Article

# Cryopreservation of immature and in-vitro matured human oocytes by vitrification

Professor Yunxia Cao obtained her MD degree in 1985, her MSc degree in 1988 and her PhD in 2000. In 1998, she established the Reproductive Medical Centre in Anhui Province, China, and produced the first IVM baby and first baby derived from frozen–thawed oocytes in Anhui Province, China. Professor Yunxia Cao is a well-known infertility specialist in China, and she is a National Board Member for Reproductive Medicine. Professor Cao research interests include the mechanisms underlying polycystic ovary syndrome, fertility preservation for women and the simplification of IVF treatment.

*Professor Yunxia Cao*

Yunxia Cao[1], Qiong Xing, Zhi-Guo Zhang, Zhao-Lian Wei, Ping Zhou, Lin Cong
Centre for Reproductive Medicine, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, P. R. of China
[1]Correspondence: e-mail: caoyunxia6@126.com

## Abstract

The objective of this study was to determine the efficacy of vitrification of human oocytes before and after in-vitro maturation (IVM). The immature oocytes recovered ($n = 472$) were divided into two groups: (i) immature oocytes ($n = 219$) vitrified at the germinal vesicle (GV) stage; and (ii) immature GV-stage oocytes ($n = 253$) that were firstly matured *in vitro* (MII-stage oocytes; $n = 178$), then vitrified ($n = 79$). The remaining oocytes ($n = 99$), which were not vitrified, were processed as controls. After warming, the oocyte survival, maturation and fertilization rates, as well as embryonic development, were compared. The results showed no significant difference between the survival rates of the oocytes vitrified at GV stage and those vitrified at MII stage (85.4% versus 86.1%). However, oocyte maturation rates were significantly reduced ($P < 0.05$) when oocytes were vitrified at immature GV stage followed by IVM (50.8%) in comparison with the control group (70.4%). Following insemination by intracytoplasmic sperm injection, there was no difference in the fertilization (62.1% versus 58.8%), cleavage (69.5% versus 67.5%) and blastocyst development (0.0% versus 0.0%) rates between these two groups. However, these results were significantly lower ($P < 0.05$) than those achieved in the control group. This suggests that better results can be achieved by vitrifying mature oocytes rather than immature oocytes.

***Keywords:*** *embryo, immature, in-vitro maturation, mature, oocytes, vitrification*

## Introduction

The development of an effective oocyte-cryopreservation programme will have a major impact on clinical practice in reproductive medicine and will serve as a powerful tool to preserve fertility for teenage girls and young women without male partners, or for those individuals who are affected by malignancies. It will also be beneficial to infertile couples who have moral or religious objections to embryo cryopreservation. In addition, a successful oocyte cryopreservation programme will eliminate the need for donor–recipient menstrual cycle synchronization and will enable the establishment of oocyte banks, which would facilitate the logistics of coordinating egg donors with recipients.

Traditionally, oocyte cryopreservation has been performed by the conventional slow-freezing method, which has been associated with relatively low survival rates and only a limited numbers of live births have been achieved (Chen, 1986; Porcu *et al*., 1997; Tucker *et al*., 1998a). Recently, the modified slow-freezing method has been introduced (Stachecki *et al*., 1998; Yang *et al*., 1998; Fabbri *et al*., 2001; Quintans *et al*., 2002; Boldt *et al*., 2003, 2006; Borini *et al*., 2004, 2006; Stachecki and Cohen, 2004; Azambuja *et al*., 2005; Bianchi *et al*., 2005; Coticchio *et al*., 2005; Levi-Setti *et al*., 2006). Although the survival and fertilization rates seem to have improved slightly, the clinical outcome has yet to be confirmed.

Theoretically, given their microstructure, immature germinal vesicle (GV) -stage oocytes should be more resistant to the damage caused by cooling and circumvent the risk of polyploidy and aneuploidies, since the chromatins are diffused and surrounded by a nuclear membrane (Cooper *et al*., 1998; Isachenko and Nayudu, 1999). Although oocyte survival rates seem to have improved, poor maturation, fertilization and embryonic development were the main problems associated with immature oocyte freezing (Toth

*et al.*, 1994a,b; Son *et al.*, 1996). So far, only one live birth has been reported following the slow freezing of immature oocytes at GV stage (Tucker *et al.*, 1998b). Therefore, cryopreservation of mature oocytes (MII stage) using the slow-freezing method seems more efficient than that of immature oocytes.

