冷凍卵子於人類生殖醫學之應用研究

計畫編號：NSC 88-2314-B-002-393

執行期限：87 年 8 月 1 日至 88 年 7 月 31 日

主持人：陳思原　執行機構：台大醫學院　執行單位：婦產科

中文摘要

冷凍卵子對於不孕症的治療有其潛在之重要價值，它可提供因為放射線、或化學治療可能失去卵巢機能的病人，或目前仍不適合懷孕的婦女保存卵子的機會，也可能提供卵子銀行，幫助需要卵子捐獻的病患。然而卵子冷凍解凍之存活率、受精率仍不高，因此經由冷凍卵子、解凍、受精、而植入胚胎成功懷孕的例子並不多。因此如果能解決這些問題，將可有效的應用於臨床幫助病人。目前各家學者所使用的冷凍方法、解凍方法不盡相同，究竟哪一種是最好仍然不清楚；卵子冷凍解凍後可能造成紡錘體及細胞質周圍顆粒的改變，而造成低的受精率及分裂率。本研究應用 ICSI 病人未成熟的卵子，他們已有多顆正常受精之胚胎，原本將丟棄不用的卵子，做一系列玻璃化冷凍卵子的研究，嘗試找尋最好的冷凍、解凍方法以提高卵子存活率，結果發現用 ethylene glycol 冷凍保護劑，以傳統的麥管做玻璃化冷凍，可達高存活率、受精率、早期胚胎分裂率，但繼續成長至囊胚期仍有障礙，需進一步的研究。

關鍵詞：冷凍卵子，玻璃化冷凍，人類卵子。

Abstract

Two hundred and forty-six immature oocytes from 110 patients undergoing intracytoplasmic sperm injection (ICSI) treatments were recruited. The immature oocytes were incubated in vitro that 180 oocytes reaching the metaphase II (MII) stage were collected. In experiment 1, the MII oocytes (n=84) were randomly pretreated with 1.5 M EG (EG1.5) for 0, 5, or 10 minutes, and then exposed to 5.5 M EG and 1.0 M sucrose (EG5.5) for 90 seconds and loaded in straws. The straws were plunged into liquid nitrogen. The vitrified oocytes were warmed and diluted in four steps with 1.0, 0.5, 0.25, and 0.125 M sucrose, for 2.5 minutes in each solution. For experiment 2, the oocytes (n=60) were pretreated with EG1.5 for 5 minutes, and then vitrified with EG5.5 in 60, 90, or 120 seconds, and diluted in four steps. In experiment 3, the oocytes (n=36) were pretreated with EG1.5 for 5 minutes, vitrified with EG5.5 in 60 seconds and diluted in three (0.5, 0.25, and 0.125 M sucrose) or four steps. The oocytes (n=18) without any treatment were used as controls. They were inseminated by ICSI and cultured in vitro in the following days. The survival rates after warming were significantly greater for oocytes pretreated with EG1.5 (65% for 0 minute, 93% for 5 minutes, and 96% for 10 minutes). The oocytes vitrified in 60 and 90 seconds had a significantly

greater rate of fertilization (63% vs. 58%) than those vitrified in 120 seconds (20%). There were no differences in survival and fertilization for vitrified oocytes diluted by three or four steps. The cleavage rates of embryos to the six- to eight-cell stage (40% vs. 38%) were comparable to that of the control group (58%). However, no blastocyst formation was observed in any vitrified oocytes, but one (8%) developed from the control oocytes. Vitrification of human oocytes with EG in straws achieves a high rate of survival, fertilization and early cleavage of embryos. Further studies should be conducted for the improvement of subsequent development and blastocyst formation.

Key Words: ethylene glycol, human oocytes, straw, vitrification

Introduction and purpose

The cryopreservation of human oocytes would make a significant contribution to infertility treatments, including its use for oocyte donation and for patients about to lose ovarian function from surgery, chemotherapy or radiotherapy. It also provides an alternative to embryo preservation for the avoidance of ethical problems. However, only a few successful pregnancies have arisen from cryopreserved human oocytes. This situation has been primarily attributed to poor survival, fertilization, and development of cryopreserved human oocytes. Therefore, improving the viability of cryopreserved human oocytes is an important topic in infertility treatment.

