The Cytoskeletal and Chromosomal Alteration in Vitrified Mouse Oocyte

Yi, B. K., E. Y. Kim, H. K. Nam, K. S. Lee, S. H. Yoon*,
S. P. Park and J. H. Lim*
*Maria Infertility Medical Institute, *Maria Obs/Gyn, Seoul*

초자화동결된 생쥐 미수정란의 cytoskeleton 및 염색체 변화

마리아 기초의학연구소, *마리아 산부인과
이봉경 · 김은영 · 남화경 · 이금실 · 윤산현* · 박세필 · 임진호*

= 국 문 초 록 =

본 연구는 동해방지제인 EFS35를 이용한 생쥐 미수정란의 초자화동결이 cytoskeleton과 염색체에 미치는 영향을 indirect immunocytochemistry와 염색체분석으로 확인하고자 실시하였다. 생쥐 미수정란은 M2 배양액에 35% ethylene glycol, 18% ficoll, 0.5 M sucrose와 10% FBS가 함유된 EFS35를 이용하여 초자화동결되었다. 본 연구에서 얻어진 결과는 다음과 같다. 초자화동결된 미수정란의 융해후 생존율은 89.3%로써 노출군 (97.7%) 과 유의한 차이가 없었다 ($p<0.05$). 초자화동결이 미수정란의 microtubule과 microfilament에 미치는 영향을 조사하였던 바, 동결군 (95.5, 100%)의 microtubule과 microfilament의 정상적인 형성율은 대조군 (97.5, 100%) 또는 노출군 (92.3, 100%) 의 결과와 비교하였을 때 유의한 차이가 나타나지 않았다 ($p<0.05$). 또한, 체외수정후 동결군에서 정상적인 염색체수를 가진 난자의 비율도 73.5%로써, 대조군 (79.5%) 과 노출군 (78.7%) 의 결과와 유의한 차이가 없었던 바 ($p<0.05$), 생쥐 미수정란을 EFS35에 노출하고 동결하는 것이 미수정란의 cytoskeleton과 염색체 성상변화에 영향을 미치지 않는다는 것을 알 수 있었다. 따라서, 본 연구에서 사용된 동해방지제인 EFS35를 이용한 초자화동결법은 생쥐 미수정란의 동결에 적합하다는 것을 시사한다고 하겠다.

INTRODUCTION

Recently, attention has been focused on cryopreservation for oocytes. For mouse oocytes in particular, cryopreservation by various slow freezing protocols has been investigated (Glenister *et al.*, 1987), additionally, ultra-rapid freezing (Sathananthan *et al.*, 1988; Surrey and Quinn, 1990) and vitrification (Kola *et al.*, 1988; Nakagata, 1989) have been introduced in the mouse system. However, it was known that the cryopreservation of mouse oocytes has been shown to induce an alteration of spindle morphology, a decreased fertility, and a significant increase of anomalies in chromosome position (Glenister *et al.*, 1987; Van der Elst *et al.*, 1988; Bouquet *et al.*, 1992). In earlier our study, Kim *et al.* (1998) demonstrated that the developmental capacity of mouse oocytes could be maintained when vitrified using the cryoprotectant EFS35. Based on this data, we tested whether the vitrification method using EFS35 has detrimental effect for cytoskeleton and chromosome constitution of mouse oocytes by immunocytochemistry and chromosome analysis (Giemsa staining).

MATERIALS AND METHODS

1. Source of oocytes

Oocytes collected from 3 to 5 weeks old female mice were induced by intraperitoneal injection of 7.5 IU PMSG and, 48 hr later, 7.5 IU hCG. The intact cumulus masses were released from excised oviducts into M2 medium containing 0.025 % hyaluronidase. After the cumulus cells had been detached from the oocytes, the oocytes were washed and were divided into three groups according to experimental purpose; control, exposure to cryoprotectant and vitrification. The exposed group was put through the same procedure as vitrification except being plunged into liquid nitrogen (LN_2).

2. Vitrification and thawing

The oocytes were vitrified by the procedures described by Kim *et al.* (1998). All manipulations of oocytes were carried out using M2 medium

containing 10% fetal bovine serum (FBS) at 25℃. Vitrification solution was used EFS35 [35% ethylene glycol (EG), 18% ficoll, 0.5 M sucrose and 10% FBS in M2 medium]. The cumulus-free oocytes were equilibrated with exposure to 10% EG for 5 min. Then, oocytes were transferred to EFS35 in a 0.25 ml straw for 30 sec before being plunged into LN_2. Thawing was achieved by agitating the straws in a 25℃ water until ice crystals disappeared. Oocytes were released into M2 medium with 0.5 M sucrose for 5 min at 25℃. Then oocytes were transferred into M2 medium for 10 min and placed in M16 medium.

