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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目:
Title of Dissertation　Studies on equilibrium vitrification of oocytes and embryos using low concentrations of cryoprotectants
(低濃度の耐凍剤を用いた卵子と胚の平衡ガラス化凍結法に関する研究)

学位論文要約:
Dissertation Summary

With the expansion of genetically engineered mouse models, cryopreservation technology of mouse oocytes/embryos is useful in order to reduce the mainentance cost of the large number of mouse variants. The protocol for cryopreserving oocytes/embryos can be divided into two categories: slow freezing and vitrification.

In original slow freezing, embryos are cooled at a slow rate to -70ºC to maintain the chemical potential of their intracellular water close to that of water in the partly frozen extracellular solution. Therefore, it is referred to as equilibrium slow freezing and embryos are frozen in 'a near-equilibrium state'. The embryos at -70ºC are dehydrated sufficiently and concentrated intracellular solutes and cell permeating cryoprotectants sufficiently. Therefore, they can survive rapid cooling in liquid nitrogen from -70ºC and rapid/slow warming after cryopreservation. Vitrification is a common strategy for the cryopreservation of mammalian embryos and has replaced the slow freeze method because it does not need a programmed freezer, and the protocol is simple and instant. Moreover, higher survival is expected because extracellular ice is not formed. Vitrification is the ultimate non-equilibrium cryopreservation because embryos are cooled under the condition in which ice forms neither in the cell nor in the surrounding medium. However, vitrification has some disadvantages. One is that oocytes/embryos are vitrified in an insufficient dehydrated/concentrated state compared to those cryopreserved in the original slow freezing. Therefore, samples are much more delicate and must be stored below -130ºC (the glass transition temperature of water), they must be handled carefully to prevent spontaneous crystallization, and must be warmed rapidly to prevent intracellular ice from forming. The other disadvantage is that high concentrations of permeating cryoprotectants must be used as the cryopreservation solution. Therefore, oocytes/embryos may be damaged by the toxicity of permeating cryoprotectants in the cryopreservation solution.

To overcome the first disadvantage of the vitrification method, we developed an equilibrium vitrification method using EFS35c, by which mouse embryos at multiple developmental stages can be vitrified in a high

dehydrated/concentrated state as those cryopreserved in original slow freezing and can be transported with dry ice. We called this vitrification method ‘equilibrium vitrification’. However, EFS35c contained high concentrations of permeating and nonpermeating cryoprotectants (35% (v/v) ethylene glycol (EG) and 0.98 M sucrose, respectively) with high osmolality (23.3 moles/kg) to promote dehydration of the cytoplasm, and the high toxicity and osmolality caused by high concentrations of cryoprotectants could damage embryos. In addition, the method is not effective for vitrification of mouse oocytes. Therefore, this method cannot be applied to embryos with high sensitivity to the toxicity of cryoprotectants and to osmolality.

To overcome the second disadvantage of vitrification, in the present study, we developed a new vitrification method by which oocytes/embryos are vitrified in a high dehydrated/concentrated state using solutions containing low concentrations of permeating (20% (v/v)) and non-permeating cryoprotectants (0.4 M sucrose).

First, we tried to select the optimum permeating cryoprotectants in FSa solution (PB1 medium containing 30% (w/v) Ficoll PM-70 and 0.5M sucrose) in which vitrified mouse oocytes can be vitrified and stored at -80°C for 4 days without decrease in survival. Four permeating cryoprotectants (EG, acetamide (AA), dimethyl sulfoxide (DMSO), and propylene glycol (PG)) and their mixture were examined. When mouse oocytes were vitrified in LN_2 with AFS20a (FSa solution containing 20% (v/v) AA), EAFS10/10a (FSa solution containing 10% (v/v) EG and 10% (v/v) AA), and EDFS10/10a (FSa solution containing 10% (v/v) EG and 10% (v/v) DMSO) solutions, the high rates of survival were obtained (96%, 96%, and 97%, respectively). When oocytes were vitrified with AFS20a and EAFS10/10a and kept at -80°C for 4 days, the rates of survival decreased significantly (63% and 60%, respectively). When oocytes vitrified with EDFS10/10a and kept at -80°C for 4 days, the survival rate was 96%, and was not significantly different from that of oocytes vitrified in LN_2 and warmed directly from LN_2. Therefore, EDFS10/10a appears to be the optimal vitrification solution for successful equilibrium vitrification.

Next, based on the results above, we tried to develop a reliable protocol in which 2-cell embryos can be stored at -80°C for 4-28 days without decrease in viability using EDFS10/10a. To examine the toxicity of vitrification solutions, 2-cell mouse embryos were incubated in EDFS10/10a or EFS35c at 25°C for 2-20 min. The developmental ability into blastocysts exposed to EDFS10/10a at 25°C for 10 min was high (87%) and was not significantly different from that of the fresh control (97%). On the other hand, the developmental ability into blastocysts exposed to EFS35c decreased significantly even after 2-min exposure (62%). Therefore, EDFS10/10a was less toxic than the original equilibrium vitrification solution (EFS35c). Next, we vitrified 2-cell embryos with 1-step or 2-step protocol in LN_2, and then kept at -80°C for various periods. The survival (97-100%) and the developmental ability into blastocysts (55-90%) were higher when using 2-step. Next, we examined the optimum