Hunter et al. performed vitrification of human oocytes using mixed cryoprotectants of dimethyl sulfoxide, propanediol, ethylene glycol and, acetamide, similar to the protocol for mouse embryo vitrification by Rall and Fahy, and achieved a survival rate of 65% and a fertilization rate of 45%. However, no cleavage of zygotes was observed. Recently, ethylene glycol (EG) combined with sucrose has been extensively studied in mammals as a cryoprotectant for the vitrification of oocytes and embryos because of its low toxicity and rapid permeation of the cell. It is thought that vitrification is superior to the conventional slow freezing method for bovine oocytes that are very sensitive to chilling. The purpose of our study was to examine the effect of vitrification with EG for human oocytes in conventional straws.

Materials and methods

A total of 1220 cumulus-corona-oocyte complexes were retrieved in 110 ICSI cycles. After the removal of granulosa cells, 927 (76%) oocytes were found to be at the metaphase II (MII) stage, 135 (11%) at the metaphase I (MI) stage, 111 (9%) at the germinal vesicle (GV) stage, and 47 (4%) were degenerative. MII oocytes with the first polar body were selected for ICSI. The

MI oocytes were incubated in vitro for 18 hours and the GV oocytes for 24 hours with human tubal fluid medium supplemented with 15% heat-inactivated maternal serum. Overall, 108 MII oocytes were recovered from the MI stage (80%) and 90 from the GV stage (81%) for this study.

Pretreatment and Vitrification Solutions

The solutions for pretreatment, vitrification, and dilution were prepared using Dulbecco's phosphate-buffered saline plus 20% fetal cord serum. The pretreatment solution contained 1.5 M EG (EG1.5). The vitrification solution consisted of 5.5 M EG and 1.0 M sucrose (EG5.5).

Experiment 1 for Assessment of the Effect of Pretreatment

The MII oocytes (n=84) were randomly allocated to be pretreated with EG1.5 for 0, 5, or 10 minutes. The oocytes not treated or pretreated with EG1.5 were transferred with minimal medium into a drop (200 μL) of EG5.5 on a Petri dish and mixed for equilibration. They were transferred to another drop (200 μL) and loaded in a 0.25-mL plastic straw. The procedures were performed at a room temperature of 22 to 24 °C. The straw was plunged into liquid nitrogen in 90 seconds since the oocytes contacted with EG5.5. After storage for 1 to 5 days, the straw was taken out of the liquid nitrogen and held in the air for 5 seconds. It was then plunged into water at room temperature for 10 seconds. The content containing oocytes was expelled into a drop (400 μL) of 1.0 M sucrose on a Petri dish. The oocytes were then transferred into 1.0, 0.5, 0.25, and 0.125 M sucrose in a four-well dish, for 2.5 minutes in each solution. Finally, the oocytes were washed and transferred into the culture medium. ICSI was performed 60 minutes after the dilution of oocytes.

Experiment 2 for Assessment of the Time of Exposure to Vitrification Solution

The oocytes (n=60) were pretreated with EG1.5 for 5 minutes and then exposed to EG5.5 for 60, 90, or 120 seconds. They were cooled, warmed, and diluted as described in the first experiment.

Experiment 3 for Assessment of the Steps of Dilution

The oocytes were pretreated with EG1.5 for 5 minutes and vitrified with EG5.5 in 60 seconds. They were warmed and diluted in three (n=18) or four steps (n=18). For the three-step dilution, the oocytes in straws were expelled into a drop (400 μL) of 0.5 M sucrose and then transferred to 0.5, 0.25, and 0.125 M sucrose, for 2.5 minutes in each solution. The oocytes (n=18) without any treatment were used as controls.

Results

The survival rate of vitrified oocytes after warming and dilution was significantly greater in the pretreatment

groups than in that without pretreatment. It was not different between groups with 5 and 10 minutes of pretreatment. After ICSI, the rates of damage, normal fertilization, and abnormal fertilization were similar among the three groups. The percentages of early cleavage to the stages of two to four and six to eight cells were also not different. No morula or blastocyst developed from these vitrified oocytes.