Recent advances in vitrification technology have markedly improved the survival rate of mature oocytes after warming and the pregnancy rate is comparable to that achieved with fresh oocytes (Kuleshova *et al.*, 1999; Kuleshova and Lopata, 2002; Katayama *et al.*, 2003; Yoon *et al.*, 2003, 2007; Chian *et al.*, 2005; Kuwayama *et al.*, 2005; Lucena *et al.*, 2006; Antinori *et al.*, 2007). It has been reported that the pregnancies and infants conceived by oocyte vitrification were not associated with an increased risk of adverse obstetric and perinatal outcomes (Chian *et al.*, 2008). All these studies were performed using in-vivo and in-vitro matured oocytes for vitrification. However, there have been no data available on oocyte survival, fertilization and early embryonic development of oocytes vitrified at the immature GV stage.

The objective of this study was to investigate whether immature and in-vitro matured human oocytes can be successfully vitrified by comparing oocyte survival rates, fertilization and embryonic development when oocytes are vitrified before and after in-vitro maturation (IVM).

# Materials and methods

## Source of immature oocytes

Immature oocytes ($n$ = 472) were obtained from 122 women with polycystic ovary syndrome (PCOS) who were undergoing IVM treatment at the infertility centre. When more than 20 immature oocytes were collected from an IVM patient, the remaining immature oocytes were donated to the present study. The study was approved by the institutional review board of the First Affiliated Hospital of Anhui Medical University. All patients agreed and signed informed consent forms in advance.

## Immature oocyte retrieval

All patients (30.2 ± 3.7 years) had no prior history of pregnancy. The patients with PCOS were primed with clomiphene citrate (CC) and human menopausal gonadotrophin (HMG; Lizhu Ltd, China) starting on day 3 of their menstrual cycle. The dose of CC was 50–150 mg per day and 70–100 IU of HMG per day for 5 days. When the size of the leading follicles in the ovaries reached 8–10 mm in diameter as observed by ultrasound scan, 10,000 IU of human chorionic gonadotrophin (HCG) (Profasi; Serono, Switzerland) was given and retrieval immature oocytes was performed 36 h later. A single lumen 17G needle was used to collect the immature oocytes via transvaginal ultrasound.

According to the experimental design, 253 immature oocytes underwent IVM and 219 immature oocytes were cryopreserved immediately by vitrification.

## IVM of immature oocytes

The first group of oocytes (219 immature GV stage) were vitrified and stored for at least 1 month in liquid nitrogen before thawing. After thawing, two or three immature oocytes were transferred to IVM medium (50 µl per droplet under mineral oil) for culture in a 5% $CO_2$ incubator at 37°C with high humidity for 36 h. Following culture for 36 h, the oocytes were denuded from cumulus cells using 80 IU/ml hyaluronidase (Sigma, USA) and mechanical disruption with fine-bore glass pipettes with the aid of an inverted microscope (IX-71; Olympus, Japan). Oocyte maturation was determined by the presence of the first polar body. The IVM medium used was tissue culture medium 199 (TCM199; Sigma) supplemented with 20% (v/v) human umbilical cord serum, 0.075 IU/ml FSH (Gonal-F; Serono), 0.5 IU/ml HCG and 0.1 µg/ml 17β-oestradiol (Sigma) (Zhang *et al.*, 2007).

The second group of oocytes (253 immature GV stage) was transferred to IVM medium (two or three oocytes per 50 µl droplet under mineral oil) and cultured in a 5% $CO_2$ incubator at 37°C with high humidity. Following culture for 36 h, the oocytes were denuded from cumulus cells using 80 IU/ml hyaluronidase and mechanical disruption with fine-bore glass pipettes with the aid of an invert microscope (IX-71, Olympus, Japan). Oocyte maturation was determined by the presence of the first polar body, after which the oocyte was processed for vitrification.

