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ASSISTED REPRODUCTIVE TECHNOLOGIES

Successful pregnancy after recryopreservation by vitrification of one blastocyst developed from a frozen embryo obtained by PESA: case report

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Introduction

The cryopreservation of human embryos in different cleavage stages has been employed as part of assisted reproduction technologies (ART), as it allows the transfer of less embryos and increases the cumulative gestation rate of a couple. Human embryos can be cryopreserved by slow or ultra-rapid freezing and vitrification. For decades, human embryos have been successfully cryopreserved using the slow rate or equilibrium freezing protocols at the 4–8 cell stage and more recently at the blastocyst stage. The freezing process includes ice crystals formation and osmotic and chilling injury, factors that may lead to damaged blastomers and zona pelucida resulting in low embryo survival. On the other hand, vitrification eliminates ice crystals formation and most protocols are very simple to perform. However, some authors consider vitrification an experimental method in human reproduction, largely because of its inconsistent reported survival rates and the variety of solutions, vessels and carriers employed [1]. A meta-analysis and systematic review found that vitrification is superior to slow freezing, which in turn is superior to ultra-rapid freezing [2] and it may be employed as an effective cryopreservation alternative to slow freezing, provided that technical and methodological details of this technique are carefully observed.

As a rule, surplus human embryos are cryopreserved after one or more good quality, fresh embryos have been transferred and they remain cryopreserved untill the couple decides to have a new transfer using the frozen or vitrified embryos. Presently, many centers are thawing cleavage stage embryos which were cryopreserved by the original equilibrium protocol [3]. After the thawing of two or three embryos, they are usually allowed one or more days *in vitro* to cleave to show signs of viability before transfer. In case all thawed embryos cleave, the practice is to allow them to develop to the blastocyst stage to select the best one for transfer.

It has already been reported that human embryos twice frozen or vitrified can result in successful deliveries ([4, 5]). Moreover, one case report describes the birth of twins after the transfer of 2 vitrified-rewarmed blastocysts generated from frozen cleavage stage embryos [6]. Here we describe a case where cleavage stage frozen embryos obtained after epidydimal aspiration and ICSI were thawed for a frozen embryo cycle. After transfer of the best embryos, one was left in culture, reached the blastocyst stage and was then vitrified. The patient did not get pregnant with the frozen embryos and subsequently received the vitrified blastocyst, of which a healthy baby has been born.

Capsule Re-cryopreservation of human embryos by two different methods results in successful pregnancy and birth.

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Case report

A 29 years old woman and her partner 45 years old underwent their first ICSI attempt due to male factor at the Nilo Frantz Research and Human Reproduction Center, in January, 2006.

The male partner was azoospermic following vasectomy 17 years ago and underwent percutaneous epididymal sperm retrieval (PESA) without any major complications earlier on the day of oocyte pick-up. After checking for the presence of spermatozoa, the aspirate was placed in a centrifuge tube and spun in fresh medium (HTF-Hepes, Irvine). The supernatant was removed and the pellet re-suspended in 0.5 ml of medium supplemented with 6% serum (SSS, Irvine) and a small droplet was placed under the microscope. Only motile (live) sperm were used for insemination by ICSI.

The woman was stimulated with 225 IU of recombinant FSH (Puregon, Organon) and 75 IU of HMG (Menopur, Ferring) for 9 days starting on day-2 of the treatment cycle, using GnRH antagonist protocol (Cetrotide). Nineteen cumulus-oocyte complexes (COCs) were collected 36 h after injection of 10.000 IU of hCG (Choragon, Ferring), eighteen MII oocytes were injected, resulting in 14 two-pronuclei zygotes. After 3 days of culture in P1 medium (Irvine), two 8-cell embryos, grade G1 [7] were selected for transfer. Seven (three grade 1 and four grade 2 and 3) embryos were cryopreserved by slow freezing [3], loaded into two plastic straws, one with four and the other with three embryos. The luteal phase was supported with 800 mg of micronized progesterone daily. Fourteen days after transfer the patient had a positive β-hCG exam. The clinical pregnancy was defined as the presence of one visualized intrauterine gestational sac, about 5 weeks later and a healthy baby boy was born at 38.5 gestational weeks by normal delivery.

Four years later, the couple came back to the clinic for a frozen embryo transfer. Seven embryos were thawed. Two 8-cell and one 5-cell embryos were transferred in the same day and the remaining four were left in culture to the blastocyst stage in Multi-Blast medium.(Irvine). One embryo reached the expanded blastocyst stage at day-7 (grade 3AB; [8]; Fig. 1) and was then vitrified with Cryotech Vitrification Kit, according to the "Cryo top" technique. The patient did not get pregnant and came back

