The Cytoskeletal and Chromosomal Constitution of Vitrified Immature Mouse Oocytes

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초자화동결된 생쥐 미성숙란의 세포골격과 염색체성상

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〓 국문초록 〓

본 연구는 동해방지제인 EFS40을 이용한 초자화동결이 생쥐 미성숙란의 cytoskeleton과 염색체의 성상에 미치는 영향을 indirect immunocytochemistry 방법과 염색체 분석법으로 알아보고자 실시하였다. 본 실험은 생쥐 미성숙란을 EFS40 (40% ethylene glycol, 18% ficoll과 0.5 M sucrose가 들어있는 M2 배양액)으로 초자화동결하여 융해한 후 16 시간동안 체외 성숙을 유도하여, 제 1극체가 나타난 성숙된 난자를 기준으로 동해제노출군 또는 대조군과 비교 조사하였다. 본 실험에서 얻어진 결과는 다음과 같다. 초자화동결된 미성숙란의 융해 후 생존율과 체외성숙율은 90.3%과 64.7%로써, 동해제노출군 (86.7%, 69.2%)과 대조군 (100%, 58.3%)에 유사하였다. 초자화동결이 미성숙란의 microtubule과 microfilament에 미치는 영향을 조사하였던 바, 동결군의 microtubule과 microfilament의 정상적인 형성율 (93.9%, 100.0%)은 동해제노출군 (94.4%, 100.0%)과 대조군 (100.0%, 100.0%)의 성적과 유사하게 나타났다. 또한, 초자화동결군에서 정상적인 염색체수를 가진 난자의 비율도 65.8%로써, 대조군 (79.6%)과 노출군 (69.0%)의 결과와 유의한 차이가 없었다. 이러한 결과로 미루어보아, 생쥐 미성숙란을 EFS40에 노출하고 동결하는 것이 미성숙란의 cytoskeleton과 염색체성상에 영향을 미치지 않으며, 본 연구에서 사용된 EFS40을 이용한 초자화동결법은 생쥐 미성숙란 동결에 적합하다는 것을 알 수 있었다.

(Key words : Immature mouse oocytes, Vitrification, EFS40, Cytoskeleton, Chromosome analysis)

INTRODUCTION

Many researchers have studied on the cryopreservation of immature oocytes, germinal vesicle (GV) stage oocytes in the mouse (Candy *et al.*, 1994), rat (Pellicer *et al.*, 1988), hamster (Mandelbaum *et al.*, 1988) and human (Son *et al.*, 1996). Also, it has known that the cryopreservation of immature oocytes has the potential to overcome problem associated with Metaphase Ⅱ stage oocytes, such as spindle disorganization (Eroglu *et al.*, 1998), disruption of cytoskeletal elements (Eroglu *et al.*, 1998) and increased abnormal chromosomes (Carroll *et al.*, 1989). Actually, the microtubules were highly sensitive to thermal changes and the last stage of meiosis seems to be altered by cryo-treatment, but immature oocytes with DNA enclosed in the nucleus and protected by the nuclear membrane might be less sensitive to the cryopreservation process than mature oocytes (Pickering *et al.*, 1987). Earlier studies on immature mouse oocyte cryopreservation have performed by slow or conventional cooling procedures using dimethylsulfoxide (DMSO) or 1,2-propanediol and variables of ultrarapid freezing methods, and reported that there is no deleterious effect on the morphology of the second meiotic spindle of the oocyte (Van der Elst *et al.*, 1992; Frydman *et al.*, 1997; Eroglu *et al.*, 1998). In our previous study (Yi *et al.*, 1999), we suggested that immature mouse oocytes can be cryopreserved successfully using EFS solution based on ethylene glycol (EG), and their developmental potential *in vivo* was high. In this study, we investigated whether the vitrification method using EFS40 freezing solution has detrimental effect on the cytoskeleton and chromosome constitution of the immature mouse oocytes by indirect immunocytochemistry and chromosome analysis.