## Vitrification of immature and mature oocytes

Immature and in-vitro matured oocytes were vitrified using a vitrification kit (MediCult Company, Jyllinge, Denmark). Briefly, oocytes were suspended in equilibration medium containing 7.5% (v/v) ethylene glycol + 7.5% (v/v) 1,2-propanediol for 5 min at room temperature and then transferred to vitrification medium containing 15% (v/v) ethylene glycol + 15% (v/v) 1,2-propanediol + 0.5 mol/l sucrose at room temperature for 45–60 s. The treated oocytes were loaded onto a McGill Cryoleaf (MediCult) and plunged immediately into liquid nitrogen where they remained in storage for at least 1 month.

## Warming of immature and mature oocytes

The warming procedure was performed as described in the warming kit (MediCult Company, Jyllinge, Denmark). Briefly, the McGill Cryoleaf was directly inserted into warming medium containing 1 mol/l sucrose for 1 min at 37°C. The warmed oocytes were transferred to diluent medium-I containing 0.50 mol/l sucrose and then moved to diluent medium-II containing 0.25 mol/l sucrose for 3 min each. The oocytes were washed twice in washing medium containing 20% human serum albumin for 3 min. Immature and mature oocyte viability was evaluated microscopically 2–3 h after culture, based on the morphology of the oocyte membrane's integrity and cytoplasm. The dead oocytes showed broken membrane and dark cytoplasm. The surviving immature oocytes first underwent IVM and then the in-vitro matured oocytes were used for insemination by intracytoplasmic sperm injection (ICSI).

## Insemination and embryo culture

Sperm samples were obtained from the male partners. Partners with severe male factors were excluded from the present study. The in-vitro matured oocytes were inseminated by ICSI. The

inseminated oocytes were transferred to G-1 medium (VitroLife, Sverige, Sweden), cultured for 3 days and then transferred to G-2 medium (VitroLife) in a 5% $CO_2$ incubator at 37°C with high humidity for 5 days. Fertilization was verified 17–19 h after ICSI. Embryo quality was evaluated on day 3 after ICSI and was based on the scoring criteria described by Tomas *et al.* (1998). The embryos were scored as follows: Grade I, equal-sized blastomeres with no fragmentation; Grade II, equal-sized blastomeres with <20% fragmentation; Grade III, unequal-sized blastomeres with <20% fragmentation; Grade IV, equal- or unequal-sized blastomeres with 20–50% fragmentation; Grade V, equal- and unequal-sized blastomeres with >50% fragmentation. Grades I and II comprised high-quality embryos. On day 5 after ICSI, the number of blastocysts in each group was also recorded.

## Experimental design

Survival rates were compared between the oocytes that were vitrified at immature GV stage and those that were vitrified at MII stage after IVM. Maturation rates were compared between the oocytes vitrified at GV stage and those that had not been vitrified. Rates of fertilization, cleavage and formation of high-quality embryos and blastocysts were compared between the oocytes that had been vitrified before and after IVM. As a control, the immature oocytes that had not been vitrified were subjected to IVM, ICSI and embryonic culture *in vitro*.

## Statistical analysis

Statistical analyses were performed using Statistics Package for Social Sciences 13.0 (SPSS Inc, Chicago). The data were analysed using the chi-squared test. A *P*-value of <0.05 was considered statistically significant.

# Results

As shown in **Figure 1**, the oocyte survival rates were 85.4% (187/219) versus 86.1% (68/79) when the oocytes were vitrified at the immature GV stage and the mature MII stage. There was no significant difference between the two groups.

The oocyte maturation rate was 50.8% (95/187) when the oocytes were vitrified at the immature GV stage and then underwent IVM after warming (**Figure 2**). This was significantly lower ($P < 0.05$) than the immature oocytes matured *in vitro* without prior vitrification (70.4% = 178/253).

