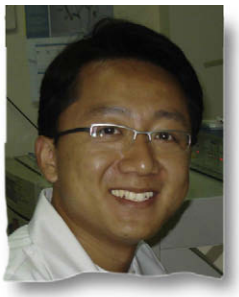
Article

Vitrification of biopsied embryos at cleavage, morula and blastocyst stage



Dr Xiao Zhang received his M.D. from Xin Xiang Medical University, China and began his career in reproductive medicine through the Ph.D. program of Peking University, China. Initially, he concentrated on oocyte slow freezing, and in 2003 he achieved the first pregnancy from frozen oocytes in China. He is currently an embryologist in the Pedieos IVF Center, specialising in vitrification and PGD. His research interests focus on the role of reactive oxygen species in female reproductive aging.

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Abstract

This study investigated the effect of vitrification on biopsied embryos at various developmental stages. After biopsy on day 3, embryos were vitrified at cleavage, morula and blastocyst stages using a commercially available kit. Non-biopsied embryos were vitrified as controls. For day-3 cleavage embryo vitrification, embryos from abnormally fertilized oocytes were randomly allocated to the biopsy and control groups. For morula and blastocyst vitrification, the embryos used in the biopsy groups were obtained from aneuploidy or affected embryos diagnosed by preimplantation genetic diagnosis (PGD). After warming, survival, blastulation and development of embryos in different groups were compared. The survival rate after warming in the non-biopsied cleavage control group was significantly higher than in the biopsied cleavage group (92.0% versus 64.0%, $P = 0.037$). Most of the biopsied embryos were destroyed due to blastomeres escaping. At the morula stage, both biopsied and non-biopsied embryos had similar survival rates. However, a significantly higher survival rate (95.6%) was observed in the biopsied blastocyst group compared with the control group (81.3%, $P = 0.035$). Biopsied embryos vitrified at an advanced stage had as high survival rates as non-biopsied embryos. Vitrification at the blastocyst stage is a practical and efficient solution for embryo cryopreservation during PGD.

Keywords: *embryo cryopreservation, IVF, preimplantation genetic diagnosis, vitrification*

Introduction

Since the first success after preimplantation genetic diagnosis (PGD) (Handyside *et al.*, 1990), PGD has been widely used in human IVF. In PGD programmes, it is important to obtain a large enough number of oocytes to provide sufficient embryos for biopsy and normal embryos available for transfer (Vandervorst *et al.*, 1998). Efficient cryopreservation of those highly precious embryos is therefore needed. Furthermore, the application of comparative genomic hybridization also requires the use of cryopreserved embryos for a subsequent menstrual cycle (Voullaire *et al.*, 2002; Wells *et al.*, 2002). However, due to the discontinuous zona and empty space consequent to the removal of blastomeres, the survival rate of biopsied human embryos was significantly lower than non-biopsied embryos when using conventional slow-freezing in which, the cooling rate was controlled at −0.2 or −0.3°C/min after seeding by a cryopreservation machine such as Planer or Minicool (Joris *et al.*, 1999; Magli *et al.*, 1999; Ciotti *et al.*, 2000).

In recent reports, vitrification, which is an alternative cryopreservation method, has been reported to be a simple, low-cost and efficient method for cryopreservation of mammalian and human oocytes and embryos at cleavage, morula and blastocyst stage (Kuwayama *et al.*, 2005a,b; Liebermann and Tucker, 2006; Al-Hasani *et al.*, 2007; Desai *et al.*, 2007; Kuwayama, 2007). Vitrification could be achieved by combining the high cryoprotectant concentration with high cooling and warming rates. In theory, crystal formation can be totally avoided (Rall and Fahy, 1985; Oktay *et al.*, 2006). Although there are fewer reports with promising results on biopsied embryo vitrification, a standard protocol for this purpose has not yet been

established (Wu *et al.*, 2005; Zheng *et al.*, 2005; Escriba *et al.*, 2006).

As routine, embryo biopsy is performed on day 3 after oocyte retrieval and biopsied embryos are cultured to days 5–6 for transfer. Therefore, cryopreservation of biopsied embryos could be performed on days 3, 4 or 5. This study compared vitrification results of biopsied embryos vitrified on day 3 (3 h post-biopsy cleavage stage), day 4 (morula stage) and day 5 (blastocyst stage), based on survival rates and continued development post-warming to evaluate the application of vitrification in PGD programme.