MATERIALS AND METHODS

1. Collection of GV-stage mouse oocytes

Oocytes collected from 3 to 5 weeks old female mice (C57BL/CBA) F1 hybrid were primed with an intraperitoneal injection of 7.5 IU pregnant mare's

serum gonadotrophin (PMSG, Sigma). Between 48 52 hr after PMSG injection, the animals were killed. The ovaries were removed after rupture of the periovarian sac and incubated subsequently in M2 medium supplemented with 10% fetal bovine serum (FBS, Gibco) and containing 0.25 mM the meiotic inhibitor dibutyryl cAMP (dbcAMP, Sigma). Fully grown GV-stage oocytes (70 80 ㎛) were selected. Oocytes were washed and divided into three groups according to experimental purpose; control, exposure to cryoprotectant and vitrification. The exposed group was put through the same procedure as vitrified group except being plunged into liquid nitrogen (LN_2).

2. Vitrification and thawing

All manipulations were carried out using M2 medium containing 10% FBS and dbcAMP at 25℃. Freezing solution was used EFS40 (40% ethylene glycol (EG), 18% ficoll, 0.5 M sucrose and 10% FBS added in M2). Before freezing, the cumulus-free oocytes were equilibrated with exposure to 20% EG for 5 min. Then, oocytes were exposed in EFS consisting of 40% EG 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 containing 0.5 M sucrose for 5 min, and then transferred into M2 medium for 10 min.

3. *In vitro* maturation and assessment

For the *in vitro* maturation, GV-stage oocytes collected from each treatment groups were cocultured in the cumulus cell monolayered drop (10㎕). After coculture for 16 hr, meiotic maturation of oocytes was assessed at extrusion of first polar body.

4. Indirect immunocytochemistry

Staining procedures were described in detail 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 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 1 μg/ml propidium iodide (Sigma) for 90 min. To detect distribution of microfilaments, the oocytes were cultured in FITC-labeled phalloidin (10 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.

5. Chromosome preparation and analysis

The procedure was modified to that of Bouquet *et al* (1992). The oocytes were placed in 0.5% pronase (Sigma) for 2 min. and a hypotonic solution of sodium citrate (1%) for 2 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 oocytes were considered when they composed of 20 separated chromosomes. Aneuploid was considered where there is an deletion (hypoploid) or addition (hyperploid). Polyploid has an addition of a set of chromosomes.

6. Statistical analysis

In each groups, the results were compared using the chi-square (X^2) test.

RESULTS

In our freezing study, the survival rates of exposed and vitrified group were 86.7% and 90.3%, respectively, there was no significant difference. Also, *in vitro* maturation rates were similar among treatment groups (control: 58.3%, exposed: 69.2% and vitrified: 64.7%). To determine the effect of freezing to the cytoskeletal configuration of immature mouse oocytes, microtubule and microfilament of vitrified-thawed immature mouse oocytes were examined by indirect immunocytochemistry after *in vitro* maturation. From examined

oocytes in each treatment group (using matured oocytes), the vast majority (>90%) displayed normal microtubules organization (control: 100%, exposed: 94.4% and vitrified: 93.9%). With respect to barrel-shaped spindle and normal chromosome alignment, no significant difference was observed among treatment groups (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 exposed (3/54: 5.6%) and vitrified groups (4/66: 6.1%) (Fig. 1B). In addition, the microfilament was observed into normal morphology indicated as a network throughout the cytoplasm (Fig. 1C), irrespective of treatment groups [control; 47/47 (100.0%), exposed; 30/30 (100.0%) and vitrified; 34/34 (100.0%)]. On the other hand, when a certain change of chromosome constitution of immature mouse oocytes according to exposed and vitrified in EFS40 was examined, the rates of oocytes containing a normal chromosome number (n=20) in exposed and vitrified group were 69.0% and 65.8%, respectively. Also, there was no significant difference compared to that (79.6%) in control (Table 2) (Fig. 2A). But, hypoploid rate of exposed (31.0%) or vitrified (34.2%) group was tend to increase than that (18.5%) of control (Fig. 2B), although there was no significant difference. In control group, 1 out of 54 (1.9%) matured oocytes was found to polyploid (Fig. 2C).

