Vitrification of Mouse Oocyte Using Open Pulled Straws Compared with Needles

Surachai Pornwiroon MD*, Somboon Kunathikom MD*,
Orawan Makemaharn MSc*, Rattanaphorn Huanaraj BSc*

** Department of Obstetrics and Gynecology, Faculty of Medicine, Siriraj Hospital, Mahidol University*

Objective: *To compare the survival rate of mouse oocytes and fertilization rate between using open pulled straws (OPS) and needles for vitrification.*
Material and Method: *Meiosis II oocytes from female C57B/6J mice aged 7-8 weeks were collected and allocated to two groups for vitrification by using OPS or needles. Vitrified oocytes were thawed, morphological survival and fertilization rate were examined.*
Results: *There was no obvious difference between the morphological survival rates of vitrified mouse oocytes using OPS and needles (66.7% vs 64.8%). Proportions Difference 1.9% (95%CI -7.1, 10.7). The vitrified oocytes from the needle had significantly higher percentages of fertilization rate than OPS (76.8% vs 62.5%). Proportions Difference -14.3% (95%CI -24.5, -3.6).*
Conclusion: *Vitrification method of mouse oocytes using needles when compared to OPS provides a similar morphological survival rate and higher fertilization rate.*

Keywords: *Mouse oocytes, Oocyte vitrification, Open pulled straws, Needles, Survival rate, Fertilization rate.*

J Med Assoc Thai 2006; 89 (12): 2015-20
Full text. e-Journal: *http://www.medassocthai.org/journal*

The cryopreservation of oocytes and embryos is a valuable method for increasing successfulness in treatment of infertility. Embryo cryopreservation is now a successful procedure, but oocyte cryopreservation achieves poorer results[1-3]. Adverse effects of cryopreservation on the integrity of several unique features of the oocytes are premature cortical granule exocytosis leading to zona hardening[4], increased parthenogenetic activation[5,6], damage of cytoskeletal elements[7-9] and disruption of the meiotic spindle[10,11]. This has primarily been ascribed to low rates of survival, fertilization, and development of cryopreserved oocytes[12].

The main biophysical factor affecting oocyte survival and subsequent embryonic development during and after cryopreservation is the intracellular ice crystal formation that generally pierces the membrane. This causes lysis, and breaks the meiotic spindle resulting in chromosomal aneuploidy. Vitrification is a process that produces a glass-like solidification of living cells that completely avoids ice crystallization during cooling and warming[13,14]. In a previous study[15-19], the effect of vitrification with ethylene glycol (EG)-based cryoprotectants on oocytes in open pulled straws (OPS) was examined. They found high post thawed survival and subsequent embryo development. The disadvantage of vitrification by using OPS is that oocytes in the vitrification solution directly contact with liquid nitrogen during cooling or storage. To protect the risk of liquid nitrogen contamination, the present study was conducted to develop an effective vitrification method by using needle and compare the survival of mouse oocytes and fertilization rate to OPS.

Correspondence to : Pornwiroon S, Department of Obstetrics and Gynecology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.

Material and Method

Preparation of oocytes

Female C57B/6J mice aged 7-8 weeks were induced to superovulate by intraperitoneal (i.p.) injection of 10 IU of pregnant mare's serum gonadotrophin (Sigma, St Louis, MO, USA). Fifty hours later, these female mice were injected intraperitoneally with 10 IU of human chorionic gonadotrophin (Organon, Oss, Netherlands) to trigger ovulation. Eighteen hours later,

the oviducts were excised, and the cumulus-oocyte complexes were collected in 0.1% Hepes buffered Earle's balanced salt solution containing 0.1% human serum albumin (HSA; Sigma) and 0.33 M pyruvate (GIBCO, NY, USA). The granulosa cells of the oocytes were removed by pipetting in Hepes buffered flushing medium containing 80 IU/ml hyaluronidase (Sigma) and washing. The mature oocytes with the first polar body were collected for the experiments. They were cultured with bicarbonate buffered Earle's balanced salt solution containing 0.5% human serum albumin (HSA; Sigma) and 0.33 M pyruvate (GIBCO, NY, USA)[20] in an atmosphere of 5% CO_2 in air at 37 C. The oocytes were randomly allocated to two groups including vitrification using OPS and vitrification using needle.