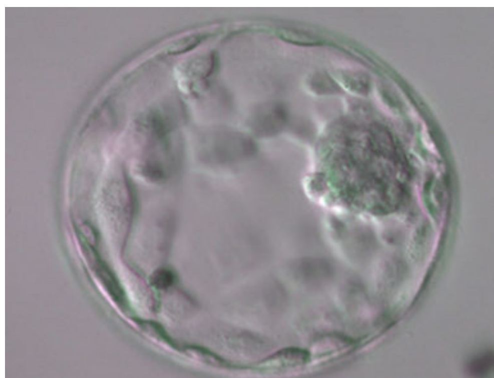

Fig. 1 Day-7 expanded blastocyst prior to vitrification

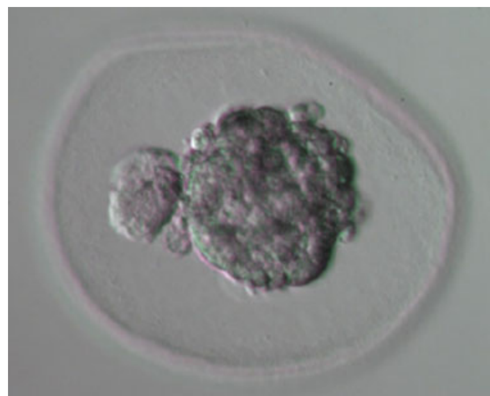

Fig. 2 Blastocyst at rewarming

to have the vitrified blastocyst transferred 4 months later. After rewarming and removal of cryoprotectants, the blastocyst was placed in Global medium (Life Global) and showed a contracted morphology for the first few hours (Fig. 2). Next day it was a nicely expanded blastocyst that started hatching just prior to transfer (Fig. 3). Twelve days after transfer the patient had a positive β-hCG exam. The clinical pregnancy was defined as the presence of one visualized intrauterine gestational sac, about 5 weeks later. The obstetrical course was uneventful and a healthy baby girl was delivered at 40 week of gestation. The weight at birth was 3.300 g, 50 cm and Apgar10.

Discussion

The present report shows that re-cryopreservation by vitrification of previously frozen embryos is feasible and may successfully establish a full term gestation. This achievement has several important consequences, first of all the avoidance of embryo wastage in cryopreserved embryo cycles. It is not uncommon that embryo survival

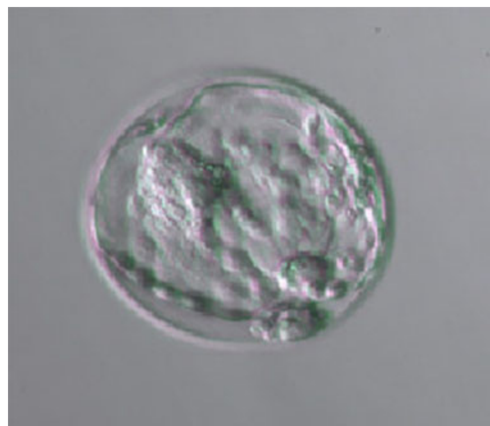

Fig. 3 Rewarmed blastocyst prior to transfer

within the same straw is not uniform. Two straws containing the 7 embryos were thawed in the same day for fear of not having enough good quality embryos for transfer After the transfer of three good-morphology embryos (two 8-cells and one 5-cells), one 7-cells embryo reached the expanded blastocyst stage in culture at day-7. The decision to vitrify this blastocyst was based on the experience of the embryologists indicating that vitrified—rewarmed blastocysts result a higher pregnancy rate than frozen-thawed ones. These observations agree with reports showing that vitrification yields higher pregnancy rates than slow freezing for the blastocyst stage [2]. Despite one previous report describing a successful pregnancy achieved after the transfer of a blastocyst developed from frozen-thawed embryos [4], in most centers it is not an established protocol to cryopreserve an embryo a second time, because it is still unclear whether this procedure would result in viable embryos capable of establishing a pregnancy. This way, many embryos are wasted after the transfer of the best ones in a cryopreseved cycle, which could either be used in further cycles or donated to research. Our case corroborates previous finding [5] and emphasizes the pregnancy potential of vitrified blastocysts generated after *in vitro* culture of frozen/thawed cleavage stage embryos.

Another point that deserves attention in this report is the fact that the embryos were obtained after PESA, a means of obtaining adequate sperm for ICSI in patients with obstructive or non-obstructive azoospermia. PESA is a safe and effective technique of sperm retrieval and reports show that successful outcomes in terms of fertilization, embryo development and pregnancy rates can be obtained particularly in cases of non-obstructive azoospermia [9, 10] as the one here described. Our case of successful combination of PESA, slow-freezing and vitrification techniques expands the possibilities of positive outcomes within a single treatment cycle, diminishing costs and unwanted repeated ovarian stimulations and oocyte-pick-ups. Also, the possibility of performing a second cryopreservation avoids embryo wastage and increases the possibility of embryo donation to research in embryo stem cell and regenerative medicine programs.

Our report is unique in describing the successful re-cryopreservation of a blastocyst generated by PESA. It also corroborates previous findings that frozen thawed embryos can be vitrified after culture to the blastocyst stage, without lacking its developmental potential to full term gestation.

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