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Clinical pregnancy derived of blastocyst culture and transfer at Permata Hati Infertility Clinic Dr Sardjito Hospital/ Faculty of Medicine Gadjah Mada University Yogyakarta. A case report

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ABSTRACT

Ita Haryadi, Amino Rahardjo, Moch. Anwar, Zain Alkaff - ***Clinical pregnancy derived of blastocyst culture and transfer at Permata Hati Infertility Clinic Dr Sardjito Hospital/Faculty of Medicine Gadjah Mada University Yogyakarta. A case report***

In the natural environment, human embryos will be only in the uterine cavity at the morula or blastocyst stage. However, *in vitro* culture of embryos in assisted reproductive technologies remain replacing back embryos at or before four cell stage due to some problems involved. In this case report we aimed to culture and transfer embryos at blastocyst stage. A sub-fertility couple, wife at 33 years old, with bilateral fallopian tube damage. Husband has normal semen analysis. Of 9 oocytes retrieved from ovum pick up (OPU), seven were fertilized. On day 3 observation, five embryos showing considerably good in grade to allow further development. On day 5, four out of 5 embryos were formed blastocysts of which 2 were transferred, and the other two blastocysts were cryopreserved. Blastocysts of this reported patient were formed at the rate of 57.1% (4/7). Pregnancy was confirmed by positive urine semi-quantitative test, two intrauterine gestational sacs with 3 heart pulsations detected by ultrasonography. We reported a pregnancy obtained from our first human blastocyst culture and transfer to the couple undergoing *in vitro* fertilization (IVF) program at Permata Hati Infertility Clinic, DR Sardjito Hospital/Faculty of Medicine Gadjah Mada University, Yogyakarta.

Key words : blastocyst culture – assisted reproductive technologies – *in vitro* fertilization

ABSTRAK

Ita Haryadi, Amino Rahardjo, Moch. Anwar, Zain Alkaff - ***Clinical pregnancy derived of blastocystculture and transfer pada Klinik Infertilitas Permata Hati Rumah Sakit Dr Sardjito/Fakultas Kedokteran Universitas Gadjah Mada Yogyakarta. Laporan kasus***

Secara alamiah, embrio manusia akan berada di dalam lingkungan uterus pada stadium morula atau blastosis. Meskipun demikian, disebabkan oleh berbagai permasalahan, kebanyakan embrio dalam program teknologi reproduksi bantuan (TRB) masih dikembalikan ke dalam uterus pada stadium 4 sel atau sebelumnya. Di dalam laporan kasus ini, kami bertujuan untuk mengkultur dan mentransfer embrio pada stadium blastosis.Pasangan suami istri (pasutri) subfertil, istri berusia 33 tahun dengan kedua tuba fallopii buntu. Suami memiliki sperma yang normal. Tujuh oosit fertilisasi dari 9 yang didapat pada prosedur pengambilan oosit. Observasi pada hari ke 3, lima embrio menunjukkan perkembangan yang baik sehingga kultur dilanjutkan untuk mendapatkan pertumbuhan lebih lanjut. Pada hari ke 5,

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empat blastosis terbentuk, di mana 2 ditranfer kembali kepada pasien dan 2 sisanya dibekukan. Angka blastosis yang didapatkan dari pasien tersebut adalah 57.1%(4/7). Kehamilan dikonfirmasi dengan tes urin semi kuantitatif, dua kantong kehamilan dengan tiga pulsasi didapatkan dari pemeriksaan ultrasonografi. Kami melaporkan kehamilan yang didapatkan dari program pertama kultur dan transfer embrio pada stadium blastosis di Klinik Infertilitas Permata Hati, RS DR Sardjito/Fakultas Kedokteran UGM, Yogyakarta.

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INTRODUCTION

Suboptimal *in-vitro* conditions, limited number of available oocytes per patient, and concern about cleavage arrest and degenerative changes due to prolonged *in vitro* culture of embryos in assisted reproductive technologies, has been addressed as a main in-vitro culture problems and lead to most centers returning the embryos to the uterine environment at or before four cell stage.

However it is not in the biological condition to replace embryos at the early stages since in the natural environment, embryos will be only in the uterine cavity at the morula or blastocyst stage. Extending culture period to 5 or 6 days after insemination allows for a more physiological synchronization of the embryo stage and uterine secretory transformation. There is also an intense concern why extended culture should be encouraged, such as production of large number of embryos exhibiting cleavage arrest and fragmentation. Furthermore selecting best embryos at a minimal number to be transferred could be achieved, because only viable and genetically normal embryos will be capable of development to the blastocyst stage and beyond.