As shown in **Table 1**, although there was no difference in the fertilization rate between the oocytes vitrified at the immature GV stage (62.1%, 59/95) and at the mature MII stage (58.8%, 40/68), the result achieved from both groups was significantly lower ($P < 0.05$) than that of the control group (76.8%, 76/99). There was no significant difference in the cleavage rate between the oocytes vitrified at the immature GV stage (69.5%, 41/59) and those vitrified at the mature MII stage (67.5%, 27/40). However, the rate obtained from both groups was significantly lower ($P < 0.01$) than those obtained using the oocytes without vitrification for IVM (88.2%, 67/76). When the oocytes were vitrified at the immature GV stage, the percentage of high-quality embryos (grades I and II) developed on day 3 was 12.2% (5/41), significantly lower ($P < 0.05$) than when the oocytes were vitrified at the mature MII stage (33.3%, 9/27). The percentage of high-quality embryos obtained on day 3 of development in both groups was notably lower ($P < 0.01$) than that achieved with the oocytes without vitrification for IVM. Although no blastocysts developed from the oocytes vitrified at the immature GV and matured MII stages, the number of blastocysts that developed in the oocytes that had not been vitrified before IVM, 46.3% (31/67) and was significantly higher ($P < 0.01$) than both vitrified groups.

# Discussion

The results demonstrate that regardless of the meiotic maturation stage of oocytes, the survival rates following vitrification are similar (**Figure 1**). The rationale for using immature GV-stage oocytes for cryopreservation was to improve the survival rate

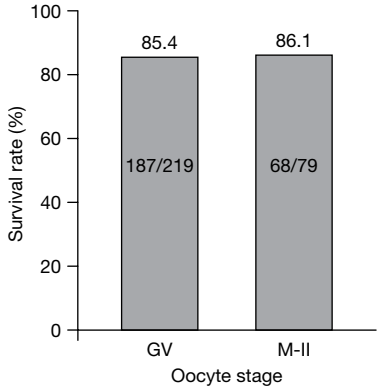


**Figure 1.** Survival rates of human oocytes vitrified at immature GV stage and at mature MII stage. Total of 298 oocytes (219 immature oocytes and 79 mature oocytes) were vitrified and warmed. There were no differences in the survival rates between the two groups.

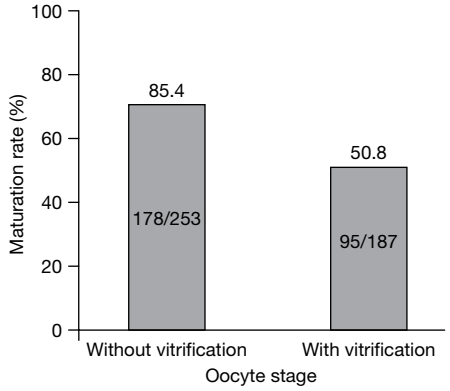


**Figure 2.** In-vitro maturation rates of immature human oocytes with or without vitrification for IVM. A total of 440 immature oocytes (253 immature oocytes without vitrification and 187 immature oocytes with vitrification) were cultured *in vitro* for maturation. There were significant differences ($P < 0.05$) between those two groups.

**Table 1.** Fertilization, cleavage and embryonic development *in vitro* of human oocytes vitrified at immature (GV) or mature (MII) stage followed by intracytoplasmic sperm injection.

| | *Control* | *Vitrified at GV stage* | *Vitrified at MII stage* | P-*value* |
|---|---|---|---|---|
| Oocytes inseminated | 99 | 95 | 68 | – |
| Oocytes fertilized | 76 (76.8) | 59 (62.1) | 40 (58.8) | <0.05 |
| Zygotes cleaved | 67 (88.2) | 41 (69.5) | 27 (67.5) | <0.01 |
| Grade I and II embryos | 33 (49.3) | 5 (12.2) | 9 (33.3) | <0.05[a], <0.01[b] |
| Blastocysts developed | 31 (46.3) | 0 (0.0) | 0 (0.0) | <0.01 |

Values are number (percentage). GV = germinal vesicle; MII = metaphase.
Controls were non-vitrified oocytes matured *in vitro*. Although there were no differences in fertilization, cleavage and blastocyst development rates between the two vitrified groups, there were significant differences between the vitrified oocytes and the control groups.
[a]Both vitrified groups versus control group; [b]vitrified at GV stage versus vitrified at MII stage.

and to reduce the risk of polyploidy and aneuploidy, due to the advantage of their microstructures resisting damage during the process of cryopreservation (Cooper *et al.*, 1998; Isachenko and Nayudu, 1999). Using a mouse model, Huang *et al.* (2008) reported that high survival rates were obtained when oocytes were vitrified at the mature MII stage and that no increased incidence of aneuploidy was observed. For this reason, it seems that high survival rates can be obtained when human oocytes are vitrified at the immature GV stage and at the mature MII stage. However, it is unclear whether the microstructure (spindles and chromosomes) of human oocytes is affected by the vitrification process and this needs further investigation.