Preparation of pretreatment, vitrification and dilution solutions

The solutions for pretreatment, vitrification, and dilution were prepared using Dulbecco's phosphate-buffered saline (DPBS) (Gibco, Grand Island, NY, USA) plus 20% fetal bovine serum. The pretreatment solution contained 1.5 mol/l ethylene glycol (Sigma). The vitrification solution consisted of 5.5 mol/l ethylene glycol and 1.0 mol/l sucrose (EG5.5)[21].The solutions for dilution were made of 0.5, 0.25 and 0.125 mol/l sucrose.

Manufacture of open pulled straws

Conventional straws were heat-softened over a Bunsen burner and pulled manually. The straws were cooled in air and then cut at the tapering end with a blade. The inner diameter of the tip decreased from 1.7 mm to 0.8 mm, and the wall thickness diminished from 0.15 to 0.07 mm[18].

Vitrification of oocytes in open pulled straws

The oocytes were pretreated with 1.5 mol/l ethylene glycol 5 mins and then mixed with EG5.5 within 1 min. Loading into the tip of the OPS was done by means of the capillary effect by simply touching a microdrop (1-2 l) of vitrification solution containing 2 oocytes. Plunging the OPS into liquid nitrogen achieved rapid cooling[18].At warming stage, the OPS was held in the air for 5 seconds, and the tip was put into a drop (400 l) of 0.5 mol/l sucrose. The vitrification medium became liquefied, and the oocytes were expelled from the OPS. They were then diluted and incubated.

Manufacture of needle

Needle No.18 G x 1" (Nipro, Japan) pierce to the top of cryovial, junction between plastic and needle were separated and then proximal end of the needle was packed with critoseal cat. no.8889-215003, Oxford, USA).

Vitrification of oocytes in needles

The oocytes were pretreated with 1.5 mol/l ethylene glycol 5 mins and then mixed with EG5.5. They were then transferred into the tip of the needle in very small amounts. The microdrop (1-2 l) of vitrification solution contained oocytes. Plunging the needle into pre-cooled cryovial and in liquid nitrogen then locked to achieve rapid cooling. Upon warming, the needles were held in the air for 5 seconds, and the tip was put into a drop (400 l) of 0.5 mol/l sucrose. The vitrification medium became liquefied, and the oocytes were expelled from the needles. They were then diluted and incubated.

Definition of morphological survival

Oocytes were defined as having morphologically survived if the cells possessed an intact zona pellucida and plasma membrane and refractive cytoplasm. They were counted and recorded.

In-vitro fertilization and culture

Spermatozoa were obtained from mature C57B/6J male mice. The vas deferens and cauda epididymides were dissected, and the spermatozoa were released into 1 ml pre-equilibrated TYH medium for 15min at 37 C[22]. After dispersion, the concentration was adjusted to a final value of 1-2x10^6 spermatozoa/ml. The insemination dishes were then incubated for 2 h to capacitate spermatozoa before the addition of oocytes. The control oocytes and surviving oocytes of the two treatment groups were transferred into the insemination medium. After 4 h of culture with spermatozoa, the oocytes were washed and then cultured in bicarbonate buffered Earle's balanced salt solution containing 0.5% human serum albumin (HSA; Sigma) and 0.33 M pyruvate (GIBCO, NY, USA). Oocytes were judged to be fertilized by the presence of two uniform blastomeres with two definite nuclei and a second polar body at 24 h after insemination[23].

Statistical analysis

The morphological survival rates of oocytes in the two treatment groups were calculated. The percentages of fertilization were compared. The 95% confidence interval analysis was used for statistical comparisons.

Results

Meiosis II Oocytes obtained from each female C57B/6J mouse aged 7-8 weeks was equally and randomly divided into two groups.There was 432 mouse oocytes. The morphological survival rates of vitrified mouse oocytes using OPS and needles are shown in Table 1. There was no obvious difference between the morphological survival rates of vitrified mouse oocytes using OPS and needles (66.7% vs 64.8%). Proportions Difference 1.9% (95%CI -7.1, 10.7).

The outcome of IVF and embryonic development of vitrified oocytes are shown in Table 2. The vitrified oocytes from the needle had significantly higher percentages of fertilization rate than OPS (76.8% vs 62.5%). Proportions Difference -14.3% (95%CI -24.5, -3.6).

