

CASE REPORT

Ongoing pregnancy after re-vitrification of cleavage stage embryos

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ABSTRACT

Cryopreservation of gametes and embryos leads to increased cumulative pregnancy rates along with restored costs of artificial reproduction techniques (ART). Vitrification, a novel method, is now being widely used in ART laboratories due to high survival rates along with low cost and simplicity. However the safety and outcome of repeated vitrification procedure has not been clearly determined in human embryos. In this report an ongoing pregnancy after re-vitrification and transfer of cleavage stage embryos was described.

Key Words: Cleavage; repeated; vitrification; pregnancy.

INTRODUCTION

Vitrification, a novel method of cryopreservation of human gametes and embryos, is mandatory and now being widely used in ART laboratories. It is a method in which not only cells but also the whole solution is solidified without the crystallization of ice (1). Today, vitrification is assumed to be the future of cryopreservation because there is not any circumstance in oocyte or embryo cryopreservation where slow freezing offers any considerable advantages compared with vitrification. As well, the majority of published data

claimed that the latest vitrification methods are seemed more efficient and reliable than any version of slow freezing (2). However data on repeated use of vitrification is not adequate yet. On contrary former methods of cryopreservation have been proved to be efficient in procedures of repeated cryopreservation on human embryos (3). So, still there are some questions those should be answered such as the safety of this novel procedure in repeated attempts due to clinical necessities.

CASE REPORT

An infertile couple, 28 years-old female with polycystic ovary syndrome (PCOS) and 31 years-old male partner with asthenoteratozoospermia, presented at outpatient ART clinic of university of Luebeck, department of gynaecology and obstetrics since June 2004. They were referred to in-vitro fertilization program after their failure to achieve pregnancy following 6 months of ovulation

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induction (OI) with dexamethasone along with natural coitus and 4 cycles of OI with clomiphene citrate along with intrauterine insemination. However the first attempt of ICSI had failed in which cryopreservation was not done. At her second ICSI cycle 7 MII oocyte were collected after a mild protocol comprising 150-225 recombinant FSH (Gonal-F, Serono, Germany) along with flexible GnRH antagonist protocol (Cetrotide 0.25 mg, Serono, Germany). Finally 2 eight cell cleavage stage embryos were transferred as well 2 pronuclear (PN) stage embryos were vitrified by using cryotops. However again the fresh embryo transfer (ET) did not result in pregnancy. Two months later, subsequent to her second ICSI cycle, she had undergone a cryopreserved ET cycle which was supported by oral estradiol valerate (E²) (Progynova, 1-6 mg/day/two weeks, Schering, Germany) plus vaginal progesterone (Crinone gel, 7.5 mg/ml, Serono, Germany). Consequently 2 PN stage vitrified embryos were thawed on 15th day of her cycle and cultured 24 hours till to cleavage stage of four cells. Nevertheless because of her psychiatric problems, that occurred quite acute on her last ICSI cycle, the couple then refused the procedure of ET. Therefore information was given to the couple concerning unproved and inadequate data on re-vitrification of cleavage stage human embryos and thawed embryos were then vitrified again to be used in further frozen cycles after taken of an informed consent from the couple. The patient then referred to the psychiatric clinic for treatment. Thereafter her psychiatric treatment the couple then accepted to undergo another frozen ET cycle with the re-vitrified cleavage stage embryos. The endometrium prepared with the induction of proliferative phase by incremental doses of oral E² (Progynova, 1-6 mg /day/two weeks, Schering, Germany) and vaginal progesterone (Crinone gel, 7.5 mg/ml, Serono, Germany) that was added on 12th day of the cycle. Thereafter 6 mg of E² and vaginal progesterone were maintained until pregnancy test. The re-vitrified cleavage stage embryos were re-thawed on 14th day of induction and then two eight cell embryos of cleavage stage both with grade 1 morphologies were transferred after culturing 24 hours in cleavage stage media (Ham's F-10 + 20% serum). The positive serum level of hCG was detected on day 14 after the ET and a 11 weeks of gestational embryo with positive heart beats were confirmed by transvaginal ultrasonography one month later. At the moment she has an ongoing pregnancy over 16 weeks of gestational age.

