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Sister Chromatid Exchange (SCE) Frequency and In Vitro Development of Mouse Zygote Cryopreserved by Vitrification

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초자화 동결에 의한 생쥐 1-세포기배의 체외 발달과 SCE 빈도

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김묘경 · 백청순* · 엄상준 · 김은영 · 윤산현** 박세필 · 정길생 * · 임진호**

= 국문초록 =

본 연구는 생쥐 1-세포기 수정란의 초자화 동결과 동결액 노출에 따른 수정란의 배발달율과 SCE 빈도를 조사하고자 실시하였다. 체외수정된 생쥐 1-세포기 수정란의 동결은 EFS40 (40% ethylene glycol, 30% Ficoll, 0.3 M sucrose)의 초자화동결법을 이용하였으며, 25℃에서 30 초동안 EFS40에 노출시킨 다음, 곧바로 액체질소에 침투시키거나, 동결액의 독성 검사를 위해 동결없이 배양하여 다음과 같은 결과를 보였다. 융해후, 2-세포기까지 생존율은 EFS40에 노출 혹은 초자화동결시 각각 95.2, 98.5% 로서 대조군의 100%와 비교했을때 큰 차이가 없었다. 그러나 배반포와 탈출배반포까지의 배발달율에 있어서 초자화 동결된 군 66.7, 50.0%는 동결액에 노출만된 군 94.0 78.8%와 대조군 93.9, 81.8%에 비해 낮았다 ($p<0.05$). 동결액에 노출 혹은 초자화동결된 생쥐 1-세포기의 체외 발달에 따른 SCE 빈도를 조사한 결과, 동결액 노출 (20.2±2.1) 혹은 초자화동결시 (21.4±3.2) SCE 빈도가 대조군 (16.8±1.5)에 비해 증가하였다.

이러한 결과를 종합하여 고찰할 때, 초자화동결된 1-세포기 수정란의 배반포 또는 탈출배반포까지의 낮은 배발달율은 동결액의 영향을 받지 않았으나, SCE 빈도는 동결액에 노출 혹은 초자화 동결시 증가 된다는 것을 알수 있다.

INTRODUCTION

Vitrification was a simple and rapid method (Fahy *et al.*, 1984) compared to conventional freezing methods that have been established over the last two decades for the preservation of mammalian embryos (Whittingham, 1977; Friedler *at al.*, 1988; Rall, 1992). Various vitrification methods by using cryoprotectants (demethylsulfoxide, propylene glycol, acetamine, polyethylene glycol and ethylene glycol) were attempt to minimize toxic injury by equilibrating embryos (Hsu *et al.*, 1986;

Rall and Fahy, 1985; Kasai *et al.*, 1990). In a previous study, we have shown that the cryopreservation of mouse zygotes was successfully performed using EFS40 (40% ethylene glycol, 30% Ficoll and 0.3M sucrose in PBS containing 10% FBS) (Kim *et al.*, 1996).

However, Kola *et al.* (1988) reported that embryos derived from vitrified mouse oocytes had an increased incidence of aneuploidy and developed poorly to fetus. Also, the cryopreservation of mouse oocytes has been shown to induce an increase of polyploid embryos and a significant proportion of the fetuses was malformed after vitrification or exposure to the vitrification solution alone (Glenister *et al.*, 1987; Carroll *et al.*, 1989; Bouquet *et al.*, 1992). Moreover, the analysis of SCE frequency has been used to determine embryotoxic and genotoxic factors in mouse embryos cultured in various conditions (Saito *et al.*, 1984; Krueger *et al.*, 1988). In the viewpoint, the use of cryopreservation of embryos obtained after in vitro fertilization must be further evaluated. The present study was undertaken specifically to investigate the possible mutagenic effects of vitrified mouse embryos.

The objective of this study is to investigate correlation between the development rate of exposed or vitrified mouse IVF zygotes and cytogenetical influence using the analysis of SCE.