Materials and methods

Human embryos and embryo culture

The age of women whose embryos were used in this study ranged from 24 to 37 years. For day-3 cleavage embryo vitrification, embryos with good quality (more than six cells with less than 20% fragmentation and even-sized blastomeres) were collected from abnormally fertilized oocytes, 3 pronuclei (PN) or 1PN, and allocated randomly to biopsy (mean age ± SD = 28.6 ± 2.9 years) and control groups (mean age ± SD = 29.7 ± 3.2 years), and then vitrified at 3 h post-biopsy. For morula and blastocyst vitrification, embryos in the biopsy group were obtained from either the aneuploid embryos found in the preimplantation genetic screening (PGS) programme or the embryos diagnosed by PGD for thalassemia and/or sickle cell disease (mean age ± SD = 28.7 ± 3.5 years). The data for the non-biopsied morulae and blastocysts in the control groups were collected from normal vitrified embryo transfer cycles performed during the same period (mean age ± SD = 30.9 ± 3.3 years). In detail, on day 4, embryos that showed complete compaction with less than 20% fragmentation were involved this study. Approximately one-third of PGD cycles in the study clinic involve day-4 embryo transfer with cryopreservation of surplus embryos when appropriate. For the blastocyst groups, blastocysts with grouped inner cell mass and trophectoderm formed from many cells or equally shaped cells for the early blastocyst were selected. Couples gave their signed consent for the use of all the embryos used in the study prior to treatment.

The embryos were cultured in the culture medium drops covered with mineral oil (Ferticult, Beernem, Belgium) in Falcon tissue culture dishes (353001; Becton Dickinson, Franklin Lakes, USA). In detail, 4 h after follicle retrieval, the oocytes were inseminated by IVF or intracytoplasmic sperm injection (ICSI) and cultured in Universal IVF medium (0.1 ml) (Medicult, Denmark) at 37°C in an atmosphere of 5% CO_2. At 15–18 h after insemination, fertilization was checked and then continuously cultured in ISM 1 medium (0.1 ml) (Medicult). After biopsy, embryos were cultured in blastocyst culture medium (0.1 ml) (ISM II medium; Medicult) until day 6.

Embryo biopsy

Embryo biopsy was performed on day 3. Only embryos on the morning of day 3 with more than six cells and less than 20% fragmentation were selected for biopsy. Embryos were biopsied on day 3 in Ca^{2+}/Mg^{2+}-free medium (Embryo Biopsy Medium; Medicult). The embryos were positioned and held in such a way that a nucleated cell was placed adjacent to the intended biopsy site and a 30–35 µm hole was opened in the zona pellucida with a series of single pulses from a 1.76-µm diode laser with pulse duration of 0.500–1.500 ms at 100% power (Saturn Active laser system; Research Instruments, Cornwall, UK). Once the hole was created, a 30-µm (outer diameter) blastomere biopsy pipette (Research Instruments) was inserted into the hole and a blastomere with a visible nucleus was carefully extracted.

Embryo vitrification cooling and warming

Embryo vitrification took place on day 3 (3 h after biopsy), day 4 and day 5 according to the different groups. The solution medium for the cryoprotectants was phosphate-buffered saline (Sigma, St. Louis, USA) supplemented with 20% synthetic serum supplement (Irvine Scientific, USA). A two-step cryoprotectant loading process was used. Embryos were transferred into equilibration medium (0.3 ml), containing 7.5% (v/v) ethylene glycol (Sigma, Steinheim, Germany) and 7.5% (v/v) dimethyl sulphoxide (Sigma, Steinheim) in the solution medium for 5–10 min at room temperature. After an initial shrinkage, embryos regained their original volume and were transferred into vitrification medium (VM) (0.3 ml) consisting of 15% (v/v) ethylene glycol and 15% (v/v) dimethyl sulphoxide and 0.5 mol/l sucrose (Sigma, Steinheim, Germany) in the solution medium and after suspension for 20 s, embryos were loaded into a McGill Cryoleaf (Medicult) and plunged into liquid nitrogen for storage.

For the warming process, the Cryoleaf was directly inserted into warming medium (1.0 ml) (phosphate-buffered saline, 20% synthetic serum supplement) containing 1 mol/l sucrose at 37°C for 1 min. The warmed embryos were then transferred through sequential dilution media (0.3 ml/each) (warming medium containing 0.5 mol/l sucrose, 0.25 mol/l sucrose or no sucrose) for 3 min each at room temperature.

Assessment of warmed embryos

After warming, embryos were placed in ISM II (0.1 ml) for blastocyst culture under mineral oil at 37°C in a humidified atmosphere of 5% CO_2 in air. After 3 h incubation, the assessment of embryo viability was carried out. For the day-3 (cleavage) and day-4 (morula) groups, embryos with more than 50% intact blastomeres were considered viable and were cultured further to day 5 or day 6 to evaluate the blastulation capability and the advanced blastocyst rate. Blastocysts were determined to have survived the vitrification/warming process if they presented an inner cell mass, trophoectoderm and a re-expanding blastocoele cavity. These blastocysts were also checked for the progression after overnight culture. The blastocysts were classified into two different categories according to the degree of expansion: the early blastocyst with a blastocoele but not expanded and the advanced blastocyst: expanded blastocysts with a blastocoele volume larger than that of the early