行政院國家科學委員會補助專題研究計畫成果報告

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計畫類別：個別型計畫

計畫編號：NSC 90-2314-B-002-458

執行期間：90 年 8 月 1 日至 91 年 7 月 31 日

計畫主持人：陳思原醫師

共同主持人：楊友仕教授

計畫參與人員：王菱霙助理

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□赴國外出差或研習心得報告一份

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□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：台大醫學院婦產科

中 華 民 國 91 年 11 月 2 日

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中文摘要

近年來囊胚植入為不孕症夫婦進行試管嬰兒治療的一種重要治療方式。而植入後剩下的胚胎，如果能有效的冷凍起來，對於病人將有很大的幫助。最近在哺乳類動物的囊胚冷凍有進一步的發展，發現應用玻璃化冷凍可以達到高的存活率及懷孕率。囊胚和較早期的胚胎明顯不同之處在於有一囊胚腔，此一腔室較不易脫水，不同發育時期大小亦不同，是否會影響冷凍的結果，值得進一步探討。本研究應用老鼠囊胚做玻璃化冷凍之研究，探討不同發育時期，包括小、中、大囊胚對於存活的影響。結果發現愈大愈不易存活，而先用顯微吸取囊胚腔液可提高大囊胚之存活率。

關鍵詞：囊胚冷凍，老鼠，玻璃化冷凍。

Abstract

Cryopreservation of blastocysts becomes an important issue; however, the result is still not satisfactory. Vitrification of blastocysts seems promising according to the results of bovine experiments. The blastocyst has different structures from earlier stages of embryos in that it has a fluid-filled blastocoel. The developmental stage such as cavitating, expanding, hatching, or hatched blastocysts may influence the survival of vitrification. Which stage of blastocysts has the highest survival from vitrification needs further studies. In this mice study, we found the size of blastocoelic cavity may influence the survival after vitrification. Micro-suction of blastocoelic fluid before vitrification improve the survival of large expanding blastocysts.

Key Words: blastocyst cryopreservation, mouse, vitrification

Introduction and purpose

Blastocyst culture using sequential medium and transfer has been recently developed to increase the implantation rate for patients undergoing in-vitro fertilization (IVF) treatments (Gardner et al., 1998). It can reduce the number of embryos transferred and multiple pregnancies. Cryopreservation of excess blastocysts turns into an important subject in the infertility treatment. Although the conventional slow freezing technique has been successfully applied for early stages of human embryos, the result for the blastocysts is still not satisfactory. Vitrification of blastocysts seems promising according to the results of some mammal experiments (Kobayashi et al., 1998; Sommerfeld et al., 1999). The value of vitrification for human blastocysts is not clear, although few successful cases have been reported

(Choi et al., 2000; Yokota et al., 2000).

Vitrification that cryoprotectants with higher concentrations turn to a glassy state during cooling and warming circumvents intracellular ice crystallization, which is a major cause of cell death from cryopreservation. Vitrification with a faster thermal change may be beneficial for embryos that are chill sensitive. The cryopreservation of blastocysts has been more difficult than that of morulae and earlier stages of embryos. The blastocyst has different structures in that it has a fluid-filled blastocoel. The permeation of cryoprotectants into the cavity essential for vitrification may be relatively insufficient. The blastocoelic cavity may be likely to be frozen, which reduces the post-warming survival rate. The developmental stage of blastocysts such as cavitating, expanding, hatching, or hatched blastocysts may influence the survival of vitrification. Which stage of blastocysts has the highest survival from vitrification needs further research.

In this project, we performed a series of experiments in mice models to investigate the effects of vitrification for blastocysts at different stages of blastocysts. The post-warming embryos are observed in vitro for further development.

Materials and methods

Preparation of embryos

Female ICR mice aged 6 to 8 weeks were induced to superovulate by intraperitoneal 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. Each female mouse was put with a male for mating. Eighteen hours later, the mating was confirmed by the presence of vaginal plug. Twenty-four hours later, oviducts were excised, and the 2-cell stage embryos were collected and cultured in human tubal fluid (HTF) medium containing 0.5% bovine serum albumin (BSA; Sigma) in an atmosphere of 5% CO_2 in air at 37 ^{o}C. The cavitating, medium expanding, and large expanding blastocysts were collected for vitrification.

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 cord serum. The pretreatment solution contained 1.5 M EG (Sigma). The vitrification solution consisted of 5.5 M EG and 1.0 M sucrose (EG5.5) (Ali et al., 1993). The solutions for dilution were made of 0.5 and 0.25 M sucrose.

