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Case Report

Vitrification of day 7 hatching blastocyst using hemistraw resulting in a full term delivery: a case study

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ABSTRACT

This is probably the first report of a successful delivery following transfer of a vitrified-warmed day 7 slow growing hatching blastocyst using hemistraw-vitriplug as an embryo carrier system. Case Report: A 26 years old Indian woman underwent controlled ovarian stimulation using short antagonist protocol and on day 11 after hCG administration, sixteen mature oocytes obtained were fertilized by ICSI procedure. On day 5, after transfer of two expanded blastocysts, three supernumerary embryos were still in the expanding stages, therefore, continued to culture in vitro. On day 7, one of them grew to be a hatching blastocyst which was cryopreserved with caution by ultra-rapid vitrification using hemistraw (HS) as carrier system. After 5 months, the vitrified slow growing day 7 hatching blastocyst was warmed and transferred in a natural cycle resulting in successful pregnancy. The women delivered a healthy male baby weighing 2820 grams at 36 weeks of gestation by caesarean section with no obvious anomalies detected. This report concludes that a day 7 hatching blastocyst can be successfully vitrified using HS and have pregnancy potential after warming.

Keywords: Hatching Blastocyst, Hemistraw, Ultra-rapid vitrification

INTRODUCTION

Despite the fact that first IVF pregnancy reported in 1976 [1] was from an early blastocyst, the transfer of cleavage stage embryos has dominated IVF for decades. This was due to the difficulties in successfully culturing embryos to the blastocyst stage as the culture media used were not complex and unable to support normal development.[2] In spite of introduction of many technical advances such as controlled ovarian stimulation, intracytoplasmic sperm injection, preimplantation genetic diagnosis and numerous modifications in the embryo culture media, the key rate-limiting factor in human IVF, the implantation rate remained relatively static for over 20 years in between 10% to 20%.[3]

Physiologically, transferring early embryo into the uterus on day 2 or 3 is a premature transfer, as the human embryo in its early stages of development doesn't resides in the uterus but rather enters the uterus only after compaction. The environment in the uterus is more acidic than the oviduct and it has been demonstrated that prior to compaction, the human embryo is unable to regulate an acid challenge resulting in both nutritional and homeostatic cellular stress.[3] Thus transferring moruale or blastocyst stage embryo was encouraged resulting in an increase in the implantation rates.

In early 1999, after the introduction of first commercial sequential media, prolonged cultivation of embryos to blastocyst stage become a routine practice in human IVF.

The advantages of blastocyst transfer are described as followed:

1. Better embryo selection having an activated genome.[4]
2. Higher predictive values for implantation on the basis of their morphological appearance as compared to early embryos.[3]
3. Reduction in the number of transferred embryos without compromising the implantation rate.[5]
4. Reduction in the incidence of multiple gestations, abnormalities in implantation and placentation (occurring due to transfer of multiple embryos), prematurity, maternal and neonatal complications.[3]
5. Better endometrial synchronity as fatal exposure to the hyperstimulated milieu and consequent endometrial contractions can be shortened.[5-7]
6. Better possibility of survival after cryopreservation as blastocyst contains a larger number of cells than an early embryo.[8]

Since the introduction of blastocyst transfer, reliable protocol for cryopreservation of supernumerary embryos was an urgent need. Results with conventional slow-freezing methods for blastocysts have been variable justifying investigation of alternative approaches.[9] In 2002, it was reported that vitrification can be more favourable than slow freezing for cryopreservation of human blastocyst.[10] Ultra-rapid vitrification involving direct contact between a reduced volume of cryoprotectant and liquid nitrogen greatly increases the cooling and thawing rates thereby reducing the formation of ice crystals and chilling injuries. Many human pregnancies have been reported with ultra-rapid vitrification of blastocyst using the cryotop, the cryoloop, electron microscope grids or the hemi-straw.[9,11-15]

In the field of human blastocyst vitrification, the clinical usefulness of later-stage cryopreservation still remains to be learned because embryos developing slowly are generally considered non-viable and are mostly discarded.[15] It has been studied that blastocysts vitrified on day 5 have a pregnancy potential similar to those of day 6 blastocysts.[8,15] Futhermore, a retrospective study following transfer of human vitrified day 5, 6 and 7 blastocysts showed that all the blastocyst have similar inherent viability with respect to mean gestational age, preterm delivery rate and mean birth weight.[16]

During the later-stage of blastocysts culture due to the increase in the fluid accumulation in the blastocoels there is a progressive enlargement of blastocyst and cavity with a consequent thinning of the zona pellucida (ZP). Finally the blastocyst reaches a stage called hatching when it breaks and the trophectoderm herniates out through a natural breach in the ZP.[2] Cryopreservation of such hatching blastocysts is technically a critical process due to the risk of damage to the exposed inner cell mass and trophectoderm cells.

