Cryopreservation of Human Multi-Pronuclear (PN) Zygote by Ultra-Rapid Freezing

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인간 다-전핵기(>2PN) 수정란의 초급속 동결에 관한 연구

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본 연구는 인간 전핵기 수정란의 동결시 electron microscope grid를 사용하는 초급속 동결방법이 유용하는지 여부를 조사하고자 실시하였다. 본 실험에서는 인간 IVF 시술시 생성되는 다-전핵기(>2PN) 수정란을 정상 2PN 수정란 대신 사용하였으며, 또한 이들은 3PN과 ≥4PN 수정란으로 나누어 전핵의 수에 따른 냉해와 융해 후 체외 배발달율을 조사하였다. 동해제는 30% ethylene glycol, 18% ficoll, 0.5 M sucrose와 10% FBS등이 D-PBS에 첨가되어 제작된 EFS30을 사용하였다. 본 연구에서 얻어진 결과는 다음과 같다. 초급속동결-융해 후, 인간 다-전핵기 수정란의 생존율은 85.5% 였다. 대조군과 동결군의 난할율을 전핵의 수에 따라 나누어 비교하였을 때, 각 군간에는 유의한 차이를 나타내지 않았다 (3PN; 81.3% 와 85.4% , ≥4PN; 90.0% 와 95.7%). 또한, 융해 후 체외발달율을 조사하였던바, ≥4PN 수정란에서는 동결군 (4.5%) 의 체외발달이 대조군 (44.4%) 보다 유의하게 낮게 나타났던 반면, 3PN 수정란에서는 동결군 (22.0%) 의 결과가 대조군 (38.5%) 에 비해 유의한 차이를 나타내지 않았다 ($p<0.05$). 따라서 인간 다-전핵기 수정란은 EM grid와 EFS30 동결액을 이용한 초급속 동결로 배발달을 유도할 수 있다는 것을 알 수 있었다.

INTRODUCTION

Cryopreservation has now become an essential assisted reproduction technique in human *in vitro* fertilization (IVF) program for the patients to have the opportunity of pregnancies from more than one embryo transfer without having to be subjected to controlled ovarian hyperstimulation and oocyte retrieval each time (Abbeel *et al.*, 1997; Baker, 1997). Human embryos have been cryopreserved at the unicellular pronuclear stage, at the multicellular cleavage stages or at the blastocyst stage using different freezing protocols with either dimethylsulfoxide (DMSO), 1,2-propanediol (PROH) or glycerol as cryoprotective agents (Trounson and Mohr, 1983; Cohen *et al.*, 1988; Feichtinger *et al.*, 1991; Veek *et al.*, 1993; Kaufmann *et al.*, 1995). Also, for these developmental stages freezing method has mainly employed slow controlled-rate cooling (Friedler *et al.*, 1988). These methods require expensive equipment and are time-consuming. However, to elude these disadvantages, simpler and faster freezing methods have been developed such as vitrification (Rall and Fahy, 1985) and ultra-rapid freezing (Trounson *et al.*, 1987). In addition, several pregnancies by ultra-rapid freezing were reported in human (Trounson, 1990; Barg and Feichtinger., 1990; Gordts *et al.*, 1990; Feichtinger *et al.*, 1991). On the other hand, Martino *et al.*, (1996) demonstrated that highly developmental capacity of bovine oocytes can be obtained by a new ultra-rapid freezing method using electron microscope (EM) grids. This fact was proved in our results (Kim *et al.*, 1998a,b). Based on these data, we tested whether the developmental capacity of human multi-pronuclear (PN) zygotes after ultra-rapid freezing using EM grids can be maintained.

MATERIALS AND METHODS

1. Recovery of human multi-PN zygotes

Human abnormally fertilized oocytes were obtained from patients undergoing IVF. Briefly, the patients, after pituitary-gonadal suppression with a luteinizing hormone releasing hormone (LHRH) agonist (Buserelin, Hoechst), were stimulated with gonadotrophin (FSH/hMG or hMG alone) followed by the administration of human chorionic gonadotrophin (hCG; Humegon, Organon) for final stage of follicular maturation. Preovulatory oocytes were retrieved at 34-36 h after hCG injection, cultured and inseminated with final concentration of 1 x 10^6 sperm/㎖. PN zygote was determined 16-18 h after insemination for the presence and number of pronuclei, and polyspermic zygotes exhibiting more than two pronuclei (multi-PN zygotes) were separated from normal fertilized oocytes to be utilized for the study. Also, the multi-PN zygotes were divided into the three-PN (3PN) zygotes or more than 3PN zygotes (≥4PN).

