be introduced to replace with coculture system in the future.

SUMMARY

The present study was carried out to evaluate whether the coculture system of human embryos with *Vero* cells can improve the quality of embryo or overcome the repetitive implantation failures in order to obtain pregnancy.

From January to December 1996, a total 202 cases which patients with the problems of repetitive implantation failures (group I) or those with the poor embryonic quality in their previous cycles (group II) was analysed. The quality of cocultured embryo, pregnancy, on-going and implantation rates between coculture and control groups were compared.

Of 93 cases in group I, coculture was performed in 34 cases and conventional IVF for the rest. Of 109 cases in group II, 36 for coculture and 73 for conventional IVF. In group I, pregnancy, on-going and implantation rates in coculture group (14/34 (41.2%), 9/34 (26.5%), 16/81 (19.8%), respectively) were higher than those of control (11/59 (18.6%), 8/59 (13.6%), 12/152 (7.9%), respectively). There is significance in the pregnancy and implantation rates ($p=0.028$ and $p=0.015$). In group II, pregnancy, on-going and implantation rates in coculture group (8/36 (22.2%), 5/36 (13.9%), 8/87 (9.2%), respectively) were higher than those of control (5/73 (6.8%), 3/73 (4.1%), 3/158 (1.9%), respectively). Like the result of group I, there is significance in the pregnancy and implantation rates ($p=0.028$ and $p=0.022$).

Coculture system with *Vero* cells works well in the groups of the two indications. Although the case of 3 day-coculture was small as 15 cases in group II, 3 day-coculture improved pregnancy rate (4/15 (26.7%)). Therefore, 3 day-coculture with assisted hatching is recommended to the patients with poor embryonic quality. In conclusion, coculture system with *Vero* cells can be suggested as an effective method which improves pregnancy rate in those who have repetitive implantation failures or whose embryonic quality was poor in their previous cycles.

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