Article

Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination

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Abstract

Survival and development of human embryos was compared following slow cooling versus vitrification involving more than 13,000 vitrified embryos. In addition, the efficacy of an open system, the Cryotop, and a closed vitrification system, the CryoTip™, were compared using human blastocysts. One hundred percent of vitrified human pronuclear stage embryos survived and 52% developed to blastocysts as compared with 89% survival and 41% blastocyst development after slow cooling. Similar survival rates were seen with vitrification of 4-cell embryos (98%) as compared with slow cooling (91%). Furthermore, 90% of vitrified blastocysts survived and resulted in a 53% pregnancy rate following transfer, as compared with 84% survival and 51% pregnancy rates following slow cooling. All corresponding values were significantly different. When the closed and open vitrification systems were compared, no difference was found with regard to supporting blastocyst survival (93 and 97% for CryoTip and Cryotop respectively), pregnancies (51 versus 59% respectively) and deliveries (48 versus 51% respectively). Vitrification is a simple, efficient and cost-effective way to improve cumulative pregnancy rates per cycle. The use of the closed CryoTip system eliminates the potential for embryo contamination during cryopreservation and storage without compromising survival and developmental rates *in vitro* and *in vivo*.

Keywords: *blastocyst, cryopreservation, pregnancy, vitrification, zygote*

Introduction

Cryopreservation of supernumerary embryos produced during human IVF provides an opportunity for patients to have repeated attempts at conception following a single drug stimulation cycle, preventing wastage of valuable genetic material and improving cumulative pregnancy rates. This approach may have several advantages for the patient (Veeck, 2003; Anderson, 2005). Firstly, it provides an opportunity to limit the numbers of embryos transferred while maximizing the usable embryo per oocyte retrieval cycle ratio at each stimulation attempt, a procedure that is costly and potentially difficult for patients. Secondly, the number of drug stimulation cycles in order to obtain oocytes can be decreased; consequently, the potential risk to the patient from exposure to anaesthesia and the possible development of hyperstimulation syndrome can be reduced. In addition, storage of embryos from a cycle allows the patient to space the timing of sibling pregnancies, and improve their potential to achieve a pregnancy at an advanced maternal age, since the eggs were retrieved when the patient was younger.

Successful cryopreservation of human embryos was first reported in 1983 by Trounson and Mohr with multicellular embryos that had been slow-cooled using dimethyl sulphoxide (DMSO). Subsequent modifications of the technique, introducing 1,2-propanediol and sucrose as cryoprotectants (Lassale *et al.*,

1985) and slow cooling to –30°C prior to plunging into liquid nitrogen, resulted in the introduction of cryopreservation as a standard method offered by virtually every full-service IVF programme world-wide (Anderson *et al.*, 2003).

The primary disadvantages to slow cooling for human embryo cryopreservation are the requirement for an expensive programmable freezing machine and the time-consuming procedure. The introduction of a technique that could be performed without the use of costly equipment and could be completed by one cryopreservation specialist within minutes would provide significant benefits for any busy IVF programme.

Vitrification of embryos and oocytes may offer a solution for this problem. Vitrification can be defined as an extreme elevation in viscosity, i.e. solidification of solutions without ice crystal formation at low temperature (Liebermann *et al.*, 2002a; Fuller and Paynter, 2004; Kasai, 2004; Liebermann and Tucker, 2004). This phenomenon can be induced by either applying an extreme cooling rate or by using high concentrations of cryoprotectant solutions. Methods developed for vitrification in embryology use a combination of these two possibilities (Liebermann *et al.*, 2003). The advantages of vitrification in embryology may be considerable. Oocytes and embryos are sensitive to ice crystal formation; consequently, the elimination of this type of injury may increase their chances for survival. The required high cooling rate can be achieved by simple methods including, for example, direct plunging into liquid nitrogen, thus the need for expensive equipment is eliminated. Additionally, the time required for equilibration and cooling is considerably reduced. On the other hand, disadvantages of vitrification are the required high cryoprotectant concentration, and consequently the increased risk of toxic and osmotic damage, and the need to use special tools permitting high cooling rate by reducing radically the volume of solutions containing the embryos.