3. Indirect immunocytochemistry

The procedures were modified with method described by Kim *et al.* (1996). In each groups, oocytes were treated with Buffer M (25% glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 1 mM EGTA and 50 mM imidazol, pH 6.7) for 3-5 min at 37℃, fixed in methanol for 10-15 min at -20℃ and stored in PBS containing 0.02% NaN_3 and 0.1% bovine serum albumin at 4℃ until before the staining. Microtubule localization was performed using anti-α-tubulin monoclonal antibody (Sigma) in which diluted 1:200 in PBS for 60 min and 1:100 of FITC-labeled goat anti-mouse antibody (Sigma) for 60 min. The chromatin was fluorescently detected by exposure to 5 μg/ml propidium iodide (Sigma) for 90 min. To detect distribution of microfilaments, the oocytes were cultured in FITC-labeled phalloidin (1 μg/ml) for 90 min. Stained oocytes were mounted under a coverslip with mounting medium (Universal Mount, Fisher Scientific Co., Huntsville, AL, USA) and were observed with fluorescence microscope.

4. *In vitro* fertilization (IVF) and *in vitro* development

A sperm suspension was prepared by placing cauda of epididymis F1 males (10 to 12 weeks old) in M16 medium and dispersed for 90 min at 37℃ in a humidified atmosphere of 5 % CO_2 in air. A final concentration of inseminated sperm was 1 2$\times 10^6$ cells/ml. After 6 8 hr incubation, the eggs were washed and transferred into M16 medium for *in vitro* development. Among them, several oocytes were examined the change of chromosome constitution after

IVF following vitrification and remainders were further cultured to observe their development *in vitro* for day 4.

5. Chromosome preparation and analysis

The procedure was modified to that of Bouquet *et al.* (1992). The inseminated eggs were cultured overnight in M16 medium containing 1 μg/ml of colcemid, thereby suppressing cytokinesis. The eggs were placed in 0.5% pronase (Sigma) for 4 min and a hypotonic solution of sodium citrate (1%) for 6 min. And then they were fixed with acetic alcohol (absolute methyl alcohol : glacial acetic acid = 3 : 1). Slides were stained with 2% Giemsa and normality was scored with microscope. The normal fertilized oocytes were considered when they composed of two haploid chromosomal sets. Aneuploid was considered where there is an addition (hyperhaploid) or deletion (hypohaploid) of $n \pm 4$ chromosomes. The zygotes added whole sets of chromosomes were considered to be polyploid.

6. Parthenogenetic activation

To assess the incidence of spontaneous parthenogenetic activation, oocytes of each treatment group were cultured in M16 medium without spermatozoa. After 24 hr of culture, the ratio was estimated as follows; number of 2-cell embryos/total number of oocytes in culture.

7. Statistical analysis

Results were compared using the chi-square (X^2) test.

RESULTS

When the *in vitro* development of exposed and vitrified oocytes in EFS35 was examined, the survival rates were 97.7 and 89.3%, there were not significantly different between two groups ($p<0.05$). Also, in vitrified group, 70% of survived oocytes was cleaved and 82.9% of them were developed to blastocysts (Table 1). This data was not significantly different from control

and exposed group ($p<0.05$). When the parthenogenetic activation was examined in individual group, it showed a little activation in control (6.9%, 2/29) and vitrified (5.7%, 2/35), except exposed group (0%, 0/21). In addition, when the effects of exposure or vitrification in EFS35 on cytoskeletal morphology of oocytes were evaluated using indirect immunocytochemistry, the majority of spindle was barrel shaped and was located peripherally (Fig. 1A). Furthermore, the proportion of normal spindle morphology in vitrified group (95.5%) was not significantly different from that in control (97.5%) and exposed group (92.3%) ($p<0.05$) (Table 2). However, there was a little abnormal microtubules morphology in all groups (Fig. 1B). As shown in Fig. 1C, the microfilaments were observed as a network throughout the cytoplasm. Thus, all of oocytes were confirmed a normal cytoskeletal morphology after vitrification.

Also, when the alteration of chromosome constitution was examined in fertilized oocytes after exposure or vitrification in EFS35, the rate of oocytes containing a normal chromosome number (2n=40) in vitrified group was 73.5%, there was not significantly different from control (79.5%) and exposed group (78.7%) ($p<0.05$). Especially, the incidence of aneuploid in each group was slightly higher than that of polyploid, but the chromosomal abnormality was not significantly different among the treatment groups ($p<0.05$). In addition, all of aneuploid were presented to hypohaploid in which the part of chromosome is deleted (Fig. 2).