2. Ultra-rapid freezing

The procedure used to freeze the multi-PN zygotes was the same as described in previous study (Kim *et al.*, 1998a,b). This experiment has two specific factors. Firstly, electron microscope grids (EM grids, Pelco international) were used as a physical support to achieve very high cooling rates when it was plunged into liquid nitrogen (LN_2). Secondly, as freezing solution, EFS30 which containing of 30% ethylene glycol, 18% ficoll, 0.5 M sucrose and 10% FBS added D-PBS was used. A mean number of multi-PN zygotes loaded on one grid were about 5. Freezing was undertaken at 22-24 h after insemination. The total time that elapsed from the immersion of multi-PN zygotes to cryoprotectants to the plunge of grid loaded multi-PN zygotes into LN_2 was about 30 sec.

3. Thawing and *in vitro* culture

For the thawing, cryoprotectants were removed by 3-step procedures at 37℃. The grids were transferred as soon as possible rapidly into 0.5 M sucrose (S) diluted in PBS. And then they were transferred into 0.25 MS-PBS and 0.125 MS-PBS. Each step needs for 1 min. After 3 min., recovered multi-PN zygotes were washed and co-cultured in cumulus cell monolayer drop added m-CR1 medium (Park *et al.*, 1996) supplemented with 10% FBS.

4. Evaluation of frozen-thawed multi-PN zygote

To analyse the cryoinjury according to freezing and thawing of multi-PN zygotes, survival, cleavage and blastocyst formation were examined (Fig. 1). Embryo survival was defined as the percentages of recovered embryos that were morphologically intact after thawing and subsequent dilution of the cryoprotectant. Especially, passage through syngamy to the first cleavage was as an indicator of developmental potential of frozen-thawed multi-PN zygotes. Final assessment of developmental capacity in this study was determined with blastocyst formation at day 6 after IVF.

5. Statistical analysis

Difference in the rates of survival and development among treatment group was compared using the Chi-square test ($p<0.05$).

RESULTS

The survival of the human multi-PN zygotes and the corresponding development rates after ultra-rapid freezing using EM grid are shown in Table 1. When the multi-PN zygotes were ultra rapidly frozen and thawed, the survival rates were all high in two groups (3PN; 81.4% and ≥4PN; 95.8%). In PN number, cleavage rates between control and freezing group were not significantly different in each group (3PN; 81.3% & 85.4% and ≥4PN; 90.0% & 95.7%). In addition, when the *in vitro* development rates after thawing were examined on day 6 post insemination, total *in vitro* development potential of multi-PN zygotes after ultra-rapid freezing (15.9%) was indicated significantly lower than that of control multi-PN zygotes (40.9%) ($p<0.05$). However, the development result of freezing 3PN group (22.0%) was not differed to that of control 3PN group (38.5%), although freezing ≥4PN group (4.5%) was significantly lower than control ≥4PN group (44.4%) ($p<0.05$).

DISCUSSION

Since the first report on a pregnancy from a frozen-thawed human embryo (Trounson and Mohr, 1983) and the first recorded birth after such a transfer (Zeilmaker *et al.*, 1984), many IVF groups have worked out their standards for a cryopreservation program. However, the results obtained are still cleary worse than with fresh embryos or in comparison with other mammalian species. In addition, there is no doubt that there is a marked advantage of ultra-rapid freezing when compared to conventional slow freezing methods in terms of cost and time. Human embryos have been frozen ultrarapidly in media containing 2.0-3.0 M DMSO and 0.24-0.5M sucrose with high rates of survival and development *in vitro* (Trounson *et al.*, 1988; Trounson and Sjöblom, 1988).

More recently pregnancies obtained by ultra-rapid freezing were reported by Trounson (1990), Gordts *et al.* (1990), Barg and Feichtinger (1990), Feichtinger *et al.* (1991) and Lai *et al.*(1996). These results commonly demonstrated that the ultra-rapid freezing method should increasingly become the method of choice for human embryo freezing in IVF programs. However, in zygotes, ultra-rapid freezing was not proved in efficient method when compared to slow freezing method (Abbeel *et al.*, 1997). In this study, we introduced a new and viable ultra-rapid freezing method at human PN stage embryo. However, we used multi-PN zygotes produced in the results of abnormal fertilization in human IVF as an alternatives of normal 2PN embryo. Production of multi-PN zygotes is not uncommon after *in vitro* fertilization; the reported incidence of polyploidy ranges from 1% to 25% in the literature, with most programs experiencing about 5% (Rudak *et al.*, 1984; Van der Ven *et al.*, 1985; Michelmann *et al.*, 1986; Wramsby *et al.*, 1987; Dandekar *et al.*, 1990). We separated the multi-PN zygotes into the 3PN zygotes or more than 3PN zygotes ($\geq$4PN) zygotes to compare the *in vitro* development and cryoinjury according to PN number of abnormal zygotes. In previous study using a new ultra-rapid freezing method (Kim *et al.*, 1998a,b), we confirmed the developmental capacity of bovine oocytes was maintained after thawing. Especially, in mature oocytes, the blastocyst formation rate was indicated similary to control data. Also, it has known that the observation of bovine protocols can be directly transferred for human system (Menezo and Zoly, 1997).

