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# Human oocyte vitrification: in-vivo and in-vitro maturation outcomes

Ching-Chien Chang obtained his BSc (1994) and MSc degrees (1996) at the National Chung-Hsing University in Taiwan. He worked as an embryologist at Lee Women's Hospital in Taiwan from 1998–1999. He joined the porcine cloning project for xenotransplantation at the Animal Technology Institute, Taiwan from 1999–2001. In 2001, he joined the Centre for Regenerative Biology at the University of Connecticut to start his PhD, and his PhD degree was conferred in 2005. At present, he is an embryologist/research fellow at Reproductive Biology Associates, Atlanta, USA. His research interests are oocyte and epigenetic reprogramming.

*Dr Ching-Chien Chang*

Ching-Chien Chang, Daniel B Shapiro, Diana Patricia Bernal, Graham Wright, Hilton I Kort, Zsolt Peter Nagy[1]
Reproductive Biology Associates, Atlanta, Georgia 30342, USA
[1]Correspondence: Tel: +404 2571 840; Fax: +404 2573 314; Fax: +404 256 1912; e-mail: peter.nagy@rba-online.com

## Abstract

This study aimed to evaluate oocyte vitrification efficiency using in-vivo matured (IVO) versus rescued in-vitro matured (IVM) oocytes. The results show that oocyte survival (85% versus 81%), fertilization (86% versus 76%) and cleavage rate (98% versus 89%) was not significantly different in IVO oocytes compared with rescued IVM sibling oocytes. The fertilized oocytes from IVO and IVM groups were cultured to blastocyst stage; however, embryo development was significantly reduced in the rescued IVM group (72% versus 15%). Embryo transfer was only performed with the embryos derived from IVO oocytes on day 5; 42 blastocysts were transferred to 18 recipients; 16 of 18 recipients had positive β-human chorionic gonadotrophin and a total of 26 fetal cardiac activities were detected in 15 recipients (implantation: 26/42, 61.9%). Ten of the 15 recipients have delivered 19 healthy babies, and the other five pregnancies are still ongoing. These data indicate that the combination of oocyte vitrification and rescued IVM not only yield a new strategy to extend the pool of total fertilizable oocytes, but also demonstrate that the efficiency of vitrified/warmed oocytes can be comparable to fresh oocytes with regard to clinical outcomes.

***Keywords:*** *cryopreservation, in-vitro maturation, oocyte, vitrification*

## Introduction

Under ovarian stimulation, a proportion (15–20%) of human oocytes remain meiotically immature, confirmed after removing the surrounding granulosa cells (Cha and Chian, 1998; Smitz and Cortvrindt, 2004). Since, in many cases, there is the possibility of abnormal embryonic development (Racowsky and Kaufman, 1992), defective cytoplasmic maturation (Smith, 2001), and the asynchronous stage of embryo development, those immature oocytes are excluded from clinical use. However, the number of mature oocytes is often restricted in patients who are low responders and who have an unsynchronized cohort of follicles; hence, those immature oocytes are clinically necessary to extend the pool of total fertilizable oocytes. In order to evaluate the maturity of oocytes accurately and conduct intracytoplasmic sperm injection (ICSI), the oocytes are often denuded immediately after oocyte collection. Hence, the in-vitro maturation (IVM) of immature oocytes without surrounding granulosa cells (so-called rescued IVM oocytes) has recently had a significant impact on clinical applications (De Vos *et al.*, 1999; Strassburger *et al.*, 2004; Vanhoutte *et al.*, 2005; Otsuki *et al.*, 2006). The oocyte is dependent on the surrounding cumulus and granulosa cells through gap junctions to provide nutrients and regulatory signals, which are necessary to promote oocyte nuclear and cytoplasmic maturation and hence the acquisition of developmental competence (Anderson and Albertini, 1976; Buccione *et al.*, 1990; Chian and Sirard, 1995; Ka *et al.*, 1997).