Recently, studies have indicated a possibility to obtain viable human blatocyst in either co-culture condition or defined sequential media system.[1,2]

In this case report, we reported a pregnancy obtained from our first blastocyst culture and transfer of couples undergoing *in-vitro* fertilization (IVF) program at Permata Hati Infertility Laboratory, DR Sardjito General Hospital/Faculty of Medicine-Gadjah Mada University Yogyakarta.

CASE REPORT

Patient presented sub fertility with bilateral tuba damage caused of ectopic pregnancy in a consecutive year, July 2002 and February 2003. Salpingectomy had been performed for both sinistral and dextral tubes. In August 2003, at age 33 years, had a controlled ovulation induction for her IVF (*In-vitro* Fertilization) program. She had long down regulation, started at 20th of menstrual cycle with 500μg busereline acetate (Suprefact, Hoechst AG, Germany) for 10 days. When down regulation criteria achieved (E2=71.5pg/ml; FSH=4.37mIU/ml;LH=2.05mIU/ml), stimulation started with 150 IU recombinant FSH (Gonal F, Serono) while busereline acetate reduced to 200μg. Monitoring of ovarian stimulation was done by transvaginal ultrasound and E2, LH level. Stimulation criteria met at E2=1390.46pg/ml; LH=5.18mIU/ml and of 12 growing follicles, two were at 17 mm in diameter, when 10.000 IU hCG (Profasi, Serono) was given for ovulation induction. Ovum Pick Up (OPU) was performed 34 hours after hCG administration. Of 12 follicles, nine oocytes were retrieved. Fresh ejaculated sperm was collected from the husband, and preparation was done with Colloidal Silica Density Gradient (45%; 90%) to harvest best quality of the sperm in term of motility, morphology, concentration and viability.

Insemination with 10.000-15.000 motile sperm/ 100μL/OCC carried on under mineral oil, then cultured in IVF-20 media (Vitrolife, Scandinavian Science) at 5% CO2, 37°C. Oocytes were mechanically denuded of corona cells for fertilization assessment 18h post insemination. Under inverted microscope, oocytes exhibiting two pronucleus (2PN) and two polar bodies (2PB) judged as fertilized. The fertilized oocytes were then placed in group of two to three in 30μL microdrops of preequilibrated IVF-20 culture media under mineral oil in Falcon 3002 (BD Labware, NJ) culture dishes.

Forty four to 45 hours after insemination, embryos were assessed for their cell number, degree of fragmentation and similarity of the blastomeres. Microdrops of culture media (IVF-

20:CCM-20, Vitrolife, Scandinavian Science, at 1:1) were set up in culture dishes under mineral oil, at 5% CO_2, 37°C for the next day.

Sixty nine to 70 hours after insemination, embryos were again assessed for their further development, in term of cell number, degree of fragmentation and similarity of the blastomeres. Appearance of cytoplasmic vacuole, dark granularity and shape anomalies of the blastomeres count as negative score for the embryos. Embryos were then placed in fresh equilibrated culture media for further development. Microdrops of culture media (CCM-20) were set up in culture dishes under mineral oil, at 5% CO_2, 37°C for the next day.

Ninety four to 95 hours after insemination, embryos were assessed for their capability to be compacted (morula) and placed in a group of two to three in fresh equilibrated culture media.

One hundred and nine to 110 hours after insemination, embryos were assessed for the presence of fluid-filled cavity (blastocoele), differentiation into outer trophoectoderm layer and inner cell mass as criteria for blastocyst formation[3]. Embryos that were still compacted morula or were early blastocyst requiring prolonged cultured to form fully expanded blastocyst, were transferred to fresh equilibrated culture media.

The best quality of blastocysts was selected for embryo transfer. The number of embryo for transfers being determined by the availability of embryos for transfer and the patient's age and previous clinical history. If the patient was > 35 years of age or if the patient had failed to achieve a pregnancy after three or more previous IVF cycles, then consideration was given to transferring three or occasionally four blastocyst if available rather than only one or two blastocyst. One or two blastocyst would be a usual recommendation to patient of younger age or with a limited IVF history.

Embryo were transferred on day 5, after selected and washed in fresh equilibrated CCM-20. With the use of a COOK Pivet Lab. Embryo Transfer Set (K-PETS-2031, Cook Australia, Eight Mile Plains, Queensland, Australia), embryos were transferred in pre-equilibrated CCM-20. Blastocysts in excess of those selected for transfer, were cryopreserved with use of vitrification with DMSO and Ethylene Glycol as cryo-protectants[4].

Luteal phase support was given by 90mg progesterone vaginal suppositoria daily (Crinone, Serono) start from the day of ovum pick up until there is laboratory evidence of pregnancy, when pregnancy occurred progesterone application continued for 30 days. Pregnancy test was done on day 16 after ovum pick up, following by ultrasound assessment to detect fetal heart at 6 weeks of gestation.