Successful pregnancies after vitrification of a completely hatched day 6 blastocyst and re-vitrified day 7 spontaneously hatched blastocyst are reported using a cryotop as a vitrification carrier system.[16,18] Successful vitrification of day 5 hatching and hatched human blastocysts is reported using hemi-straw as embryo carrier.[19] However, as far as we known, there are no reports on pregnancies following transfer of a vitrified and warmed slow-growing day 7 hatching blastocyst using hemi-straw as vitrification carrier system.

CASE REPORT

A 26 year-old Indian women (BMI - 21 kg/m^2) with her 28 year-old overweighed Indian husband (BMI – 27 kg/m^2) presented at our Out Patient Department for primary infertility trying to conceive since 2 years. Thorough female physical examinations revealed hypertrophid cervix. Transvaginal ultrasonography showed normal characteristics except bilateral polycystic ovaries. Routine hormonal investigations were normal. The husband's semen analysis was severe oligo-astheno-teratospermic according to the World Health Organization criteria. Blood investigations of the husband showed elevated FSH levels. Husband was advised for specific life style modifications to reduce weight and prescribed with appropriate nutritional supplements. Inspite of conservative treatment offered to the husband, semen analysis again came to be severe oligo-astheno-teratospermic. Therefore, the couple was advised and posted for IVF-ICSI treatment cycle.

Controlled ovarian stimulation

The controlled ovarian stimulation was achieved using short antagonist protocol. Recombinant follicle stimulating hormone (rFSH, Foligraf, Bharat Serum and Vaccines Limited, India) was started from day 2 of the cycle and total dose given to the patient was 1950 IU. The decapeptide analogue of gonadotropin-releasing hormone antagonist cetrorelix acetate (Ovurelix, Sun Pharma Industries Limited, India) at a dose of 0.25 mg was started from day 8 to postpone the luteinizing hormone (LH) surge till day 11. Human chorionic gonadotropin (hCG, Ovitrelle, Merck Sereno Europe Limited, UK) at a dose of 250 mg was administered once the dominant cohort of follicles reached diameter of 18mm on day 11 of the cycle.

In vitro fertilization procedure

Transvaginal ultrasound-guided ovum pick-up was done 35 hours after the hCG administration. Seventeen oocytes were retrieved out of which sixteen were mature oocytes and one was immature oocyte (Germinal vesicle). Sperm preparation was done using discontinuous gradient (Isolate®, Irvine Scientific, USA) technique. All the sixteen mature oocytes obtained were fertilized by ICSI procedure using a micromanipulator (Integra 3™, RI, UK). On day 3 check, three pre-morulae stage, three 10-12 cells stage and two 4-6 cells stage embryos were

observed. On day 5, two expanded blastocyst were transferred using embryo replacement catheter (K-JETS-7019-SIVF, Cook Incorporated, USA). The remaining one expanding blastocyst, one cavitating embryo and one morulae were continued to culture in vitro. In our routine practice we cryopreserve only better quality expanded blastocysts, therefore cryopreservation was not done on day 5. On day 7, one of these surplus embryos grew to be a hatching blastocyst while other two embryos were arrested (Figure 1).

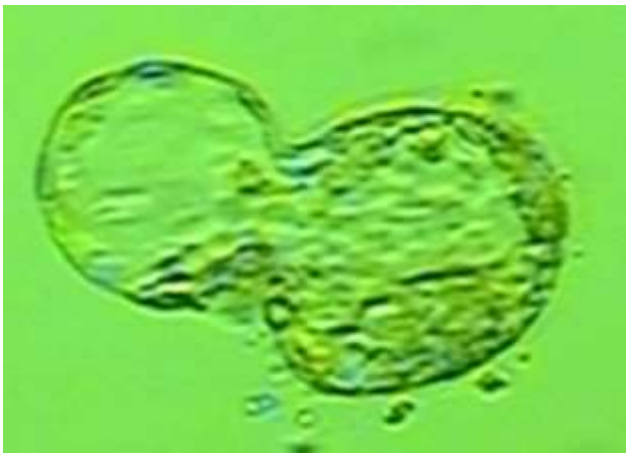

Figure 1: Hatching blastocyst showing a large, compact ICM at the 5 o'clock position in this view. Approximately 70% of the blastocyst has herniated from the natural breach in the thinned ZP at the 8-10 o'clock position in this view. Both the ICM and TE contain many cells and are tightly compact.