The probability of embryo implantation and clinical pregnancy is clearly an important indicator of clinical efficiency for any assisted reproduction treatment program; however, these indicators may not provide enough information to evaluate the efficiency of oocyte cryopreservation. Therefore, it is necessary to include the starting number of thawed/warmed oocytes as a denominator (Edgar and Gook, 2007). To assess the clinical efficiency of oocyte cryopreservation, the minimum

number of starting oocytes to achieve successful implantation should also be analysed. Recently, clinical outcomes for oocyte cryopreservation have improved dramatically because the clinical efficiency of oocyte cryopreservation (about 13 oocytes/implantation) is comparable with that for fresh oocytes (about 10 oocytes/implantation) (Antinori *et al.*, 2007). Oocyte cryopreservation using a vitrification protocol has less detrimental effects on oocyte physiology (Gardner *et al.*, 2007; Larman *et al.*, 2007) than slow freezing. Since human oocyte cryopreservation has recently become increasingly efficient in the field of human ART (Kuwayama *et al.*, 2005; Lucena *et al.*, 2006; Oktay *et al.*, 2006; Antinori *et al.*, 2007; Barrit *et al.*, 2007; Bianchi *et al.*, 2007; Borini *et al.*, 2007; Cobo *et al.*, 2007), oocyte cryopreservation may provide the potential solution to conquer many application hurdles, including restricted quantity and asynchronous schedule for rescued IVM oocytes. However, there are few data regarding the survival and developmental potential of rescued IVM oocytes after thawing/warming. Therefore, the first objective of this study was to evaluate oocyte cryopreservation efficiency comparing IVO versus rescued IVM oocytes using the vitrification method. The second objective was to assess how many oocytes are required to achieve successful implantation in the current oocyte cryopreservation method.

## Materials and methods

The oocytes were obtained from young and healthy women (mean age: 28.0 ± 2.2 years) who donated anonymously in the study. They underwent ovarian stimulation using a daily dose of 200 IU of recombinant FSH (Follistim; Organon, Roseland, NJ, USA). Gonadotrophin-releasing hormone (GnRH) antagonist (ganirelix acetate; Organon) was administered, starting on day 6, for LH surge prevention. Recombinant human chorionic gonadotrophin (HCG; Ovidrel; Serono, Rockland, MA, USA) was administered to trigger nuclear maturation of oocytes when two or more follicles reached 18 mm. The oocytes were denuded by 30 s exposure of cumulase (Halozyme therapeutics, San Diego, CA, USA) followed by mechanical denudation 1–2 h after oocyte retrieval. The maturity of denuded oocytes was judged by the presence of the first polar body (PB), which is defined as the metaphase II (MII) stage. The in-vivo MII stage oocytes were cryopreserved by vitrification about 3–4 h after oocyte retrieval.

### Rescued IVM

The immature oocytes, either at the germinal vesicle (GV) stage or at the MI stage, were incubated in Quinn's advantage fertilization medium (SFM, Cooper/Sage, Bedminster, NJ, USA) with 10% serum protein substitute (SPS) for 24–28 h. After 24–28 h of rescued in-vitro maturation, the oocytes with a polar body were regarded as mature and were subject to vitrification (**Figure 1**).

### Oocyte vitrification

The basal medium used for oocyte cryopreservation was HEPES-buffered embryo culture medium (Cooper/Sage) supplemented with 20% (v/v) SPS (Cooper/Sage). The denuded oocytes were vitrified by the minimum volume cooling (MVC) method described by Kuwayama *et al.* (2005). Briefly, the oocytes were equilibrated in equilibration medium [basal medium with 7.5% (v/v) ethylene glycol and 7.5% (v/v) dimethyl sulphoxide (DMSO)] at room temperature for 15 min. Oocytes were transferred to the vitrification medium [basal medium with 15% (v/v) ethylene glycol, 15% (v/v) DMSO, and 0.5 mol/l sucrose] at room temperature for 45–60 s. The cryoprotectant-treated oocytes were placed onto a fine polypropylene strip (Cryotop®, Kitazato Bio Pharma Co., Japan). Then the polypropylene strip carrying the oocytes was submerged into liquid nitrogen and ready for storage.

### Oocyte warming

The polypropylene strip with vitrified oocytes was immersed directly into 5.0 ml of warming solution [HEPES-buffered embryo culture medium with 20% (v/v) SPS and 1.0 mol/l sucrose] at 37°C for 1 min. Oocytes were then transferred to 1.0 ml of the dilution solution [HEPES-buffered embryo culture medium with 20% (v/v) SPS and 0.5 mol/l sucrose] for 3 min at room temperature. The oocytes were subsequently washed in 1.0 ml washing solution [HEPES-buffered embryo culture medium with 20% (v/v) SPS] for 10 min at room temperature. Oocytes were incubated in Quinn's advantage fertilization medium (SFM, Cooper/Sage) with 10% SPS for 3 h before ICSI.