A total of 9 oocytes were retrieved on the day of ovum pick up. Fertilization was shown by presence of two pronucleus on 7 out of 9 oocytes (66.7%). The rest 3 oocytes were either unfertilized (1) or exhibiting polyspermia (2), on which those oocytes were culture separately to observed their further changes of development. However, these normally failed fertilization oocytes were either arrested, totally fragmented or failed to form blastocyst on their further development.

Of 7 fertilized oocytes, one had pronucleus in different size on which embryo arrested at day four of development and failed to be compacted with 40-50% fragmentation. Whereas one normally fertilized oocytes was still on its one cell stage on the following day of observation, thus there were five remaining normally fertilized oocytes to be cultured and selected for embryo transfer.

On day two of observation, one embryos was at 2 cell stage and four embryos were at 4 cell stage with minimal cytoplasmic fragmentation (<5%), and on the following day developed to 6-8 cell stage. One embryo exhibited a bit more fragmentation (10-15%) compare to the rest four (<5%), but all were cultured to allows their capability in forming blastocyst.

On day four, all five embryos were compacted to form morula. Early day five, three embryos were form good quality of blastocyst and were assessed for transfer using several markers according to the blastocyst scoring criteria reviewed by Bongso, et al[3]. Good quality blastocyst that give the best implantation chance would be cavitated or expanded on day 5, have distinct inner cell mass (ICM), a well laid down trophoectoderm (TE) with sickle shape cell, a thin zona pellucida and a high total.

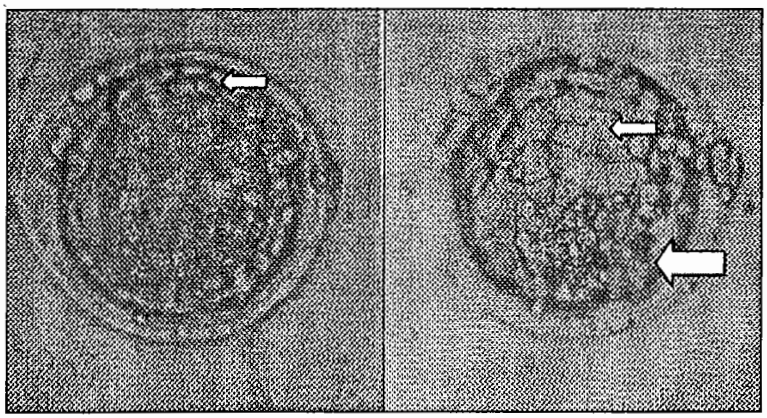

FIGURE 1. D5 Grade 1 Blastocyst, focused on TE cell (left & right, small arrow) and ICM (right, big arrow)

cell number (TCN) when focusing carried out at different planes at the surfaces and depths of the blastocyst (small arrow). According to blastocyst grading, all three blastocysts were judged as grade 1 (FIGURE 1), with distinct and big inner cell mass (big arrow). Two blastocyst considered transferred, the excessive three embryos were two cryopreserved for further employment when needed and one embryo was discharged because failed to cavitated and degenerated at the following day of observation.

On day 16 after ovum pick up (OPU), pregnancy was confirmed by positive urine semi-quantitative pregnancy test (Test Pack, Abbot), and 1 week later ultrasound showed intrauterine 2 gestational sac with unclear either 2 or 3 heart pulsations. A repeated ultrasound investigation on 7 weeks of gestation, confirmed of 2 gestational sac and 3 heart pulsation of embryos with consistent dimensions by embryo transfer dates (FIGURE 2).

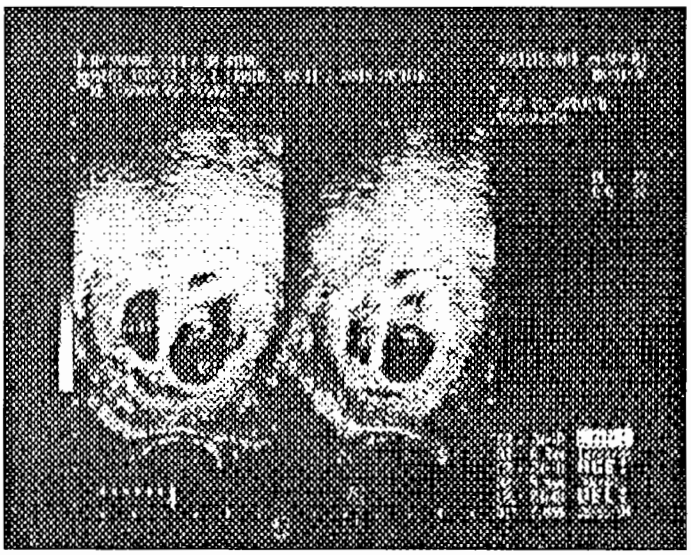

FIGURE 2. Ultrasound investigation image of 7 weeks gestation.Note, 2 gestational sacs, 3 fetuses. Two fetuse were within 1 gestational sac (right)