Vitrification / warming protocol

The vitrification/warming protocol was the same in both studies according to the one

mentioned by Kuwayama et al. (4). The PN (2PN) stage zygotes (German study, according to the rules of German legislation) were incubated in Equilibration Solution (ES) 7.5% Ethylene Glycol (EG) (Sigma-Aldrich, Steinheim, Germany) and 7.5% Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, Steinheim, Germany) in Ham's F-10 media supplemented with 20% patient serum for 5-15min (according to the time needed for re-expansion of the vitrified material) at room temperature. After an initial shrinkage and recovery they were aspirated and placed into the Vitrification Solution (VS) (15% EG, 15% DMSO and 0.5 M Sucrose) (Merck, Darmstadt, Germany) for less than 60 sec at room temperature. After having observed that cellular shrinkage has been taken place, oocytes and zygotes were aspirated and placed on the tip of the Cryotop (Kitazato, Japan). No more than two oocytes or zygotes were placed on each Cryotop. Cooling of the oocytes and zygotes was done by direct contact with the liquid nitrogen (LN₂). The Cryotops were stored in liquid nitrogen for at least two months.

Warming of PN zygotes was performed by placing the Cryotop in Thawing Solution (TS) (1M Sucrose) for less than 60 sec at 37°C and then into Dilution Solution (DS) (0.5 M Sucrose) for three min. followed by other DS of 0.25 M Sucrose for three min. The warmed oocytes and zygotes were placed 4-5 times into Washing Solution (WS) (Ham's F-10 + 20% serum) before incubation or culture. The intact zygotes were cultured in Ham's F-10 for 24 hours prior to embryo transfer. The embryo quality was scored according to Steer et al. (5).

DISCUSSION

Cooling and cryopreserving of oocytes, zygotes and embryos of humans at subzero temperatures is an unphysiological situation that shows significant necessity of external support in order to survive these cells for professional use. As well warming or thawing procedures, the reverse action of cooling, of the cryopreserved human cells are also such critical unphysiological events. Severe injuries may occur at all phases of these procedures. Therefore techniques those mentioned to be used in professional applications should

balance these unphysiological effects of the freezing and thawing (1). Understanding the causes and mechanisms of damage may help the development of new methods of cryopreservation to avoid lethal or irreversible injuries.

Alike of the fate of all former techniques, cryopreservation with slow freezing method has begun to be left due to some critical problems. For example requirement of long time for freezing, low viability of cells after thawing, high rates of intracellular crystal formation and also high costs of freezing with this technique were all urged innovations in cryopreservation area. Thereafter the year 2000, vitrification technique became to be more popular and claimed to a novel method that maintained comparable results to former slow freezing protocols (2). Furthermore by the year 2005, after three large comparative studies it had been concluded that vitrification is a more efficient way for cryopreservation of human embryos leading the highest survival rates rather than slow-rate freezing (4, 6, 7). Survival rates of 4-cell stage and blastocyst stage human embryos were significantly higher than those after slow freezing. As well vitrification with cryotop was found to be superior for cryopreservation of PN stage embryos in regard to the rates of survival, cleavage, and of further development (4). Besides the application of vitrification, a rapid technique of cooling, have also some advantages such as simplicity, inexpensiveness, and being a time consuming procedure along with higher survival and developmental rates when compared to former methods. As well even cryopreservation of more sensitive embryos such as expanded blastocysts could be resulted in successful pregnancy. Therefore vitrification is seemed to be more protective method rather than slow freezing method especially in preventing of intracellular damage (8).

On the other hand there is still not adequate data that has been published concerning the safety of multiple repeats of vitrification procedure on human oocyte and embryos. Conversely it has been reported that slow freezing has the advantage of being a repeatable method on human cells and embryos (3). There has been only two recent reports indicated a twice vitrification or re-vitrification of embryos those derived by in-vitro

matured oocytes (9, 10). One of them described a re-vitrification procedure of embryos at blastocyst stage whereas the other one described a twice vitrification of embryos at PN stage and at cleavage stage (Table 1). However both of them reported successful pregnancy with the day 5 transfer of the re-vitrified blastocyst stage embryos. In parallel, the current report also described a twice vitrification of embryos at PN and cleavage stage with a total culture period of 48 hours. Thereafter a successful pregnancy was also obtained by transfer of two cleavage stage re-vitrified embryos. As well another report has also been indicated a successful pregnancy after repeated cryopreservation by vitrification of cleavage stage embryos those were previously frozen by slow freezing method (8).

In conclusion these small data support the idea of the safety of the repeated vitrification and indicate that human embryos could be re-vitrified. As well the current data also suggested that re-vitrification of human embryos could be done regardless of stage of embryos and of previous method of cryopreservation. However advanced projects those evaluating the effects and safety of re-vitrification of human embryos in regard of evidence based medicine should be studied.

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