Vitrification procedure

As per our routine practice we cryopreserve embryos using VitriFreeze ES™ - VitriThaw ES™ kits (Fertipro N.V., Belgium) using hemistraw-vitriplug as a carrier system (Figure 2). The same vitrification system was used to cryopreserve the hatching blastocyst. The Hemistraw (Astra-Med-tec, Salzburg, Austria) is an embryo carrier consisting of a larger gutter on which small quantity of cryoprotectant (<1 µl) with embryos can be deposited and ultra-rapid cooling rate of >20,000^{0}C/min can be achieved by allowing direct contact of the embryo with liquid nitrogen (LN_2). The Hemi-straw (HS) can be subsequently inserted into a larger pre-cooled 0.5ml straw (Vitriplug, Cryo Bio System, Grenoble, France) under LN_2. [20]

The hatching blastocyst was initially equilibrated in pre-warmed 1 ml of pre-incubation medium in a petri dish at 37^{0}C. The blastocyst was then exposed to different combinations of the freezing medium at room temperature. Drops of 300µl each of freezing medium 1, 2 and 3 in a four-well dish were pre-incubated at room temperature. The hatching blastocyst was first exposed to freezing medium 1 containing 5% dimethyl sulfoxide (DMSO) and 5% ethylene glycol (EG) for 2 minutes, then shifted to freezing medium 2 containing 10% DMSO and 10% EG for 4 minutes and finally exposed to freezing medium 3 containing 20% DMSO and 20% EG for 30 to 60 seconds. The hatching blastocyst was shifted to subsequent medium using pipette of 600µm diameter (RI, UK). An approximate volume of 0.3µl of the freezing medium 3 containing the hatching blastocyst was deposited on the pre-flushed tip of the trough or gutter of the HS using pipette of 600µm diameter (RI, UK) within 30 seconds. In routine practice, for expanded blastocyst pipette of 290µm diameter is used and for fully expanded or hatched blastocyst, pipette of 600µm diameter is used for deposition onto the HS. The HS was then instantly plunged in a dewar of LN_2 and with the aid of forceps inserted into a larger pre-cooled, pre-labelled vitriplug straw. Finally the labelled straw was deposited in the LN_2 storing can. The proper shrinkage of the hatching blastocyst was assessed during the whole procedure under x200 magnification.

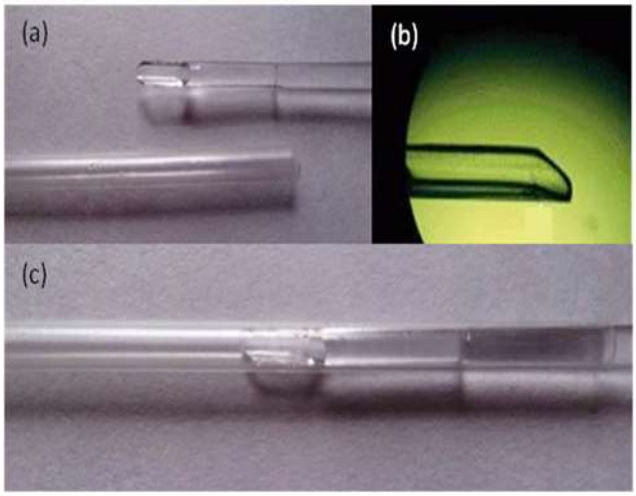

Figure 2: Hemistraw - vitriplug. (a) A hemistraw and vitriplug, (b) The trough or gutter of hemistraw where the embryo with cryoprotectant is deposited, (c) Hemistraw inserted into the vitriplug is showed.

Warming procedure

The urine pregnancy test came to be negative done 12 days after embryo transfer. After a period of 5 months, natural cycle frozen embryo transfer was planned. The Vitrithaw ES™ kit (Fertipro N.V., Belgium) was used for the warming process. A dewar of LN_2 containing the HS was placed near to the microscope. The HS was removed from the dewar and with the aids of forceps the outer larger vitriplug straw was removed and instantly the tip of the HS was placed in prewarmed 1 ml thawing medium 1 containing 20-30% sucrose in a petri dish at room temperature. Once the hatching blastocyst detached from the gutter of the HS, it was shifted in prewarmed 1 ml thawing medium 2 containing 10-20% sucrose in a petri dish at 37^{0}C for 2 minutes using a pipette of 600µm diameter (RI, UK) to accelerate the re-expansion process. The blastocyst was then subsequently transferred in prewarmed 300 µl of thawing medium 3 containing 5-

10% sucrose and thawing medium 4 containing 1-5% sucrose at an interval of 4 minutes at room temperature in a four-well dish. Finally the blastocyst was washed in thawing medium 5 for 1 minute and transferred in blastocyst medium (Cook, Brisbane, Australia). The proper re-expansion of the hatching blastocyst was assessed during the procedure using x200 magnification.