For infertile women with PCOS, IVM is an effective treatment (Chian *et al.*, 2004; Zhang *et al.*, 2007; Wei *et al.*, 2008) and several hundred healthy babies have been born (Edwards, 2007). An attractive strategy of fertility preservation for women without a male partner is to cryopreserve their oocytes. It has been reported that immature oocyte retrieval followed by IVM and vitrification is an effective option for fertility preservation in women undergoing cancer treatment (Rao *et al.*, 2004), because immature oocyte retrieval followed by IVM and vitrification involves minimal delay in cancer treatment and does not require ovarian stimulation. More importantly, immature oocyte retrieval followed by IVM and vitrification will help some cancer patients with hormone-sensitive tumours, because it eliminates the risk of ovarian stimulation in oestrogen-receptor-positive breast cancer patients.

Besides the negative impact on hormone-sensitive cancer patients, the side effects of gonadotrophin stimulation have also caused concern. Apart from ovarian hyperstimulation syndrome, the long-term side effects of gonadotrophin stimulation have also been noted (Venn *et al.*, 1999, 2001). A standard ovarian stimulation protocol requires several weeks to desensitize the pituitary with gonadotrophin-releasing hormone agonist or antagonist, which is then followed by gonadotrophin stimulation.

In recent reports, Chian *et al.* (2009a,b) have provided proof-of-principle evidence that the novel strategy of fertility preservation involving immature oocyte retrieval, IVM and vitrification of human oocytes can lead to successful pregnancies and healthy live births. In their studies, oocytes were first subjected to IVM and those that had matured *in vitro* were vitrified. The results of the present study provide important information to demonstrate that mature oocytes survive vitrification better than immature GV-stage oocytes.

The results of the present study indicate that there was no difference between the fertilization and cleavage rates when oocytes were vitrified at the immature GV stage and at the mature MII stage, although these rates were considerably lower than those obtained using in-vitro matured oocytes that had not been vitrified (**Table 1**). The results of the present study also indicate that the percentage of high-quality embryos developed on day 3 from oocytes vitrified at the mature MII stage was higher than that of oocytes vitrified at the immature GV stage. In addition, no blastocysts developed from any of the oocytes that were vitrified at both GV and MII stages. In contrast, 46.3% (31/67) blastocysts developed from in-vitro matured oocytes that had not been vitrified. It is not clear why vitrified oocytes have less capacity for fertilization and embryonic development. As reported by Huang *et al.* (2008) using the mouse model, it is possible that the reduced developmental competence of in-vitro matured and vitrified oocytes may be due to DNA fragmentation. However, this result has yet to be confirmed with human oocytes.

In conclusion, it has been shown for the first time with human oocytes that there is no difference in the survival rate between oocytes vitrified at immature GV stage and those vitrified at the mature MII stage and that the potential of oocyte maturation is reduced by the vitrification of immature oocytes at the GV stage. Therefore, the data suggest that oocytes should be vitrified at mature MII stage following IVM rather than at the immature GV stage.

## Acknowledgements

The authors thank Dr Ri-Cheng Chian from the Division of Reproductive Biology, Department of Obstetrics and Gynecology, McGill University, Montreal, Canada, for his assistance and critical reading this manuscript and valuable comments.

## References


Antinori M, Licata E, Dani G *et al*. 2007 Cryotop human oocytes vitrification results in high survival rate and healthy deliveries. *Reproductive BioMedicine Online* **14**, 72–79.