Manufacture of pulled straws

The straws (I.V.M., l’Aigle, France) are heat-softened over a hot plate, and pulled manually. The straws are cooled in air, and then cut at the narrowest point

with a blade. The inner diameter and the wall thickness decrease from 1.7 mm to approximately 0.8 mm, and from 0.15 mm to approximately 0.07 mm, respectively (Chen et al., 2001).

Vitrification of embryos in CPS

The embryos (3-5 at a time) were pretreated with 1.5 M EG and then exposed to EG5.5. The tip of the pulled straw was loaded with 2 mm of vitrification medium, 2 mm of air, 2 mm of vitrification medium containing embryos, 2 mm of air, and 2 mm of vitrification medium using a syringe (Chen et al., 2001). The total exposure to EG5.5 lasted for one minute. They were then plunged into liquid nitrogen for cooling. The procedures were performed at a room temperature of 22 to 24 ^{o}C.

After storage for from one to five days, the CPS was removed from the liquid nitrogen for warming. The opposite end of the pulled straw was sealed using the index finger. The content was then expelled into a drop of 0.5 M sucrose (400 μL) by using the increasing air pressure in the tube caused by the thermal change. They are diluted in the stepwise (0.5, 0.25M) sucrose solutions 5 min for each step, and then transferred to culture medium.

Micro-suction of blastocoelic fluid

The medium or large expanding blastocysts were immobilized by the holding pipette. The area of inner cell mass was positioned at the 12-o'clock direction. The aspirating needle used for micro-suction was aimed to the blastocoel from the three-o'clock direction. The aspirating needle punctured the zona. After breaking the membrane with a negative pressure, the blastocoelic fluid was aspirated completely. The blastocoel became collapsed.

Definition of survival and observation of developmental capacity in vitro

Embryo survival was assessed as re-expanding rates at 24 and 48 h after thawing respectively.

Statistics

The morphological survival rates of blastocysts in the various groups were calculated. The Chi-square test was used for statistical comparisons. A *p* value less than 0.05 was considered significantly different.

Results

In blastocysts at various stages of development, survival rates decreased as the blastocoel enlarged for cavitating blastocysts of 92%, medium expanding blastocysts of 62%, and large expanding blastocysts of 41%. Micro-suction before vitrification significantly increased the survival rate to 88% for medium expanding blastocysts, and 83% for large expanding blastocysts.

Discussion

After vitrification and warming, the survival rate of blastocysts decreased when the blastocoelic cavity enlarged. The access of cryoprotectants into the

cavity necessary for vitrification may be inadequate for large expanding blastocysts. Micro-suction of blastocoelic fluid is a simple way to evacuate the cavity that facilitate the vitrification procedures and increase the survival rate.

Various stages of mouse oocytes and embryos have been used as models for the cryopreservation of human oocytes and embryos. Nonetheless, the species differences between mice and human should be considered. Further studies for human blastocyst should be performed before clinical application of this technique.

References

1. Kobayashi S, Takei M, Kano M, Tomita M, Leibo SP. (1998) Piglets produced by transfer of vitrified porcine embryos after stepwise dilution of cryoprotectants. Cryobiology 36:20-31.
2. Sommerfeld V, Niemann H. (1999) Cryopreservation of bovine in vitro produced embryos using ethylene glycol in controlled freezing or vitrification. Cryobiology 3:95-105.
3. Choi DH, Chung HM, Lim JM, Ko JJ, Yoon TK, Cha KY. (2000) Pregnancy and delivery of healthy infants developed from vitrified blastocysts in an IVF-ET program. Fertil Steril 74:838-9.
4. Gardner DK, Vella P, Lane M, Wagley L, Schlenker T, Schoolcraft WB. (1998) Culture and transfer of human blastocysts increases implantation rates and reduces the need for multiple embryo transfer. Fertil Steril 69:84-8.
5. Yokota Y, Sato S, Yokota M, Ishikawa Y, Makita M, Asada T, Araki Y. (2000) Successful pregnancy following blastocyst vitrification: case report. Hum Reprod 15:1802-3.
6. Chen SU, Lien YL, Cheng YY, Chen HF, Ho HN, Yang YS. (2001) Vitrification of mouse oocytes using closed pulled straws (CPS) achieves a high survival and preserves good patterns of meiotic spindles, when compared with conventional straws, open pulled straws (OPS), and grids. Hum Reprod 16:2350-6.