Frozen embryo transfer procedure

As per routine practice warmed blastocyst are incubated for re-expansion for 2 hours before embryo transfer. But the warmed hatching blastocyst was incubated for 1 hour and 30 minutes only. The re-expanded, warmed hatching blastocyst was of optimum quality. During a natural cycle with endometrium thickness of 9 mm, the embryo was transferred using an embryo transfer catheter (K-JETS-6019-SIVF, Cook Incorporated, USA). On day 12 after embryo transfer, pregnancy was confirmed with a positive urine pregnancy test; 5 weeks later, an ultrasound showed one clear and distinct gestational sac with healthy fetal heartbeats. The women delivered a healthy male baby weighing 2820 grams at 36 weeks of gestation by caesarean section. No obvious anomalies were detected.

DISCUSSION

We report a case of successful vitrification of a slow growing day 7 hatching blastocyst using hemistraw-vitriplug as embryo carrier system. Embryo transfer carried out after thawing resulted in a full term delivery of a healthy baby with no obvious anomalies.

It has been studied that hatching of the human blastocyst by day 6 is a favourable prognostic factor for IVF outcome having a higher implantation potential.[21] Therefore the day 7 hatching blastocyst which we vitrified was concluded to be a slow growing blastocyst as on day 5 it was still in the expanding stage. The reason for such delayed development of the blastocyst is not known but it is hypothesized that cytoplasmic immaturity of oocytes, chromosomal abnormalities in blastomeres and suboptimal culture conditions can cause longer intermitotic periods and consequently slower embryo development.[22] It has been demonstrated that, although more slowly growing blastocyst may be innately compromised to some extent, day 7 blastocyst have similar implantation potential to day 5 and day 6 blastocyst and can be vitrified without impairing their ability to implant.[23] In the present case, no effect of such slow growth was observed on the quality of the blastocyst, before and after the vitrification process and following post warming transfer resulted in a successful pregnancy.

The handling of a hatching blastocyst is critical which needs technical expertise. The approximate diameter of a hatching blastocyst ranges from 300µm to 450µm depending upon the amount of blastocyst hatched out of the ZP.[24] In the present case, during the process of vitrification, deposition on HS and warming, the hatching blastocyst was successfully handled with a pipette of 600µm diameter (RI, UK). It reduced the risk of damage or breakage of the hatching blastocyst.

The present study demonstrates successful use of HS as an embryo carrier for cryopreservation of day 7 hatching blastocyst. On the tip of the HS, the blastocyst is encased in a very small volume (0.3µl) of cryoprotectant. This enhances uniform heat exchange during cooling while reducing the thermo-insulating effect of the conventional straw. It has been stated that the thermo-insulating effect occurs due to the nitrogen vapour bubbles forming around the straws because of greater thermal mass of the conventional straws.[25] Considerable thinning of the straw wall and reduction of inner diameter of the straw minimizes the volume of cryoprotectant resulting in capillary lift of the embryo.[26] During warming, the micro-drop is instantly warmed and the blastocyst is immediately expelled in the dilution solution reducing the cytotoxic and osmotic effects. [9]

It has been studied in an in vitro physiological model of human embryo implantation on endometrial stroma that a hatched blastocyst after attaching the stroma appears to undergo contraction. Further, it becomes less expanded before entering the invasive stage.[27] This was the reason why the warmed hatching blastocyst was transferred before becoming fully re-expanded i.e., 1 h 30 mins after the warming process to improve the chances of implantation.

Many investigators have reported that pregnancy rate of vitrified slow growing day 6 and day 7 blastocyst transfers were significantly higher than that of fresh day 6 and day 7 slow growing blastocyst transfers.[16,28,29] These may be due to asynchrony with the endometrial receptivity associated with the fresh transfers of slower growing embryos. Thus embryo cryopreservation which enables synchronization between embryonic and endometrial development is an effective alternative for slower growing embryos.[16] The present case also demonstrates optimum synchronization of endometrial receptivity and transfer of warmed hatching blastocyst in a natural cycle resulting in full term pregnancy.

CONCLUSION

We report a case of successful delivery following transfer of a vitrified-warmed slow growing day 7 hatching blastocyst using hemistraw-vitriplug as an embryo carrier system. It can be concluded that even if on day 5 the developmental stage of the embryo is not up to the mark, strategy to continue culturing the embryo in vitro till day 7 can be considered to evaluate and prevent wastage of slow growing higher quality supernumerary embryos. Ultra-rapid vitrification using HS can be considered an optimal method for cryopreservation of such slow growing day 7 hatching blastocysts. Thus, this case report

focuses the need of more data and research on such type of slow growing later-stage embryo cryopreservation on IVF outcomes.

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