Azambuja R, Badalotti M, Teloken C *et al*. 2005 Successful birth after injection of frozen human oocytes with frozen epididymal spermatozoa. *Reproductive BioMedicine Online* **11**, 449–451.

Bianchi V, Coticchio G, Fava L *et al*. 2005 Meiotic spindle imaging in human oocytes frozen with a slow freezing procedure involving high sucrose concentration. *Human Reproduction* **20**, 1078–1083.

Boldt J, Tidswell N, Sayers A *et al*. 2006 Human oocyte cryopreservation: 5-year experience with a sodium-depleted slow freezing method. *Reproductive BioMedicine Online* **13**, 96–100.

Boldt J, Cline D, McLaughlin D 2003 Human oocyte cryopreservation as an adjunct to IVF-embryo transfer cycles. *Human Reproduction* **18**, 1250–255.

Borini A, Sciajno R, Bianchi V *et al*. 2006 Clinical outcome of oocyte cryopreservation after slow cooling with a protocol utilizing a high sucrose concentration. *Human Reproduction* **21**, 512–517.

Borini A, Bonu MA, Coticchio G *et al*. 2004 Pregnancies and births after oocyte cryopreservation. *Fertility and Sterility* **82**, 601–605.

Chen C 1986 Pregnancy after human oocyte cryopreservation. *Lancet* **327**, 884–886.

Chian RC, Gilbert L, Huang JYJ *et al*. 2009a Live birth after vitrification of in-vitro matured human oocytes. *Fertility and Sterility* **91**, 372–376.

Chian RC, Huang JYJ, Gilbert L *et al*. 2009b Obstetric outcomes following vitrification of in-vitro and in-vivo matured oocytes. *Fertility and Sterility* **91**, 2391–2398.

Chian RC, Huang JYJ, Tan SL *et al*. 2008 Obstetric and perinatal outcome in 200 infants conceived from vitrified oocytes. *Reproductive BioMedicine Online* **16**, 608–610.

Chian RC, Son WY, Huang JY *et al*. 2005 High survival rates and pregnancies of human oocytes following vitrification: Preliminary report. *Fertility and Sterility* **84**, S36.

Chian RC, Buckett WM, Tan SL 2004 In-vitro maturation of human oocytes. *Reproductive BioMedicine Online* **8**, 148–166.

Cooper A, Paynter SJ, Fuller BJ, Shaw RW 1998 Differential effects of cryopreservation on nuclear or cytoplasmic maturation *in vitro* in immature mouse oocytes from stimulated ovaries. *Human Reproduction* **13**, 971–978.

Coticchio G, Bonu MA, Bianchi V *et al*. 2005 Criteria to assess human oocyte quality after cryopreservation. *Reproductive BioMedicine Online* **11**, 421–427.

Fabbri R, Porcu E, Marsella T *et al*. 2001 Human oocyte cryopreservation: new perspectives regarding oocyte survival. *Human Reproduction* **16**, 411–416.

Huang JYJ, Chen HY, Park JYS *et al*. 2008 Comparison of spindle and chromosome configuration in in-vitro- and in-vivo-matured mouse oocytes after vitrification. *Fertility and Sterility* **90** (4 Suppl.), 1424–1432.

Isachenko EF, Nayudu PL 1999 Vitrification of mouse germinal vesicle oocytes: effect of treatment temperature and egg yolk on chromosomal normality and cumulus integrity. *Human Reproduction* **14**, 400–408.

Katayama KP, Stehlik J, Kuwayama M *et al*. 2003 High survival rate of vitrified human oocytes results in clinical pregnancy. *Fertility and Sterility* **80**, 223-224.

Kuleshova LL, Lopata A 2002 Vitrification can be more favorable than slow cooling. *Fertility and Sterility* **78**, 449–454.

Kuleshova L, Gianaroli L, Magli C *et al*. 1998 Birth following vitrification of a small number of human oocytes: case report. *Human Reproduction* **14**, *3077–3079*.

Kuwayama M, Vajta G, Kato O, Leibo SP 2005 Highly efficient vitrification method for cryopreservation of human oocytes. *Reproductive BioMedicine Online* **11**, 300–308.