Successful vitrification of mammalian embryos was first reported by Rall and Fahy in 1985. Since then, a number of cryoprotectant solutions have been investigated for human use including the use of DMSO, glycerol, ethylene glycol, propanediol and sugars in various combinations (Chen *et al.*, 2000; Mandelbaum, 2000; Shaw *et al.*, 2000; Wright *et al.*, 2004). In addition, numerous carrier systems have been tried, including electron microscope grids, open pulled straws, denuding pipettes, open hemi-straws, and cryoloops (Martino *et al.*, 1996; Arav and Zeron, 1997; Vajta *et al.*, 1998a,b; Lane *et al.*, 1999; Kuleshova and Shaw, 2000; Park *et al.*, 2000; Vanderzwalmen *et al.*, 2000; Vandervorst *et al.*, 2001; Yeoman *et al.*, 2001; Liebermann and Tucker, 2002; Liebermann *et al.*, 2002a,b; Mukaida *et al.*, 2002, 2003; Selman and El-Danasouri, 2002; Vanderzwalment *et al.*, 2002, 2003; Isachenko *et al.*, 2003; Son *et al.*, 2003, 2005; Cremades *et al.*, 2004; Walker *et al.*, 2004). While all of these systems have their advantages and disadvantages, the primary concern for many authorities and scientists is the potential risk of contamination for patients. As the rapid cooling in all these systems requires direct contact of the embryo containing solution and liquid nitrogen, there is a potential risk of disease transmission through contaminated liquid nitrogen during cooling and storage (Bielanski *et al.*, 2000).

The purposes of this study were: (i) to compare the efficacy of vitrification versus traditional slow cooling for cryopreservation of human embryos, and (ii) to investigate the possibility of replacing an earlier open system (Cryotop) with a newly introduced closed method (Cryotip™) to eliminate the potential danger of contamination.

Materials and methods

Patient treatment and embryo culture

Experiments were conducted in patients following informed consent and IRB approval. Patients were stimulated during an IVF cycle by the use of clomiphene citrate (Clomid; Shionogi Co. Ltd, Osaka, Japan). Clomiphene administration (50 mg/day) was initiated on day 3 of the cycle and continued until the rise in LH, caused by the nasal administration of 300 μg of gonadotrophin-releasing hormone agonist (GnRHa) (Suprecur; Mochida Pharmaceutical Co. Ltd, Tokyo, Japan). Human menopausal gonadotrophin (HMG) (Humegon; Organon Co. Ltd, Netherlands) was initiated on day 8 (150 IU) and continued every other day until the administration of GnRHa.

Oocytes were recovered by using a transvaginal ultrasound-guided device (GM07M05V110; Mochida Pharmaceutical). After retrieval, oocytes were cultured in TCM199 medium buffered with 11 mmol/l Hepes, 9 mmol/l Na-HEPES and 5 mmol/l $NaHCO_3$ (referred further as TCM 199) supplemented with 10% Synthetic Serum Substitute (SSS; Irvine Scientific, Santa Ana, CA, USA) in 5% CO_2 in air at 37°C. After 2 h, insemination was performed by intracytoplasmic sperm injection (ICSI). The following day, fertilized oocytes with two pronuclei (2PN) and two polar bodies were transferred into 0.5 ml droplets of Quinn's Advantage Cleavage Medium (Sage BioPharma, USA) supplemented with 10% SSS and cultured further as described above. Embryos that developed beyond the 4-cell stage on day 5 were transferred into 100-μl droplets of blastocyst medium (Irvine Scientific) and cultured under identical conditions to day 6 after ICSI.

Vitrification of embryos

Equilibration, vitrification, thawing, dilution and washing solutions were equivalents to those in Vit Kit (Irvine Scientific). At the time of vitrification embryos were transferred into equilibration solution (ES) consisting of 7.5% (v/v) ethylene glycol and 7.5% (v/v) DMSO dissolved in TCM199 supplemented with 20% SSS at 27°C for 5–15 min. After an initial shrinkage, embryos regained their original volume, and were transferred into three 20 μl drops of vitrification solution (VS) consisting of 15% (v/v) EG and 15% (v/v) DMSO and 0.5 mol/l sucrose dissolved in TCM199 supplemented with 20% SSS, After incubation for 20 s in each drop respectively, embryos were loaded into CryoTips™ (Irvine Scientific) or on Cryotops and plunged into liquid nitrogen.

CryoTip consists of a plastic straw with a thin part (250-μm inner diameter, 20-μm wall thickness and 3-cm length) connected to a thick part (2000-μm inner diameter and 150-μm wall thickness, 4.5-cm length) and equipped with a movable protective metal sleeve (**Figure 1**). Embryos were loaded in approximately 1-μl solution into the narrow part of the CryoTips without any air bubbles by aspiration of medium, embryo and medium, and medium by a connected syringe. Subsequently, the straw was heat-sealed at both ends, the protective sleeve was pulled over the narrow part and the device was plunged into liquid nitrogen.

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