DISCUSSION

Study has indicated that transferring embryos at blastocyst stage would be advantage since it is giving more synchronization between the endometrium and the embryo, and the possible selection of embryos with higher implantation potential. Further more, blastocyst stage transfer would reduce multiple pregnancy and improve human IVF outcome[2,5,6].

Delayed transfer at the blastocyst stage, could only be suitable procedure when some criteria are met. First, culture condition has to be excellent enough to allow development of the intrinsically competent embryos to the blastocyst. Second, embryos should be develop in sufficient numbers to allow most patients to have an ET, and finally, and most important, the blastocyst that grow in vitro must be viable[7] . In this case report, patient had 9 oocytes on the retrieval day. Fertilization was achieved on 7 oocytes (66.7%), and good development rate was observed on 5 embryos at day 3. These embryos were either assessed as grade 1 or 1-2, according the day 3 assessment criteria described elsewhere. This finding confirmed that our culture condition could support embryo development at considerable rate, as when at least three good grade 8 cell embryo present at day 3 of culture, blastocyst culture and transfer could be offered to the patients[7,8] .

With the advent of sequential culture media that respect the changing metabolic requirement of embryos, the blastocyst derived of good-quality cleaving embryos is considerably high[8,9,10] . Studies indicated, when culture in vitro blastocyst development rate ranging between 25-53%[2,3,7,11,12,13]. In this our first blastocyst transfer program, blatocyst were formed at the rate of 57.1% (4/7). One expanded blastocyst was formed on early day 5, two followed to formed expanded blastocyst at the observation just before embryo transfer procedure, and the last one was formed expanded blastocyst at the following day (day 6). This fact suggested that our finding was similar to that reported by other center, thus that the culture conditions used in our laboratory are optimized to a point where most of the zygote capable to continued development to the blastocyst stage are able to realize their full developmental potential.

With regard to the many studies that had found women < 40 years, and have more than three good quality embryos at day 3 of culture are at risk of high order multiple pregnancy, two blastocyst were selected for transfer on the day 5 to our patient, both are graded as grade 1 blastocyst with big distinct inner cell mass (ICM) and well sickle cell shape of trophoectoderm (TE). Milki *et al*[12] reported two blastocyst transfer giving considerably reduced multiple pregnancy in blastocyst transfer program. Instead of single blastocyst transfer that we had not confident enough to do so, we transferred two.

On going pregnancy was considered when transvaginal ultrasound showed two gestational sac, one with an embryo with a crown-rump length (CRL) of 6.3 mm and the other containing two embryos with crown-rump length of 7.8 mm and 5.9 mm. All of the embryos had a heart beat. To our knowledge, monozygotic twinning are more likely evidence in blastocyst transfer program related to zona pellucida (ZP) manipulation such as subzonal insemination (SUZI), intracytoplasmic sperm injection (ICSI), and assisted hatching[14]. However, Peramo et al reported in his two cases of IVF program[15] resulted monozygotic twinning. We do not know at this moment, at what stage or by which mechanism the inner cell mass segregated in the IVF program forming monozygotic twinning pregnancy. But some suggestions revealed to genetic origin, and probably trauma occur during transfer procedure or changes in the ZP caused by culture media.

In conclusion, an acceptable blastocyst rate can be obtained using defined sequential media system and there are some reasons for better pregnancy rate of blastocyst stage transfer; human cleavage stage embryos normally resides in the oviduct and does not enter the uterine cavity until after compaction[16], the oviduct and uterus provide different nutritional environment for the embryo[17], uterine contractility decreases progressively at the time of blastocyst transfers compare to transfer procedures at the earlier stages[18], further more, by

culturing embryos to the blastocyst stage, embryos with limited development capability may be identified and avoided to be transferred such as chromosomally abnormal embryos[19,20]. However, some criteria have to be met when considering patients recruited to the blastocyst culture and transfer program.

In this report, we culture embryos to the blastocyst stage and transfer, resulted with on going pregnancy of three heart pulsation embryos within two gestational sac and all are consistent in dimension crown rump length (CRL) to embryo transfer date.

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Note Added in Proof:

While this manuscript was being reviewed, three healthy baby boys were born at Sardjito General Hospital by Sectio Cesarian. One baby was 2200g of weight, the other two were 2150g and 2050g.

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