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ARTICLE

Vitrification of human embryos previously cryostored by either slow freezing or vitrification results in high pregnancy rates

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Abstract Occasionally, clinical scenarios arise where embryos, previously cryostored and warmed, need to be recryopreserved. The outcome of 30 such transfer cycles from 25 women where embryos were recryopreserved is detailed. In 16 cases, embryos were initially cryopreserved by slow freezing and in 14 cases by vitrification. The cryopreservation stages were the pronuclear stage (n = 16), day-3 cleavage stage (n = 12), blastocyst (n = 1) and oocytes (n = 1). All recryopreservation was by Cryotop-based vitrification. From this mixed source, 30/31 twice-cryopreserved embryos survived warming and were transferred, resulting in 13 pregnancies, 11 deliveries with normal gestational age and birthweight, one pre-term birth at 33 weeks and two miscarriages. There were no malformations reported for the live births. Recryopreservation using vitrification by CryoTop has been used in a variety of clinical scenarios to preserve surplus cryopreserved embryos. The current study, although limited in numbers, resulted in high survival rates, clinical pregnancy rates similar to once-cryopreserved embryos and healthy live births independently of the initial stage and cryopreservation method. The technique may increasingly be applicable to elective single-embryo transfer and blastocyst transfer to maximize the pregnancy rate while minimizing the number of cryopreserved embryo transfers.

KEYWORDS: blastocysts, cryopreservation, oocytes, revitrification, slow freezing, vitrification

Introduction

Recryopreservation is a powerful tool for the embryologist. The ability to vitrify and warm embryos multiple times allows patients to micromanage their cryostored embryos. Recent trends in IVF towards single-embryo transfer and blastocyst transfer create scenarios where recryopreservation may be a useful tool for clinicians and scientists in recommending various treatment options to couples. As an increasing number of cycles have only a single embryo transferred, more embryos are becoming available for cryopreservation. If the embryos are cryopreserved at the blastocyst

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stage, then rarely will recryopreservation be appropriate. However, many clinics do not culture all embryos to the blastocyst stage because of the uncertainty in the number of day-5 embryos that will be available or because of reduced embryo quality. Where numerous embryos are cryopreserved at the cleavage stage, couples may be faced with decisions in how to use the cryopreserved embryos, either by repeated cleavage-stage transfers or by bulk warming and extended culture to identify the best embryo for transfer. If after culture, surplus good-quality embryos remain, these may be recryopreserved. The ability to successfully recryopreserve surplus good-quality embryos allows the patient to explore such options to maximize their chances of pregnancy with minimal treatment. Knowing that healthy embryos are not wasted is a key parameter in their decision-making process and a valuable option for clinicians when discussing treatment options. Other scenarios reported here include cases where embryo transfer needs to be rescheduled after embryo warming and when oocytes rather than embryos have previously been cryopreserved. The later situation may become more common as oocyte cryopreservation activity increases.

When occasional embryos have been refrozen and thawed (Baker et al., 1996) using conventional protocols (Testart et al., 1986), there has been cryoinjury sufficient to largely preclude its widespread employment. Vitrification, at least in the animal models, allows for multiple freezing with minimal damage (Sheehan et al., 2006) and there may be an opportunity for it to be applied in a clinical environment. There have been several recent reports detailing successful revitrification, including embryos (Kumasako et al., 2009), oocytes (Chang, 2008) and after biopsy (Peng et al., 2011); however, these are isolated cases. This article reports on the outcome of 30 cases where embryos have been revitrified after an initial freezing/vitrification event.