Discussion

Vitrification is a procedure that requires a high concentration of cryoprotectant and an elevated cooling for no ice crystal formation, which is one of the major causes of cryoinjury. For this reason, vitrification has emerged as a possible alternative. Novel techniques and a variety of different types of cryocontainers to improve the results of oocyte and embryo freezing have been developed.

Vitrification of mammalian oocytes using the method of OPS was developed by Vajta in 1998 for vitrification of bovine oocytes and reported a favorable capacity for development[18]. However, the survival ability of oocytes is not clear. It is considered that a portion of oocytes in OPS might come into contact directly with liquid nitrogen. This may have an adverse effect on their survival and risk liquid nitrogen contamination.

In the present study, the authors developed a technique that avoids direct contact between liquid nitrogen and the oocytes. In practice, any technology in reproductive biology and especially in a therapeutic medical approach must ensure and guarantee the full protection of biological objects from microorganisms[24,25]. Liquid nitrogen, which is used for storage of frozen material, can be a source of contamination by these microorganisms[26,27]. Filtration or ultraviolet treatment of liquid nitrogen cannot guarantee the absence of contamination of biological material by virus. Different types of simple and cryo-stable viruses may increase their virulence after direct plunging and storage in liquid nitrogen, like hepatitis virus[28], papova virus[29], vesicular stomatitis virus[30] and herpes virus[31].

Two previous studies[32,33] found that vitrified mouse oocytes from OPS had morphological survival 62% and 63% respectively. In the present study, vitrified mouse oocytes from OPS and needle had morphological survival rate of 66.7% and 62.5% and fertilization rate of 64.8% and 76.8% respectively. It is possible that direct contact with liquid nitrogen on a portion of the oocytes in OPS may have a negative effect on the meiotic spindle that results in lower fertilization. This deserves further study.

The advantages of needles beyond OPS other than preventing liquid nitrogen contamination are the

Table 1. Morphological survival rate of mouse oocytes after vitrification using open pulled straws (OPS) and needles (n = 432)

Method	Oocytes	Intact morphology
OPS	216	144 (66.7)*
Needles	216	140 (64.8)*
Proportions Difference	1.9 %	95%CI [-7.1,10.7]

* Values in parentheses are percentages

Table 2. IVF and fertilization rate of mouse oocytes after vitrification using OPS and needles

Method	Oocytes	Fertilization 2-cells
OPS	144	90 (62.5)*
Needles	140	107 (76.8)*
Proportions Difference	-14.3 %	95%CI [-24.5,-3.6]

* Values in parentheses are percentages

price as it is cheaper and simpler to manufacture. Further studies are needed to improve the survival rate of oocytes vitrified in needles and study cleavage and blastocyst rate before applying to use for human oocytes.

Conclusions

Vitrification method of mouse oocytes using needles provides a similar morphological survival rate and higher fertilization rate than open pulled straws.

Acknowledgements

This study was funded by the Faculty of Medicine Siriraj Hospital.