### ICSI fertilization, embryo culture and embryo replacement

Sperm from the husbands of the matched recipients was prepared by density gradient centrifugation and ICSI fertilization was performed as previously described (Nagy *et al.*, 1994). Eighteen hours after ICSI, oocytes were assessed for the presence of pronuclei and switched to Quinn's advantage cleavage medium with 15% SPS for further culture. Embryo cleavage was assessed at 72 h after insemination. At 80 h after insemination, embryos were transferred to Quinn's advantage blastocyst medium with 15% SPS. At 116 h afterinsemination, blastocyst formation was assessed and graded for embryo replacement. Embryo replacements were only performed with the embryos derived from in-vivo-matured (IVO) oocytes after cryopreservation.

## Results

### Oocyte survival and fertilization after warming

A total of 240 IVO oocytes were vitrified from 10 oocyte donors, and 63 of 79 sibling immature oocytes were matured *in vitro* and vitrified on the next day. A total of 117 of 137 from the IVO group (85%) and 51 of 63 rescued IVM (81%) oocytes survived after warming (**Table 1**; **Figure 1**). After 3 h of warming, all the surviving oocytes were subjected to ICSI with sperm from the corresponding recipient couples, and fertilization was judged by the formation of two pronuclei after 18 h of ICSI. No significant difference was found between the fertilization rates of IVO and IVM oocytes (**Table 1**; 86% versus 76%).

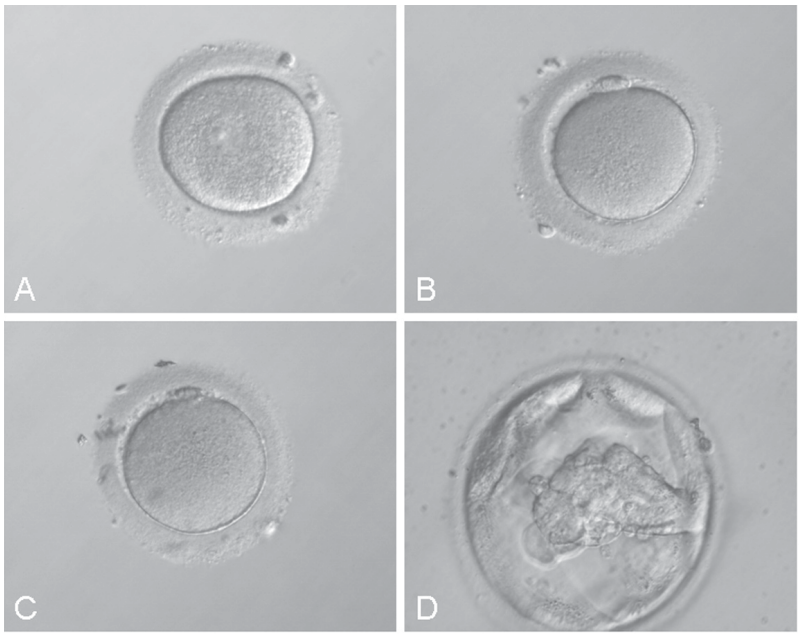


**Figure 1.** Rescued in-vitro maturation, oocyte cryopreservation, and preimplantation development of a representative human oocyte. (**A**) A denuded oocyte at germinal vesicle stage. (**B**) The same oocyte extruded a polar body after 26 h of rescued in-vitro maturation. (**C**) The oocyte survived vitrification and warming. (**D**) A blastocyst embryo at 116 h after intracytoplasmic sperm injection derived from the oocyte that had been in-vitro matured, vitrified/warmed, and fertilized with a spermatozoon.

