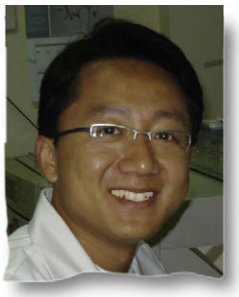
Article

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

embryo, with a thinning zona. Hatching or hatched embryos were also considered as advanced embryos.

Statistical analysis

For the embryo survival, blastulation and the development of the blastocyst rates were analysed using the Fisher's exact test. Statistical significance was defined as a P value <0.05.

Results

In total, 50 abnormally fertilized embryos from 39 patients were obtained and allocated randomly to the control and biopsy groups, and 24 discarded morulae from 12 PGD cycles and 25 morulae from 15 conventional ICSI cycles from women of similar age were selected as the biopsy and control morula groups, respectively. For blastocyst groups, 59 blastocysts from 32 conventional ICSI cycles and 47 blastocysts from 15 PGD cycles were selected. There was no significant difference in the age of the blastocysts between these groups. As shown in **Table 1**, the survival rate after warming in the non-biopsied control group (92.0%) was significantly higher than in the biopsied group (64.0%, $P = 0.037$). A total of 164 out of 186 (88.2%) blastomeres survived in the control group, which was significantly higher than for the biopsy group (143/190, 75.3%, $P < 0.01$). Six out of nine destroyed embryos were due to blastomeres escaping (**Figure 1a**) during the cooling and warming procedures and did not progress after further culture.

For the embryos vitrified at the morula stage, both the biopsied and non-biopsied embryos had similar survival rates (87.5% versus 92.0%). On the contrary, biopsy significantly improved the post-warming results of vitrification at the blastocyst stage (95.7%), compared with the counterpart control group (81.4%, $P = 0.035$). There was no statistical significance difference in the survival rate between the morula stage groups and biopsied blastocyst groups. Because embryos of the cleavage groups were derived from abnormally fertilized oocytes, data for the cleavage groups were not compared with the other groups.

After warming, the records of embryo progress are summarized in **Table 2**. There was no significant difference between control and biopsied cleavage groups with respect to blastulation rate. In the morula groups, no significant difference was found in the blastulation and advanced blastocyst rate. After overnight culture, the progression of survived blastocysts was observed. From the biopsied blastocyst group, 28 embryos (62.2%, $n = 45$) showed progression of hatching, while from the control blastocyst group, 22 embryos (45.8%, $n = 48$) showed further expansion or hatching.

Discussion

Since the first human pregnancy from the transfer of frozen embryos in, 1983 (Trounson and Mohr, 1983), cryopreservation of embryos has been a critical technology to increase IVF success rates. Although slow freezing has been the primary method of cryopreservation following PGD, biopsy increased the chance of cell lysis or blastomeres escaping during the slow-freezing procedure (Joris *et al.*, 1999; Magli *et al.*, 1999; Ciotti *et al.*, 2000; Zheng *et al.*, 2005). Recently, with high survival rates and low rates of cooling injury, vitrification has greatly improved the survival rate of biopsied embryos either at the cleavage stage or at the blastocyst stage, compared with slow freezing (Zheng *et al.*, 2005; Escriba *et al.*, 2006). This study employed the vitrification protocol based on ethylene glycol and dimethyl sulphoxide as the cryoprotectants, which has been proven to be successful in oocyte, cleavage and blastocyst vitrification (Mukaida *et al.*, 2003; Kuwayama *et al.*, 2005a,b; Liebermann and Tucker, 2006; Desai *et al.*, 2007).

Although human embryos obtained after abnormal fertilization may not reflect the same cryobiological properties and developmental potential as embryos obtained after normal fertilization, embryos derived from 1PN and 3PN oocytes may be used as a model to evaluate potentially negative effects of the cryopreservation procedure on the developmental capacity of these manipulated embryos (Liebermann *et al.*, 2002; Molina *et al.*, 2006). The data from the control group of the cleavage stage also confirmed the value of this model with 92.0% survival rate, which is similar to previous studies reporting 85% and 95.3% survival rates with normal embryos (Rama Raju *et al.*, 2005; Desai *et al.*, 2007).