References

1. Mandelbaum J, Belaisch-Allart J, Junca AM, Antoine JM, Plachot M, Alvarez S, et al. Cryopreservation in human assisted reproduction is now routine for embryos but remains a research procedure for oocytes. Hum Reprod 1998; 13(Suppl 3) 161-74.
2. Porcu E, Fabbri R, Seracchioli R, Ciotti PM, Magrini O, Flamigni C. Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. Fertil Steril 1997; 68: 724-6.
3. Emiliani S, Van den BM, Vannin AS, Biramane J, Englert Y. The outcome of cryopreserved human embryos after intracytoplasmic sperm injection and traditional IVF. J Assist Reprod Genet 1999; 16: 405-9.
4. Moller CC, Wassarman PM. Characterization of a proteinase that cleaves zona pellucida glycoprotein ZP2 following activation of mouse eggs. Dev Biol 1989; 132: 103-12.
5. Trounson A, Kirby C. Problems in the cryopreservation of unfertilized eggs by slow cooling in dimethyl sulfoxide. Fertil Steril 1989; 52: 778-86.
6. Vincent C, Pickering SJ, Johnson MH. The hardening effect of dimethylsulphoxide on the mouse zona pellucida requires the presence of an oocyte and is associated with a reduction in the number of cortical granules present. J Reprod Fertil 1990; 89: 253-9.
7. Bos-Mikich A, Wood MJ, Candy CJ, Whittingham DG. Cytogenetical analysis and developmental potential of vitrified mouse oocytes. Biol Reprod 1995; 53: 780-5.
8. Hotamisligil S, Toner M, Powers RD. Changes in membrane integrity, cytoskeletal structure, and developmental potential of murine oocytes after vitrification in ethylene glycol. Biol Reprod 1996; 55: 161-8.
9. Le Gal F, Massip A. Cryopreservation of cattle oocytes: effects of meiotic stage, cycloheximide treatment, and vitrification procedure. Cryobiology 1999; 38: 290-300.
10. Van der EJ, Van den AE, Jacobs R, Wisse E, Van Steirteghem A. Effect of 1,2-propanediol and dimethylsulphoxide on the meiotic spindle of the mouse oocyte. Hum Reprod 1988; 3: 960-7.
11. Sathananthan AH, Ng SC, Trounson AO, Bongso A, Ratnam SS, Ho J, et al. The effects of ultrarapid freezing on meiotic and mitotic spindles of mouse oocytes and embryos. Gamete Res 1988; 21: 385-401.
12. Gook DA, Schiewe MC, Osborn SM, Asch RH, Jansen RP, Johnston WI. Intracytoplasmic sperm injection and embryo development of human oocytes cryopreserved using 1,2-propanediol. Hum Reprod 1995; 10: 2637-41.
13. Fahy GM. Vitrification. In: McGrath JJ, Diller KR, editors. Low temperature biotechnology: emerging applications and engineering contributions. New York: American Society of Mechanical Engineers; 1988: 313-46.
14. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. Nature 1985; 313: 573-5.
15. Vajta G, Kuwayama M, Booth PJ, Holm P, Greve T, Callesen H. Open Pulled Straw (OPS) vitrification of cattle oocytes. Theriogenology 1998; 49: 176.
16. Hurtt AE, Squires EL, Seidel GE, Revista GE. Vitrification of equine and bovine oocytes in an ethylene glycol, ficoll and sucrose using open pulled straws. Theriogenology 1999; 51: 166.
17. Hurtt AE, Landim-Alvarenga F, Seidel GE Jr, Squires EL. Vitrification of immature and mature equine and bovine oocytes in an ethylene glycol, ficoll and sucrose solution using open-pulled straws. Theriogenology 2000; 54: 119-28.
18. Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, et al. Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol Reprod Dev 1998; 51: 53-8.
19. Vieira AD, Mezzalira A, Barbieri DP, Lehmkuhl RC, Rubin MI, Vajta G. Calves born after open pulled straw vitrification of immature bovine oocytes. Cryobiology 2002; 45: 91-4.
20. Purdy JM. Methods for fertilization and embryo culture in vitro. In: Edwards RG, Purdy JM, editors. Human conception in vitro. London: Academic Press; 1995: 135.

21. Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol Reprod 1996; 54: 1059-69.
22. Toyoda Y, Yokoyama M, Hosi T. Studies on the fertilization of mouse eggs in vitro. I. In vitro fertilization of eggs by fresh epididymal sperm. Jpn J Anim Reprod 1971; 16: 147-51.
23. Fraser LR, Drury LM. The relationship between sperm concentration and fertilization in vitro of mouse eggs. Biol Reprod 1975; 13: 513-8.
24. Gardner DK. Development of serum-free media for the culture and transfer of human blastocysts. Hum Reprod 1998; 13 Suppl 4: 218-25.
25. Bielanski A, Bergeron H, Lau PC, Devenish J. Microbial contamination of embryos and semen during long term banking in liquid nitrogen. Cryobiology 2003; 46: 146-52.
26. Tedder RS, Zuckerman MA, Goldstone AH, Hawkins AE, Fielding A, Briggs EM, et al. Hepatitis B transmission from contaminated cryopreservation tank. Lancet 1995; 346: 137-40.
27. Bielanski A, Nadin-Davis S, Sapp T, Lutze-Wallace C. Viral contamination of embryos cryopreserved in liquid nitrogen. Cryobiology 2000; 40: 110-6.
28. Hawkins AE, Zuckerman MA, Briggs M, Gilson RJ, Goldstone AH, Brink NS, et al. Hepatitis B nucleotide sequence analysis: linking an outbreak of acute hepatitis B to contamination of a cryopreservation tank. J Virol Methods 1996; 60: 81-8.
29. Charles CR, Sire DJ. Transmission of papovavirus by cryotherapy applicator. JAMA 1971; 218: 1435.
30. Schaffer TW, Everen J, Silver GH, Came PE. Biohazard: virus contaminated liquid nitrogen. Science 1976; 192: 25-6.
31. Jones SK, Darville JM. Transmission of virus particles by cryotherapy and multi-use caustic pencils: a problem to dermatologists? Br J Dermatol 1989; 121: 481-6.
32. Chen SU, Lien YR, Chen HF, Chao KH, Ho HN, Yang YS. Open pulled straws for vitrification of mature mouse oocytes preserve patterns of meiotic spindles and chromosomes better than conventional straws. Hum Reprod 2000; 15: 2598-603.
33. Chen SU, Lien YR, Cheng YY, Chen HF, Ho HN, Yang YS. Vitrification of mouse oocytes using closed pulled straws (CPS) achieves a high survival and preserves good patterns of meiotic spindles, compared with conventional straws, open pulled straws (OPS) and grids. Hum Reprod 2001; 16: 2350-6.