**Table 1.** Comparison of survival rate, fertilization rate and embryo development between in-vivo (IVO) and rescued in-vitro maturation (IVM) oocytes after oocyte cryopreservation.

| | *Total no. of oocytes warmed* | *No. survived (%)* | *No. of 2PN (% per survival)* | *No. of cleaved (% per 2PN)* | *Embryo grade[a] A/B* | *C/D* | *No. of blastocyst Day 5/Day 6 (% per 2PN)* |
|---|---|---|---|---|---|---|---|
| IVO | 137 | 117 (85) | 101 (86) | 99 (98) | 70 (71) | 29 (29) | 73 (72) |
| IVM (MI–MII) | 34 | 28 (82) | 25 (89) | 23 (92) | 10 (43) | 13 (57) | 4 (16) |
| IVM (GV–MII) | 29 | 23 (79) | 14 (61) | 12 (86) | 4 (33) | 8 (67) | 2 (14) |

[a]Grade A, excellent quality, all blastomeres were of an equal size and without anucleate fragments. Grade B, good quality, embryos had blastomeres of equal or unequal size and maximum of 20% of anucleate fragments. Grade C, fair quality, anucleate fragments were present in 20–50% of the embryo. Grade D, poor quality, embryos had more than 50% of the anucleate fragments.
GV = germinal vesicle, MI = metaphase I, MII = metaphase II, 2PN = 2 pronuclei.

## Preimplantation embryo development

At 72 h after insemination, embryo cleavage was assessed. There was no significant difference in the cleavage rates between IVO and IVM oocytes (**Table 1**; 98% versus 89%). At 80 h after insemination, embryos were transferred to Quinn's advantage blastocyst medium with 15% SPS. At 116 h after insemination, blastocyst formation was assessed and graded, and the blastocyst formation rate was significantly different between IVO and IVM groups (**Table 1**; **Figure 1**; 72% versus 15%).

## Clinical outcome of oocyte cryopreservation

Embryo replacements were only performed with the embryos derived from IVO oocytes after cryopreservation. A total of 18 consenting women received a total of 137 vitrified/thawed oocytes (mean number of oocytes/recipient: 7.61). A total of 42 blastocyst stage embryos were replaced in 18 embryo transfers, with a mean number of 2.33 embryos per transfer. On day 11 after embryo replacement, positive serum β-HCG was detected in 16 of 18 recipients. A total of 15 recipients had detectable fetal heartbeats confirmed by ultrasound 30 days after embryo replacement. Of the 42 transferred embryos 26 implanted, corresponding to an implantation rate of 61.9%. The clinical pregnancy rate was 83.3% per recipient (**Table 2**). Ten of the 15 recipients have delivered 19 healthy babies, and five pregnancies are ongoing.

## Discussion

In order to discover a new strategy to extend the pool of total fertilizable oocytes from rescued IVM oocytes, this study evaluated oocyte cryopreservation, comparing IVO and rescued IVM oocytes using the vitrification method. The results show that oocyte survival, fertilization and cleavage rate were not significantly different in the rescued IVM oocytes compared with

**Table 2.** Clinical outcome of oocyte vitrification using in-vivo-matured oocytes

| | |
|---|---|
| No. of embryo transfers | 18 |
| No. of transferred embryos | 42 |
| No. of embryo per transfer | 2.33 |
| Positive β-HCG | 88.8% (16/18) |
| Clinical pregnancy | 83.3% (15/18) |
| Implantation rate per transferred embryo | 61.9% (26/42) |
| No. of ongoing pregnancies | 5 |
| No. of deliveries | 10 |
| No. of live births | 19 |

IVO sibling oocytes. The fertilized oocytes from the in-vivo and in-vitro groups were cultured to blastocyst stage; however, the developmental potential of the embryo was significantly reduced in the rescued IVM group. The clinical outcomes in this study also provide valuable information about the efficiency of oocyte cryopreservation when it was used routinely as an adjunct to a human IVF program.

Even with controlled ovarian stimulation, a proportion (15–30% or occasionally more) of human oocytes remain meiotically immature on oocyte retrieval; however, those immature oocytes are discarded in routine IVF procedures. If the potential of the immature oocytes can be preserved, it may become a way of extending the oocyte source for IVF patients, or even for embryonic stem cell study. Theoretically, there are two approaches for preserving the immature oocytes: oocyte cryopreservation at the immature stage (before IVM); and oocyte cryopreservation at the mature stage (after IVM). Initial reports revealed that maturation, fertilization and development were significantly impaired when oocytes were cryopreserved at the immature stage (Toth *et al.*, 1994a,b; Son *et al.*, 1996; Park *et al.*, 1997). Additionally, in routine clinical cases, the companion cumulus cells of the oocytes have to be stripped in order to evaluate the maturity of the oocyte accurately before further ICSI. In this study, the oocytes were cryopreserved at the mature stage when they had been rescued IVM, which may offer greater possibilities for integration into clinical applications.