Materials and methods

A total of 25 couples had 30 treatment cycles involving the transfer of embryos that had initially been cryopreserved by either slow freezing or vitrification and subsequently revitrified. The details of patient demographics, embryology and outcomes of the cycle in which embryos or oocytes were initially cryopreserved are presented in **Table 1**. Nine couples were aged <35 years, nine were aged 35–39 and seven were aged ≥40 years. Three cases involved donated embryos and one case involved donated oocytes that had been cryopreserved as oocytes before warming, followed by insemination and revitrification of surplus embryos. The patient demographics and the basic embryology in the original IVF cycle in the couples with revitrification were similar to all other cases involving the transfer of vitrified embryos in 2009–2010 (**Table 2**), although the number of oocytes and embryos were higher in the study group.

There were 10 cases where all embryos had been cryopreserved due to risk of ovarian hyperstimulation and two cases where the original IVF embryo transfer produced a live birth and the couples, returning for a second pregnancy, elected to warm and culture all the cryopreserved embryos to identify the most viable embryo for transfer. These bulk warmings resulted in surplus embryos that were recryopreserved. Two cases involved recryopreservation of the embryos on the same day as the warming: the first where the embryo transfer was cancelled after the slow-frozen embryo had been thawed and the embryos were vitrified 2 h later; the second where the client had wished all her vitrified blastocysts to be warmed, one to be transferred and the balance discarded with the intention of ceasing all further treatment – one surplus embryo was of sufficient quality to be cryopreserved and the client changed her management and requested it to be revitrified. While most of the initial warming cycles involved relatively large numbers of embryos with a view towards embryo selection (i.e. including extended culture to identify the best embryo), all recryopreserved–warmed embryos were transferred as single embryos.

Some initial cryopreservation was by slow freezing but all recryopreservation was by vitrification on either day 3 or day 5/6. Sixteen cases had embryos initially cryopreserved using conventional propanediol/sucrose protocols (Testart et al., 1986) and 14 cases were initially vitrified by the CryoTop method as described by Kuwayama et al. (2005a,b). In 16 cases, embryos were initially cryopreserved at the pronuclear stage, in 12 cases at the day-3 stage, in one case as blastocysts and in one case as oocytes (**Table 1**). The same vitrification procedure was employed for cleavage- and blastocyst-stage embryos and the initial and second cryopreservation events. Media was prepared fortnightly and tested for quality using immature oocytes which were not subsequently used after IVF. Each media batch required a survival rate >90% for approval for clinical use.

The vitrification protocol used preparations based on M199 media (Sigma, Australia) and supplemented prior to use with either 10% heat-inactivated patient serum or 10 mg/ml human serum albumin (HSA; Sage Biopharma, Gytech, Australia) if serum was unavailable. Embryos were held at 37°C in cleavage media until vitrification. Each embryo was individually vitrified by transferring to initial cryosolution containing 7.5% dimethylsulphoxide (DMSO; Sigma), 7.5% ethylene glycol (Sigma) in M199 plus serum/HSA for 15 min at room temperature before transfer to the final cryosolution (15% DMSO, 15% ethylene glycol in M199 plus serum/HSA) also at room temperature. The embryos were held in the final solution for 50 s before being rapidly loaded onto the CryoTop tool and excess cryoprotectant was removed to leave a fine meniscus of fluid over the embryo before plunging into virgin liquid nitrogen. The tool was then encased in the protective sheath before cryostorage. The warming process involved very rapid transfer to the first solution (1 mol/l sucrose (Sigma) for 50 s at 37°C, followed by 3 min in the dilution media (0.5 mol/l sucrose) at room temperature then 5 min in washing solution (no sucrose) also at room temperature. The embryos were then warmed in a HD Scientific Work Station before washing in cleavage (PN stage) or blastocyst media (day-3 or day-5 stage; Sage). Embryo culture was in 10 μl cleavage/blastocyst media under mineral oil at 37°C in MINC incubators (Cook, Australia) with 6% CO_2, 5% O_2 and nitrogen balance. Prior to transfer, a day-3 embryo or blastocyst was moved to transfer solution, containing blastocyst culture media enhanced with 10% HSA (Sage) before embryo transfer in 10–20 μl fluid. When the transfer was on the same day as the warming,