DISCUSSION

This study demonstrated that exposure in EFS40 freezing solution and temperature lowering (vitrification) during the GV-stage have no deleterious effect on the morphology of the second meiotic spindle, microfilament and chromosome constitution of immature mouse oocyte. It has been well known that cryopreservation of GV-stage mouse oocytes is advantageous to circumvent the spindle damage and increased chromosome abnormalities noted in cryopreservation of Metaphase Ⅱ oocytes (Eroglu *et al.*, 1998). The GV-stage oocytes are theoretically less susceptible to this kind of freezing injury, because the chromatin at GV-stage is in a decondensed stage and few

microtubule- organizing centers are found at the perinuclear site and most of the microtubular system is not organized (Rime *et al*., 1987). A normal spindle appeared as fine microtubules traversing the metaphase plate, forming the classic barrel shape. Recently, many researchers have reported that there is no significant abnormalities in cytoskeletal organization on GV-stage oocytes after cryopreservation (Van der Elst *et al*., 1992; Baka *et al*., 1995; Frydman *et al*., 1997; Eroglu *et al*., 1998). Also, this study indicated that there was no detrimental cooling effect at microfilament (data not shown) which are presented a normal distribution that is located mainly in the cell cortex, regardless of treatment groups (Fig. 1C).

We have further studied the effect of cryopreservation at the GV-stage on the chromosomal constitution as counting the number of chromosome in oocytes from each group. In all treatment groups, although morphological maturation rates were similar, the chromosome abnormality after exposure to freezing solution and vitrification of immature mouse oocytes showed a tendency to increase aneuploid, especially hypoploid. It has known that the disruption or depolymerization of the spindle after exposure to low temperatures gives rise to aneuploidy or polyploidy (Van der Elst *et al*., 1992; Sarananthan *et al*., 1988). Taken altogether, these results indicated that exposure to cryoprotectant or freezing has not effect on the alteration of cytoskeleton morphology and the chromosome constitution of immature mouse oocytes and that our vitrification method using EFS40 freezing solution was suitable for the cryopreservation of immature mouse oocytes.

V. SUMMARY

This study was to confirm whether the vitrification method using EFS40 freezing solution has detrimental effect on the cytoskeleton and chromosome constitution of the immature mouse oocytes by indirect immunocytochemistry and chromosome analysis. Immature mouse oocytes were vitrified using EFS40 (40% ethylene glycol, 18% ficoll, 0.5 M sucrose diluted in M_2 medium), thawed and then survived oocytes were *in vitro* matured for 16 hr. When the

microtubule morphology and microfilament distribution in vitrified-thawed immature mouse oocytes were examined, normal percentage of two cytoskeleton in vitrified group (93.9 and 100.0%) was not significantly different from that in control (100.0 and 100.0%) and exposed group (94.4 and 100.0%). The rate of oocytes containing a normal chromosome number in vitrified group was 65.8%, this result was not significantly different from that in control (79.6%) and exposed group (69.0%). These results indicated that exposure to cryoprotectant or freezing has not effect on the alteration of cytoskeleton morphology and the chromosome constitution of mouse oocytes and that our vitrification methods using EFS40 freezing solution was suitable for the cryopreservation of immature mouse oocytes.

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Table 1. The spindle morphology of immature mouse oocytes vitrified in EFS40

Treat-ment	No. of (%)*				Spindle morphology (%)		
	vitri-fied	recov-ered	survi-ved	matur-ed	barrel -shaped	acti-vated	no spindle
Control	-	-	96	56 (58.3)	56 (100.0)	-	-
Exposed	90	90 (100.0)	78 (86.7)	54 (69.2)	51 (94.4)	3 (5.6)	-
Vitrified	120	113 (94.2)	102 (90.3)	66 (64.7)	62 (93.9)	4 (6.1)	-

Table 2. Incidence of chromosome abnormalities of immature mouse oocytes vitrified in EFS40

Treat-ment	No. (%) of oocytes				
	scored	normally matured	aneuploid		polyploid
			hypoploid	hyperploid	
Control	54	43 (79.6)	10 (18.5)	-	1 (1.9)
Exposed	42	29 (69.0)	13 (31.0)	-	-
Vitrified	38	25 (65.8)	13 (34.2)	-	-

Figure 1. Immunofluorescence localization of microtubules (A, B) and microfilaments (C) (Green: microtubules, microfilament; red or yellow: chromatin). A. normal: barrel-shaped, P indicates the chromatin of polar body, x600, B. abnormal: activated, x600, C. normal, x400.

Figure 2. Chromosomal constitution of oocyte matured *in vitro*, A. normal (n=20) x 1200, B. hypoploid (n=17) x800, C. polyploid (2n=40). ×800.