In contrast to the cleavage control in the data, the biopsied cleavage embryos had one-third lower survival rate. Most

Table 1. Survival of embryos vitrified at different developmental stages.

Group	*No. of embryos vitrified*	*No. of surviving embryos (%)*
Cleavage control	25	23 (92.0)[a]
Cleavage biopsy	25	16 (64.0)[a]
Morula control	25	23 (92.0)
Morula biopsy	24	21 (87.5)
Blastocyst control	59	48 (81.4)[b]
Blastocyst biopsy	47	45 (95.7)[b]

Values with the same superscript letter are significantly different.
[a] $P = 0.037$.
[b] $P = 0.035$.

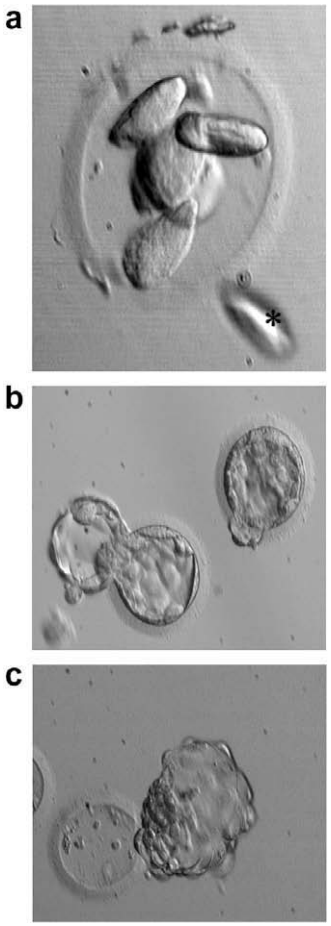

Figure 1. (**a**) The blastomeres shrunk greatly after incubation in vitrification medium and escaped from the zona of the biopsied embryo (asterisk). (**b**) After warming, hatching embryos were observed on the morning of day 6. (**c**) Blastocyst totally hatched despite a thick zona.

of the destroyed embryos were due to blastomeres escaping from their zonae, which was also a major culprit in the slow-freezing procedure reported by other scholars (Joris *et al.*, 1999; Magli *et al.*, 1999). In slow freezing, the damaged zona is likely to trigger ice crystal formation in the vicinity of blastomeres. But unlike slow freezing, vitrification can completely avoid ice crystal formation and cell lysis was seldom observed in the vitrification procedure. The high osmotic potential of the medium caused the blastomeres to shrink dramatically. As a result, blastomere escape usually occurred when the embryos were suspended in the vitrification medium, during loading or in the first-step warming medium, which had a higher sucrose concentration (**Figure 1a**). Also the loose cell connection of blastomeres after biopsy contributes greatly to the unsuccessful vitrification of biopsied cleavage embryos. However, this result seems to be inconsistent with another study reporting a 94% survival rate without blastomeres escaping (Zheng *et al.*, 2005). One potential explanation for this discrepancy is that the biopsy procedure that involved a slit opening on the zona, which was produced by partial zona dissection (PZD) and could block blastomeres escaping (Cieslak *et al.*, 1999). Similar to previous reports, there was no difference in the blastulation rates from the survived embryos. Together, these demonstrate that ultra-rapid vitrification imposes less stress on the embryo (Lane *et al.*, 1999; Sheehan *et al.*, 2006) and that it is an efficient way to cryopreserve cleavage embryos but, due to a round hole in the zona, may not be applicable for biopsied embryos at the cleavage stage.