The capacity of a mature oocyte to support the initial stage of embryo development and implantation is termed oocyte developmental competence and is a measure of intrinsic oocyte quality. Many factors affect oocyte competence, including communication between the oocyte and its surrounding cumulus cells, which is necessary for the development of a competent oocyte (Krisher, 2004). The oocyte-secreted factors, paracrine factors such as BMP15 and GDF9 regulating follicular proliferation, steroidogenesis, differentiation, apoptosis and expansion (Eppig, 2001; Gilchrist *et al.*, 2004; Hussein *et al.*, 2005) have been shown to enhance oocyte development competence during IVM (Hussein *et al.*, 2006). In addition to the local production of soluble factors that act in an autocrine and paracrine fashion during pre-antral follicle development, the oocyte communicates with and modifies its surroundings via direct physical contact with cumulus cells (Plancha *et al.*, 2005). The foundation of this relationship lies partly in highly specialized oocyte–cumulus cell contacts called trans-zonal projections (TZP) that are established at the onset of folliculogenesis (Hutt and Albertini, 2007). Without the TZP between companion cumulus cells and oocytes, the rescued IVM oocytes have a lower developmental competence compared with IVO oocytes, in part owing to compromised micro-environments to support complete oocyte maturation.

To date, few studies have investigated the potential of oocyte cryopreservation on rescued IVM oocytes. Even though the first live birth resulting from rescued IVM was reported in 1996 (Nagy *et al.*, 1996), rescued IVM has not been applied to daily IVF practices. This limited application is mostly due to the depleted developmental competence in rescued IVM. The present study has shown that rescued IVM oocytes have comparable survival, fertilization and cleavage rates to in-vivo oocytes, although the rescued IVM oocytes have less developmental competence than in-vivo ones. If the developmental competence of oocytes in the rescued IVM process can be reversed, it is more likely that the oocyte cryopreservation will be also applicable to the oocytes from the immature source.

One of the most important goals in oocyte cryopreservation is to use the minimum number of oocytes to achieve a successful implantation/clinical pregnancy. The minimum number of thawed/warmed oocytes per implantation can largely represent the overall efficiency of oocyte cryopreservation. However, the efficiency has varied considerably among previously published reports (Tucker *et al.*, 1998; Porcu *et al.*, 1999; Boldt *et al.*, 2003; Fosas *et al.*, 2003; Yoon *et al.*, 2003; Kuwayama *et al.*, 2005; Chamayou *et al.*, 2006; La Sala *et al.*, 2006; Levi Setti *et al.*, 2006; Lucena *et al.*, 2006; Selman *et al.*, 2006; Antinori *et al.*, 2007; Barrit *et al.*, 2007; Bianchi *et al.*, 2007; Borini *et al.*, 2007; Cobo *et al.*, 2007; De Santis *et al.*, 2007); the range can be from 6 to 100 oocytes per implantation. In this study, 5.3 vitrified-warmed oocytes were required to achieve an implantation. The number of oocytes used represents the efficiency of oocyte cryopreservation, and also provides an important message that may impact on future ART treatments. If these results remain consistent, then it may be a better strategy for managing fertility treatments before invasive medical treatment is required. The efficient oocyte cryopreservation will be used to delay childbearing, avoid embryo cryopreservation with its associated moral and ethical concerns, and upgrade oocyte donation programs allowing immediate donor–recipient matching and scheduling after complete infectious disease screening.

## Acknowledgements

The authors would like to thank Ms Jaclyn Friedman for her help with revising this manuscript. The authors gratefully acknowledge Organon NV (Oss, The Netherlands), a part of Schering-Plough Corporation, for the support of this study.

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*Declaration: The authors report no financial or commercial conflicts of interest.*

*Received 18 February 2008; refereed 13 March 2008; accepted 20 June 2008.*