DISCUSSION

Vitrification provides a simple and rapid method that has been established for the preservation of mammalian oocytes and embryos. In earlier our study, Kim *et al.* (1998) showed that mouse oocytes could be effectively vitrified using EFS35. In this study, we examined whether irreversible changes in the morphology of cytoskeleton and chromosomal constitution by vitrification can be induced. In most mammalian species, the ovulated oocytes have a spindle arrested in metaphase of second meiotic division. This spindle is composed of microtubules attached the maternal chromosomes. It has known that

microtubules and microfilaments are the major cytoskeletal components in the mammalian ova and provide the framework for the chromosomal movement and cell division (Kim *et al*., 1996). Also, it has been known that factors such as temperature lowering and exposure to cryoprotectant can induce deterimental changes of the meiotic spindle of mouse oocyte (Van der Elst *et al*., 1988; Pickering *et al*., 1990). In this study, the majority of vitrified oocytes with EFS35 presented a normal spindle morphology in which is similar to control group (Fig. 1A). However, there was a few abnormal appearance in which two small clusters were seen at the spindle poles apart from the equatorial plate (=activated) in all groups (Fig. 1B). On the other hand, it has been demonstrated that the domain rich in microfilaments seems to be responsible for the maintenance of the meiotic spindle and chromosomes in a peripheral position (Webb *et al*., 1986). In this study, microfilaments are presented a normal distribution that is located mainly in the cell cortex, regardless of treatment groups (Fig. 1C). The organization of microfilaments was shown to be unaffected by exposure and vitrification in EFS35. When the chromosome constitution of vitrified mouse oocytes with EFS35 was examined, the rates of normally fertilized oocytes (2n=40) were not significantly different among the treatment groups (Table 3). Several studies reported that the incidence of polyploid is high in mouse and human embryos (Al-Hasani *et al*., 1987; Glenister *et al*., 1987). However, our results were similar to that described by Kola *et al*. (1988), who reported that embryos derived from vitrified mouse oocytes had an increased incidence of aneuploid. There were not significantly different in the incidence of chromosomal abnormality among the treatment groups. In conclusion, these results demonstrated that the vitrification using EFS35 had no detrimental effect on the morphology of cytoskeleton and the chromosome constitution of mouse oocytes before and after IVF.

SUMMARY

The objective of this study was to confirm whether the vitrification method using EFS35 has detrimental effect for cytoskeleton and chromosome

constitution of the mouse oocytes by indirect immunocytochemistry and chromosome analysis. Mouse oocytes were vitrified using EFS35 which consisted of 35% ethlylene glycol, 18% ficoll, 0.5 M sucrose and 10% FBS in M2 medium. The results obtained in this experiment were summarized as follows: When the survival rates after exposed or vitrified in EFS35 were examined, there was not significantly different between two groups (97.7 and 89.3%) ($p<0.05$). Also, when the microtubule morphology and microfilament distribution in vitrified oocytes were examined, normal percentage of two cytoskeleton in vitrified group (95.5 and 100 %) was not significantly different from that in control (97.5 and 100%) and exposed group (92.3 and 100%) ($p<0.05$). In addition, the rate of oocytes containing a normal chromosome number in vitrified group (73.5%) after IVF was not significantly different from that in control (79.5%) and exposed group (78.7%) ($p<0.05$). These results indicated that cryoprotectant (EFS35) or freezing have not effect on the alteration of cytoskeleton morphology and the chromosome constitution of mouse oocytes. Therefore, our vitrification methods using EFS35 was suitable for cryopreservation of mouse oocytes.

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Table 1. Developmental capacity of mouse oocytes exposed or vitrified in EFS35

Treatment	No. of oocytes examined	No. of oocytes survived (%)	No. of (%)	
			2-cell	day 4 blastocyst
Control	53	-	42 (79.2)	33** (78.6)
Exposed	43	42* (97.7)	29 (69.0)	23** (79.3)
Vitrified	56	50* (89.3)	35 (70.0)	29** (82.9)

*,** No significant difference ($p<0.05$).

Table 2. Morphology of microtubules in mouse oocytes exposed or vitrified in EFS35

Treatment	No. of oocytes	Barrel-shaped	Activated†	No spindle
Control	40	39 (97.5)*	1 (2.5)	-
Exposed	39	36 (92.3)*	3 (7.7)	-
Vitrified	44	42 (95.5)*	2 (4.5)	-

† Two small clusters were seen at the spindle poles apart from the equatorial plate

* No significant difference ($p<0.05$).

Table 3. Incidence of chromosome abnormalities in mouse oocytes exposed or vitrified in EFS35 after IVF

Treat-ment	No. of oocytes examined	No. of oocytes					
		Fertilized[¶] (%)	Scored	Normally fertilized (%)	Aneuploid (%)		Polyploid (%)
					Hypo-haploid	Hyper-haploid	
Control	62	46[*] (74.2)	44	35[**] (79.5)	8 (18.2)	-	1 (2.3)
Exposed	72	47[*] (65.3)	47	37[**] (78.7)	9 (19.1)	-	1 (2.1)
Vitrified	54	35[*] (64.8)	34	25[**] (73.5)	9 (26.5)	-	-

[¶] Fertilization was defined as 2 pronuclear formation.

[*,**] No significant difference ($p<0.05$).

Figure 1. Immunofluorescence localization of microtubules (A, B) and microfilaments (C) (green: microtubules, microfilaments; red or yellow: chromatin). A. normal: barrel-shaped, x600 ; B. abnormal: activated, C. normal, ×300.

Figure 2. Chromosomal constitution of fertilized eggs. A. normal (2n=40), B. hypohaploid (2n=36), C. polyploid (3n=60) : ×300.