The survival rates after warming and dilution were not different among the three groups. The damage rates after ICSI were similar. The oocytes exposed to EG5.5 for 60 or 90 seconds had significantly greater rates of fertilization than that exposed for 120 seconds. The cleavage rate of zygotes appeared lower in the 120-second group. The cleavage rate for oocytes exposed for 60 seconds tended to be higher than that for those exposed for 90 seconds, but the difference was not significant. The embryos arrested at the stage of six to eight cells.

There was no difference in survival for vitrified oocytes between three- and four-step dilutions. The rates of damage after ICSI, fertilization, and cleavage were not different between the two groups. The percentages of normal fertilization and early cleavage of zygotes from vitrified oocytes were lower than those of non-vitrified oocytes, but the difference was not significant. No blastocyst formation was observed from the vitrified oocytes, and one blastocyst developed from the control oocytes.

Discussion

The present study demonstrated a high survival rate of cryopreserved human oocytes vitrified with 5.5 M EG and 1.0 M sucrose in straws. This morphological survival rate appeared to be greater than that of the conventional slow freezing method reported in the literature. The rates of normal fertilization and early cleavage of vitrified oocytes were comparable to those of the control. However, the subsequent development and blastocyst formation seemed impaired. Nonetheless, considering the high rate of morphological survival and early development of vitrified oocytes, further studies are warranted to improve the growth potential of vitrified human oocytes.

One-step vitrification with brief exposure to a very concentrated cryoprotectant was successfully used for the cryopreservation of bovine oocytes by Martino et al.. Otoi et al. found that the three-step addition of cryoprotectants was superior to one-step vitrification for the survival of cryopreserved bovine oocytes. In the vitrification of human oocytes, we found that oocytes pretreated with EG1.5 had a significantly higher survival rate than those without pretreatment. This indicates that the permeation of EG in the pretreatment enhances intracellular

vitrification. That would reduce the time needed for exposure to a vitrification solution of EG5.5 that could be toxic for oocytes.

In this study, we found that oocytes exposed to EG5.5 for 60 or 90 seconds prior to cooling had a higher rate of fertilization and cleavage than those exposed for 120 seconds. This indicated that prolonged exposure to the concentrated cryoprotectant might have induced a toxic effect. Whether shortening the time of pretreatment and exposure to vitrification solution can improve the growth potential and preserve the survival rate of vitrified oocytes deserves further research.

The vitrified oocyte is sensitive to osmotic changes after warming. A stepwise dilution is usually used to prevent excessive swelling and lysis of the oocyte as the cryoprotectant leaves the cell. Using the same vitrification solution of EG5.5, Martino et al. diluted vitrified bovine oocytes in three steps, from 0.5 to 0.25 to 0.125 M sucrose. Hong et al. performed a four-step dilution for vitrified human oocytes and found a better growth potential for oocytes kept 2.5 minutes in each step than those kept 5 minutes in each step. In our study, there was no difference in survival and growth capacity for vitrified human oocytes after three- or four-step dilution.

References

1. Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultrarapid cooling. Biol Reprod 1996;54:1059-69.
2. Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H. Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol Reprod Dev 1998;51:53-8.
3. Otoi T, Yamamoto K, Koyama N, Tachikawa S, Suzuki T. Cryopreservation of mature bovine oocytes by vitrification in straws. Cryobiology 1998;37:77-85.
4. Hunter JE, Fuller BJ, Bernard A, Jackson A, Shaw RW. Vitrification of human oocytes following minimal exposure to cryoprotectants; initial studies on fertilization and embryonic development. Hum Reprod 1995;10:1184-8.
5. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at −196 °C by vitrification. Nature 1985;313:573-5.
6. Hong SW, Chung HM, Lim JM, Ko JJ, Yoon TK, Yee B, et al. Improved human oocyte development after vitrification: a comparison of thawing methods. Fertil Steril 1999;72:142-6.