In this study, we reconfirmed the usability of a new ultra-rapid freezing method using EM grids when it was applied to the human multi-PN zygotes. As shown in Table 1, after ultra-rapid freezing and thawing, the high survival rate (85.5%) and cleavage rate (88.7%) were obtained, although total blastocyst formation (15.9%) was significantly lower than that of control (40.9%). However, the development results of

two treatment group were not differed in PN number. Although the specimens used in this experiment were abnormally fertilized embryos and the numbers are still too small to fully evaluate of the method, this study demonstrated that the new ultra-rapid freezing method can be used as a method of human PN zygotes cryopreservation.

Therefore, these results demonstrate that developmental capacity of human multi-PN zygotes can be maintained by ultra-rapid freezing method using EM grid and EFS30.

SUMMARY

The objective of this study was to test whether the developmental capacity of human multi-pronuclear (PN) zygotes after ultra-rapid freezing using EM grid can be maintained. For this experiment, multi-PN zygotes which produced in human IVF program were used as an alternatives of normal 2PN zygotes, and they were separated into 3PN or ≥4PN zygotes to compare their *in vitro* development and cryoinjury according to PN number. As freezing solution, EFS30 which consisted of 30% ethylene glycol, 18% ficoll, 0.5M sucrose and 10% FBS added D-PBS was used. The result obtained in this experiment was summarized as follows; When the multi-PN zygotes were ultrarapidly frozen and thawed, the high mean percentages (85.5%) were survived. Also when the cleavage rates between control and freezing group were compared with PN number, there were not significantly different in each group (3PN; 81.3% & 85.4% and ≥4PN; 90.0% & 95.7%). When the *in vitro* development rates after thawing were examined, freezing 3PN group (22.0%) was not differed to control 3PN group (38.5%), although the development result of freezing ≥4PN group (4.5%) was significantly lower than that of control ≥4PN group (44.4%)($p<0.05$). These results demonstrate that developmental capacity of human zygote can be obtained by ultra-rapid freezing method using EM grid and EFS30.

REFERENCES

Abbeel van den E, Camus M, Waesberghe van L, Devroey P, Steirteghem van AC: A randomized comparison of the cryopreservation of one-cell human embryos with a slow controlled-rate cooling procedure or a rapid cooling procedure by direct plunging into liquid nitrogen. *Hum Reprod* 1997, 12(7), 1554-1560.

Baker AF: Use of the one-step method to freeze and thaw human pronuclear stage and 2-8 cell embryos. *ASRM 13rd Annual postgraduate program Course VII* 1997, 31-37.

Barg PE, Feichtinger W: Ultrarapid freezing of mouse and human embryos: a modified approach. *J Vitro Fert Embryo Transfer* 1990, 53, 469-472.

Cohen J, DeVane GW, Elsner CW, Fehilly CB, Kort HI, Massey JB, Turner TG Jr: Cryopreservation of zygotes and early cleaved human embryos. *Fertil Steril* 1988, 49(2), 283-289.

Dandekar PV, Martin MC, Glass RH: Polypronuclear embryos after *in vitro* fertilization. *Fertil Steril* 1990, 53, 510-514.

Feichtinger W, Hochfellner C, Ferstl U: Clinical experience with ultra-rapid freezing of embryos. *Hum Reprod* 1991, 6(5), 735-736.

Friedler SH, Giudice LC, Lamb EJ: Cryopreservation of embryos and ova. *Fertil Steril* 1988, 49, 743-763.

Gordts S, Roziers P, Campo R, Noto V: Survival and pregnancy outcome after ultrarapid freezing of human embryos. *Fertil Steril* 1990, 53(3), 469-472.

Kaufmann RA, Menezo Y, Hazout A: Co-cultured blastocyst cryopreservation: experience of more than 500 transfer cycle. *Fetil Steril* 1995, 64, 1125-1129.

Kim EY, Kim NH, Yi BK, Yoon SH, Park SP, Chung KS, Lim JH: Developmental capacity of bovine follicular oocytes after ultra-rapid

freezing by electron microscope grid. I. Cryopreservation of immatured bovine oocytes. *Kor J Fertil Steril* 1998a, 25(1). 71-76.