Levi-Setti PE, Albani E, Novara PV *et al*. 2006 Cryopreservation of supernumerary oocytes in IVF/ICSI cycles. *Human Reproduction* **21**, 370–375.

Lucena E, Bernal DP, Lucena C *et al*. 2006 Successful ongoing pregnancies after vitrification of oocytes. *Fertility and Sterility* **85**, 108–111.

Porcu E, Fabbri R, Seracchioli *et al*. 1997 Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. *Fertility and Sterility* **68**, 724–726.

Quintans CJ, Donaldson MJ, Bertolino MV, Pasqualini RS 2002 Birth of two babies using oocytes that were cryopreserved in a choline-based freezing medium. *Human Reproduction* **17**, 3149–3152.

Rao GD, Chian RC, Son WS *et al*. 2004 Fertility preservation in women undergoing cancer treatment. *Lancet* **363**, 1829–1830.

Son WY, Park SE, Lee KA *et al*. 1996 Effects of 1,2-propanediol and freezing-thawing on the in-vitro developmental capacity of human immature oocytes. *Fertility and Sterility* **66**, 995–999.

Stachecki JJ, Cohen J 2004 An overview of oocyte cryopreservation. *Reproductive BioMedicine Online* **9**, 152–163.

Stachecki JJ, Cohen J, Willadsen SM 1998 Cryopreservation of unfertilized oocytes: the effect of replacing sodium with choline in the freezing medium. *Cryobiology* **37**, 346–355.

Tomas C, Orava M, Tuomivaara L, Martitainen H 1998 Low pregnancy rate is achieved in patients treated with intracytoplasmic sperm injection due to previous low or failed fertilization in in-vitro fertilization. *Human Reproduction* **13**, 65–70.

Toth TL, Lanzendorf SE, Sandow BA *et al*. 1994a Cryopreservation of human prophase I oocytes collected from unstimulated follicles. *Fertility and Sterility* **61**, 1077–1082.

Toth TL, Baka SG, Veeck LL *et al*. 1994b Fertilization and in-vitro development of cryopreserved human prophase I oocytes. *Fertility and Sterility* **61**, 891–894.

Tucker MJ, Morton PC, Wright G *et al*. 1998a Clinical application of human egg cryopreservation. *Human Reproduction* **11**, 3156–3159.

Tucker MJ, Wright G, Morton PC, Massey JB 1998b Birth after cryopreservation of immature oocytes with subsequent in-vitro maturation. *Fertility and Sterility* **70**, 578–579.

Venn A, Jones P, Quinn M, Healy D 2001 Characteristics of ovarian and uterine cancers in a cohort of in-vitro fertilization patients. *Gynecologic Oncology* **82**, 64–68.

Venn A, Watson L, Bruinsma F *et al*. 1999 Risk of cancer after use of fertility drugs with in-vitro fertilization. *Lancet* **354**, 1586–1590.

Wei Z, Cao Y, Cong L *et al*. 2008 Effect of metformin pretreatment on pregnancy outcome of in-vitro matured oocytes retrieved from women with polycystic ovary syndrome. *Fertility and Sterility* **90**, 1149–1154.

Yang DS, Winslow KL, Blohm PL 1998 Improved survival rate after cryopreservation of human fresh and aged unfertilized oocytes using a specially developed oocyte cryopreservation regime. *Fertility and Sterility* **70** (Suppl. 1), S86.

Yoon TK, Lee DR, Cha SK *et al*. 2007 Survival rate of human oocytes and pregnancy outcome after vitrification using slush nitrogen in assisted reproductive technologies. *Fertility and Sterility* **88**, 925–926.

Yoon TK, Kim TJ, Park SE *et al*. 2003 Live births after vitrification of oocytes in a stimulated in-vitro fertilization-embryo transfer program. *Fertility and Sterility* **79**, 1323–1336.

Zhang ZG, Zhao JH, Wei ZL *et al*. 2007 Human umbilical cord blood serum in culture medium on oocyte maturation *in vitro*. *Archives of Andrology* **53**, 303–307.


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

*Received 5 June 2008; revised and resubmitted 8 December 2008; refereed 9 December 2008; accepted 30 March 2009.*