protocol for 2-cell embryos vitrified in LN_2 and then kept at -80°C for 7 days. When embryos were pretreated with ED5/5 (PB1 solution containing 5% (v/v) EG and 5% (v/v) DMSO) at 25°C for 2 min and then treated with EDFS10/10a at 25°C for 1 or 0.5 min, the survival (92-100%) and developmental ability into blastocysts (69-75%) were relatively high. Lastly, we examined whether 2-cell embryos vitrified with EDFS10/10a and then kept at -80°C for 4 days remain viable after being recooled with LN_2. We assessed the developmental ability by *in vivo* using C57BL/6J mice. The rates of implantation and birth of vitrified/transported/recooled embryos were high (79% and 58%, respectively). The vitrified 2-cell mouse embryos with EDFS10/10a had the ability to develop to term. Therefore, 2-cell mouse embryos can be vitrified in a high dehydrated/concentrated state using EDFS10/10a solution.

Then, based on the results in 2-cell embryos, I examined whether this method is effective for other developmental stages. When 4- and 8-cell embryos were vitrified and then kept at -80°C for 4-28 days, the survival (97-100%) and developmental ability into morulae (92-97%) were relatively high. However, the blastocyst formation slightly decreased as the keeping period at -80°C was extended (68-89%). This indicated that 4- and 8-cell embryos were vitrified in a high dehydrated/concentrated state, However, since water in the embryos was devitrified without recrystallization at -80°C, the embryos would be damaged by the toxicity of permeating cryoprotectants during a long-time storage at -80°C. When morulae were vitrified and warmed directly from LN_2, the rates of survival and blastocyst formation were quite high (100% and 90%, respectively). However, when vitrified morulae were kept at -80°C for 4-28 days, the survival markedly decreased (19-55%). Therefore, morulae were able to vitrified with this protocol but were vitrified in an insufficient dehydrated/concentrated state. When blastocysts were vitrified and warmed directly from LN_2, the ability to re-expand and to hatch were quite low (57% and 13%, respectively), and no blastocysts re-expanded after being vitrified and then kept at -80°C for 4 days. However, when blastocysts were shrunk by puncturing the blastocoel, vitrified, and then kept at -80°C for 4-28 days, the ability to re-expand (65-90%) and to hatch (35-71%) increased markedly. Therefore, blastocysts shrunken artificially were vitrified in a high dehydrated/concentrated state using this method.

Finally, we examined whether this method can be applied to mouse mature oocytes. First, we examined the optimal pretreatment time with ED5/5. When oocytes were pretreated with ED5/5 at 25°C for 1-5 min, treated with EDFS10/10a for 1 min, and vitrified in LN_2, the survival rates were high after beening kept at -80°C for 4 day (92-100%). When oocytes were pretreated at 25°C for 2 min and *in vitro* fertilized, the rates of fertilization and blastocyst formation were relatively high (80% and 71%, respectively). Next, we examined whether oocytes

with or without zona dissection before *in vitro* fertilization affect the fertilization ability aftaer being vitrified. When vitrified-warmed oocytes were not dissected the zona before *in vitro* fertilization and then inseminated, the rates of fertilization and blastocyst formation were low (2% and 0%, respectively). On the other hand, with zona dissection, the fertilization rate was high (88%) and the blastocyst formation rate was relatively high (68%). Therefore, vitrified-warmed oocytes can be fertilized and develop into blastocysts by zona dissection before *in vitro* fertilization. When oocytes were vitrified with EDFS10/10a, then kept at -80ºC for 4-28 days, and dissected the zona, the rates of survival, fertilization and blastocyst formation were also high (88-99%, 80-90% and 55-70%, respectively). These results suggest that most of the vitrified oocytes retained their ability to be fertilized, and to develop into blastocysts. However, the blastocyst formation slightly decreased as the keeping period at -80ºC was extended, suggesting that oocytes were damaged by the toxicity of permeating cryoprotectants in a devitrified vitrification solution during a long-time storage at -80ºC. Lastly, we compared the cell number in blastocysts. The cell number of blastocysts derived from oocytes vitrified and then kept at -80°C for 4 days group was not significantly different from that derived from intact oocytes (44.6±10.9 and 47.1±9.5, respectively). However, there was an overall delay in blastocyst formation. These results suggested that vitrification would reduce the development speed, but the blastocyst formation rate and cell number were not affected. Therefore, this equilibrium vitrification method can be used for mouse oocytes, without the effect on the subsequent fertilization and embryonic development.

In conclusion, the new equilibrium vitrification method developed in this study is effective for mouse oocytes and embryos at multiple stages. Our new method has several advantages as follows: 1) The vitrification medium (EDFS10/10a) contains lower concentrations of permeating and non-permeating cryoprotectants, and thus is less chemical toxicity and osmolality (6.43 moles/kg). 2) As this method does not require ultra-rapid cooling/warming, skilled techniques or minute devices (for ultra-rapid vitrification) are not need. 3) The process is instant and quick. 4) The procedure can be performed at room temperature without a programmable freezer. 5) This method is effective for oocytes, 2-, 4-, 8-cell embryos, and shrunken blastocysts. 6) Vitrified oocytes/embryos can be transported in dry ice. 7) This method can be easily applied to “closed systems” because the embryos are vitrified in a near-equilibrium state and do not require rapid warming.