It has been described that the presence and extension of the blastocoele severely affects survival of embryos after cryopreservation. In this study, there was a non-significant trend towards a lower survival rate in the non-biopsied blastocysts compared with the compacted embryos (81.4% versus 92.0%). A possible explanation is that because blastocysts have more blastocoelic fluid than other stages, they may be more susceptible to ice crystal formation during cooling (Cremades *et al.*, 2004; Hiraoka *et al.*, 2004; Mukaida *et al.*, 2006), but more studies are needed in order to confirm this. Unlike biopsied cleavage embryos, no blastomeres escaped from the zona due to the tight cell connections at the morula and blastocyst stages (Sathananthan *et al.*, 1999). Considering there was no difference in blastulation and advanced blastulation rates between the compaction groups, compaction is a suitable stage for biopsied embryo vitrification.

On the contrary, the biopsied blastocysts showed an unexpectedly higher survival rate than their controls. Compared with other reports, this study confirmed that an opening in the zona has no negative impact on blastocyst vitrification (Hiraoka *et al.*, 2007). Another potential indication is that

Table 2. Blastulation and development to advanced blastocysts of surviving embryos.

Group	*No. of blastocysts (%)*	*No. of hatching or expanded blastocysts (%)*
Cleavage control	4 (17.4)	0 (0.0)
Cleavage biopsy	3 (18.8)	2 (66.7)
Morula control	12 (52.2)	8 (66.7)
Morula biopsy	10 (47.6)	6 (60.0)
Blastocyst control	48 (NA)	22 (45.8)
Blastocyst biopsy	45 (NA)	28 (62.2)

NA, not applicable, progressed blastocysts after overnight culture. There were no statistically significant differences between biopsied and control embryos for any of the groups.

biopsy allows better exposure of the expanded blastocoele to the cryoprotectant and results in better dehydration of the blastocoele (Cervera and Garcia-Ximenez, 2003). Zech *et al.* (2005) reported that blastocysts with a larger blastocoelic cavity survived vitrification better when they had partially or completely hatched. Also this hypothesis was supported by a recent report that vitrification of blastocysts results in lower DNA damage to the blastomeres following zonal hatching before vitrification (Kader *et al.*, 2007). Furthermore, the progress capability was proven by the overnight embryo culture after warming, after which 28 (62.2%) blastocysts from the biopsy group progressed. Therefore, vitrification of biopsied embryos at the blastocyst stage has potential benefits both on survival rate and clinical outcome.

Based on these data, vitrification at advanced embryo stages is an efficient method for biopsied embryo cryopreservation. Practically, vitrification at the blastocyst stage is optimal. First, this strategy provides an opportunity to select viable embryos for transfer. Second, a blastocyst has more cells than at other stages and it has a greater capability of withstanding cell loss in the vitrification procedure. In addition, each Cryoleaf can only contain one embryo, to keep embryo records in a PGD programme, and occupies more space than usual cryopreservation items, such as straws (Huang *et al.*, 2007), so they are available in fewer numbers and it is much more efficient to vitrify biopsied embryos at the blastocyst stage.

There is one issue with vitrification that needs further discussion. The vitrification system is based on minimized loading solution and ultra-cooling procedure. With the Cryoleaf (Huang *et al.*, 2007), the embryos were in contact with liquid nitrogen directly. During the procedure, there have been concerns about the possible risk of cross-contamination with liquid nitrogen contact (Bielanski *et al.*, 2000; Balaban *et al.*, 2007), especially for biopsied embryos, which have inconsistent zonae. One possible solution is filtering the liquid nitrogen with a 0.2-μm filter, which can eliminate bacteria and fungi and store it in a vapour phase of liquid nitrogen tank (Cobo *et al.*, 2007) to minimize the risk of contamination.

In conclusion, vitrification at morula and blastocyst stages with high survival rate could be a primary solution for biopsied embryo cryopreservation. Considering the workload and space needed for Cryoleaf, it is more practical to vitrify biopsied embryos at the blastocyst stage in PGD programmes. This preliminary finding is worthy of further investigation in clinical application.

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