Kim EY, Kim NH, Kim MK, Yi BK, Yoon SH, Park SP, Chung KS, Lim JH: Developmental capacity of bovine follicular oocytes after ultra-rapid freezing by electron microscope grid. II. Cryopreservation of *in vitro* matured bovine oocytes. *Kor J Ani Reprod* 1998b, 25(1). 1-10.

Lai ACH, Lin BPH, Chang CC, Tsai HD, Hwang VWS, Lo HY: Pregnancies after transfer of ultrarapidly frozen human embryos. *J Assist Reprod and Gen* 1996, 13(8), 625-628.

Martino A, Songsasen N, Leibo SP: Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. *Biol Reprod* 1996, 54, 1059-1069.

Michelmann HW, Bonhoff A, Mettler L: Chromosome analysis of polyploid human embryos. *Hum Reprod* 1986, 1, 243-246.

Menezo Y, Joly CG: Cryopreservation of blastocysts : The *in vitro* vs. *in vivo* situation in bovine and human. *ASRM 13rd Annual postgraduate program Course Ⅶ* 1997, 47-55.

Park SP, Kim SE, Uhm SJ, Kim EY, Kim T, Yoon SH, Chung KS and Lim JH: Effect of a simple serum-free medium, CR1, on the development of IVM/IVF bovine embryos. *Kor J Fertil Steril* 1995, 22(2), 105-108.

Rall WH, Fahy GM: Ice-free cryopreservation of mouse embryos at -196 ℃ by vitrification. *Nature* 1985, 313, 573-575.

Rudak E, Dor J, Mashiach S, Nebel L, Goldman B: Chromosome analysis of multipronuclear human oocytes fertilized *in vitro*. *Fertil Steril* 1984, 41, 538-545.

Trounson A: Cryopreservation. *Br Med Bull* 1990, 46, 695-708.

Trounson A, Mohr L: Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature* 1983, 305,

707-709.

Trounson A, Peura A, Kirby C: Ultrarapid freezing: a new low-cost and effective method of embryo cryopreservation. *Fertil Steril* 1987, 48, 843-850.

Trounson A, Peura A, Freeman L, Kirby C: Ultrarapid freezing of early cleavage stage human embryos and eight-cell mouse embryos. *Fertil Steril* 1988, 49, 822-826.

Trounson A, Sjöblom P: Cleavage and development of human embryos *in vitro* after ultrarapid freezing and thawing. *Fertil Steril* 1988, 50, 373-376.

Van der Ven HH, Al-Hasani S, Diedrich K, Hamerich U, Lehmann F, Krebs D : Polyspermy in *in vitro* fertilization of human oocytes: frequency and possible causes. *Ann NY Acad Sci* 1985, 442, 88-95.

Veeck LL, Amundson CH, Brothman LJ, DeScisciolo C, Maloney MK, Muasher SJ, Jones HW Jr: Significantly enhanced pregnancy rates per cycle through cryopreservation and thaw of pronuclear stage oocytes: a five year clinical study. *Fertil steril* 1993, 1202-1207.

Wramsby H, Fredga K, Liedholm P: Ploidy in human cleavage stage embryos after fertilization *in vitro*. *Hum Reprod* 1987, 2, 223-236.

Zeilmaker GH, Alberda A, Th., van Gent I, Rijkmans CMPM, Drogenkijk AC: Two pregnancies following transfer of intact frozen-thawed embryos. *Fertil steril* 1984, 42, 293-296.

Table 1. Cryopreservation of human multi-PN zygotes by ultra-rapid freezing using EM grid

Treat.	No. of 3- or ≥4-PN		Repli.	No. of survived	No. of cleaved (on day2)	No. of blastocyst* (on day6)		
						Cavitation	≥ErB**	Total
Control	3PN	16	2		13 (81.3)	2	3	5 (38.5)[a]
	≥4PN	10	2		9 (90.0)	1	3	4 (44.4)[a]
	Total	26	4		22 (84.6)	3	6	9 (40.9)[c]
Freezing	3PN	59	11	48 (81.4)	41 (85.4)	6	3	9 (22.0)[a,b]
	≥4PN	24	7	23 (95.8)	22 (95.7)		1	1 (4.5)[b]
	Total	83	18	71 (85.5)	63 (88.7)	6	4	10 (15.9)[d]

* Blastocyst was defined as blastocoel cavity formation ** ErB; Early blastocyst

[a-b,c-d] Means in the column without common superscripts are significantly different ($p<0.05$)

Fig. 1. ***In vitro*** **developmental morphology of human multi-PN zygotes after freezing and thawing ultra rapidly. (A) Multi-PN zygotes just before freezing (B) Cleaved embryos at 24 h after thawing. It showed also uncleaved multi-PN zygotes. (C) Early or hatching blastocyst developed from multi-PN zygotes at day 6 after thawing. x200.**