การแช่แข็งไข่หนูด้วยการลดอุณหภูมิอย่างรวดเร็วโดยการใช้หลอดแช่แข็งไข่เปรียบเทียบกับเข็ม

สุรชัย พรวิรุฬห์, สมบูรณ์ คุณาธิคม, อรวรรณ เมฆมหรรณพ์, รัตนาภรณ์ หัวนะราษฎร์

วัตถุประสงค์: *จุดประสงค์ในการศึกษาครั้งนี้ เพื่อที่จะศึกษาเปรียบเทียบอัตราการอยู่รอดและปฏิสนธิของไข่หนูที่ผ่านกระบวนการแช่แข็งด้วยการลดอุณหภูมิอย่างรวดเร็วโดยเปรียบเทียบการแช่แข็งระหว่างการใช้หลอดแช่แข็งไข่และเข็ม*

วัสดุและวิธีการ: *ได้ทำการศึกษาโดยนำไข่ระยะไมโอซิสที่ 2 จากหนูเพศเมียพันธุ์ C57B/6J อายุ 7-8 สัปดาห์มาสุ่มแบ่งเป็น 2 กลุ่ม โดยนำไปแช่แข็งด้วยวิธีการลดอุณหภูมิอย่างรวดเร็วโดยใช้อุปกรณ์ในการแช่แข็งต่างกันคือการใช้หลอดแช่แข็งไข่และเข็มจากนั้นนำไข่ที่ผ่านกระบวนการแช่แข็งมาละลายแล้วทำการศึกษาอัตราการอยู่รอดและการปฏิสนธิ*

ผลการศึกษา: *จากจำนวนไข่หนูที่ทำการศึกษาทั้งหมด 432 ฟอง พบว่าอัตราการอยู่รอดของไข่หนูที่ผ่านการแช่แข็งด้วยการลดอุณหภูมิอย่างรวดเร็วโดยใช้หลอดแช่แข็งไข่ไม่มีความแตกต่างกันอย่างมีนัยสำคัญเมื่อเทียบกับการใช้เข็ม โดยในกลุ่มที่ใช้หลอดแช่แข็งไข่มีอัตราการอยู่รอด 144 ฟองและกลุ่มที่ใช้เข็มอัตราการอยู่รอด 140 ฟองจากจำนวนไข่หนู 216 ฟอง (95%CI -7.1, 10.7) และพบว่าอัตราการปฏิสนธิของไข่หนูในกลุ่มที่ใช้เข็มสูงกว่าในกลุ่มที่ใช้หลอดแช่แข็งไข่อย่างมีนัยสำคัญ โดยมีการปฏิสนธิในกลุ่มที่ใช้เข็ม 107 ฟองจากจำนวนไข่หนู 140 ฟองและในกลุ่มที่ใช้หลอดแช่แข็งไข่ 90 ฟองจากจำนวนไข่หนู 144 ฟอง (95%CI -24.5, -3.6)*

สรุป: *การแช่แข็งไข่หนูด้วยวิธีการลดอุณหภูมิอย่างรวดเร็วโดยใช้เข็มเป็นภาชนะในการเก็บไข่นั้นเมื่อเปรียบเทียบกับวิธีเดิมคือการใช้หลอดแช่แข็งไข่ (open pulled straws) พบว่ามีอัตราการอยู่รอดของไข่โดยดูจากลักษณะรูปร่างใกล้เคียงกันและมีอัตราการปฏิสนธิสูงกว่า*