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[5,13]. 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.

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% CO2 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. 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 needle. 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 15 min 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>
<thead>
<tr>
<th>Method</th>
<th>Oocytes</th>
<th>Intact morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPS</td>
<td>216</td>
<td>144 (66.7)*</td>
</tr>
<tr>
<td>Needles</td>
<td>216</td>
<td>140 (64.8)*</td>
</tr>
<tr>
<td>Proportions Difference</td>
<td>1.9 %</td>
<td>95%CI [-7.1,10.7]</td>
</tr>
</tbody>
</table>

* Values in parentheses are percentages

<table>
<thead>
<tr>
<th>Method</th>
<th>Oocytes</th>
<th>Fertilization 2-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPS</td>
<td>144</td>
<td>90 (62.5)*</td>
</tr>
<tr>
<td>Needles</td>
<td>140</td>
<td>107 (76.8)*</td>
</tr>
<tr>
<td>Proportions Difference</td>
<td>-14.3 %</td>
<td>95%CI [-24.5,-3.6]</td>
</tr>
</tbody>
</table>

* 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

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.
การแช่แข็งไข่ด้วยการลดอุณหภูมิอย่างรวดเร็วโดยการใช้หลอดแช่แข็งไข่เปรียบเทียบกับเข็ม

สุรชัย พวิรุฬห์, สมบูรณ์ คุณาธิคม, อาวรม แม่ยวนทรพ์, รัตนาภรณ์ หัวเรำกุล

วัตถุประสงค์: จุดประสงค์ในการศึกษาครั้งนี้ เพื่อที่จะศึกษาเปรียบเทียบการอยู่รอดและปฏิสนธิของไข่หนูที่ผ่านกระบวนการแช่แข็งไข่ด้วยการลดอุณหภูมิอย่างรวดเร็วโดยใช้หลอดแช่แข็งไข่และเข็มวัสดุและวิธีการ: ได้ทำการศึกษาโดยนำไข่ระยะไมโอซิสที่ 2 จากหนูเพศเมียพันธุ์ C57B/6J อายุ 7-8 สัปดาห์มาสุ่มแบ่งเป็น 2 กลุ่ม โดยนำไข่มาแช่แข็งด้วยวิธีการลดอุณหภูมิอย่างรวดเร็วโดยใช้หลอดแช่แข็งไข่อย่างน้อย 216 ฟอง พร้อมทั้ง grupo ที่ใช้เข็มแช่แข็งด้วยวิธีการลดอุณหภูมิอย่างรวดเร็วโดยใช้หลอดแช่แข็งไข่ 144 ฟอง โดยการลดอุณหภูมิจะอยู่ในกลุ่มที่ใช้เข็ม และกลุ่มที่ใช้หลอดแช่แข็งไข่ส่วนใหญ่จะอยู่ในกลุ่มที่ใช้เข็มและกลุ่มที่ใช้หลอดแช่แข็งไข่ 107 ฟอง จากจำนวนไข่ 140 ฟอง และในกลุ่มที่ใช้หลอดแช่แข็งไข่ 90 ฟองจากจำนวนไข่ 144 ฟอง (95% CI -24.5, -3.6)

สรุป: การแช่แข็งไข่ด้วยวิธีการลดอุณหภูมิอย่างรวดเร็วโดยใช้หลอดแช่แข็งไข่เปรียบเทียบกับการแช่แข็งไข่ด้วยวิธีการแช่แข็งไข่ด้วยหลอดแช่แข็งไข่ (open pulled straws) พบว่ามีความแตกต่างกับกันอย่างมีนัยสำคัญมากที่เกิดจากการแช่แข็งไข่ด้วยหลอดแช่แข็งไข่ 107 ฟองจากจำนวนไข่ 140 ฟอง และในกลุ่มที่ใช้หลอดแช่แข็งไข่ 90 ฟองจากจำนวนไข่ 144 ฟอง (95% CI -24.5, -3.6)