MATERIALS AND METHODS

1. Production of zygotes *in vitro*

Hybrid F1 female mice (C57BL/6 × CBA/N) were superovulated by intraperitoneal injection of 5 I.U. pregnant mare serum gonadotrophin (PMSG ; Sigma), followed by 5 I.U. human chorionic gonadotrophin (hCG ; Sigma) 50 h later. At approximately 13.5 h post-hCG injection, the cumulus-oocyte complexes (COCs) recovered were inseminated with capacitated sperm. After *in vitro* fertilization, the embryos were cultured inM16 medium for 8 – 10 hrs. When embryos were identified as 2 pronuclei and second polar body, these were used in experiment for vitrification.

2. Vitrification studies

Zygotes were vitrified by a method of Kim *et al.* (1996) with some modifications. Briefly, as vitrification solution, EFS40 [containing 40% (v/v) ethylene glycol, 30% (w/v) Ficoll 70 (average molecular weight 70,000, Sigma) plus 0.3 M sucrose in modified Dulbecco's phosphate-buffered saline (mDPBS, Gibco BRL)] were used.

1) Exposure in EFS40

Zygotes were exposed to EFS40 for 30 sec. Without being cooled, the contents of each straw were expelled into a well containing 0. 8ml of 0.75 M S-DPBS.

2) Vitrification

Zygotes were directly suspended in EFS40 and transferred to straw loaded vitrification solution for 30 sec. at room temperature (25℃). The straw was then slowly immersed into liquid nitrogen (LN_2).

3. Thawing and assessment

The straws storaged in LN_2 warmed rapidly in water bath at 25℃. The contents of each straw were expelled into dish containing 0.8ml of DPBS containing 0.75 M sucrose (S-DPBS) by pushing the cotton plug and then put into fresh 0.75 M S-DPBS for 4 min. The embryos were transferred to 0.5 M, 0.25 M S-DPBS at room temperature (25℃), respectively. Finally, at 5 min. after recovery in fresh DPBS, zygotes were cultured in a 50 μl droplet of M 16 medium supplemented with 4 mg/ml BSA at 37℃, 5% CO_2 incubator. The survival of the zygotes was assessed by blastocysts at day 4 and hatching blastocysts at day 5.

4. Procedure for SCE analysis

For the detection of SCE, the embryos were cultured for two cycles of DNA replication in a

medium containing 5-bromodeoxyuridine (BrdU), a thymidine analogue. The method used was similar to that previously described by Perry and Evans (1975). After IVF, the 2-cell embryos were transferred to M16 medium containing BrdU at a final concentration of 30 μM. The cultures were then kept in the dark to minimize the number of SCE caused by photolysis of DNA containing BrdU. After 87 h insemination in 16-cell and morula stage, colcemid (0.2 μM) was added and at 6 h afterwards, chromosome preparations were made according to the method of Tarkowski (1966). The embryos were placed in a hypotonic solution of sodium citrate (2%) for 10-12 min. and then fixed individually. Freshly prepared acetic alcohol was used as the fixative (3: 1, absolute ethyl alcohol and glacial acetic acid). The slides were incubated for 24 h at 50℃ and stained with the Hoechst 33258 (0.5 μg/ml Söresen buffer) for 15 min., rinsed with distilled water (DW), mounted in the S rensen buffer (pH 6.8) and exposed through coverslips to long-wave UV light for at least 4 h. The slides were then rinsed with DW and stained with 2.5% Giemsa solution for 15 min.

For each of the embryos, SCE scoring was done for all the analyzable metaphase blastomeres, and the the observed SCE count for one randomly chosen blastomere was used of the global analysis. In each of the groups (control, vitrified-thawed and cryoprotectant exposed zygotes), the frequency of exchanges was expressed as the mean SCE/blastomere ± the standard error of the mean (SEM).

5. Statistics

The data were analyzed by means of chi-square analysis.

RESULTS AND DISCUSSION

1. Development of zygotes exposed to or vitrified in EFS40

After IVF at 8 – 10 h, produced zygotes were vitrified or exposed to EFS40. The *in vitro* survival of each groups was compared with control embryos (Table 1). Survival rates of exposed to EFS40 (98.5%) and vitrified embryos (95.2%) which cleaved to the 2-cell stage were not significantly lower than that of control (100%). However, the developmental rates upto blastocyst and hatching blastocyst in vitrified group (66.7, 50.0%) were lower than those in control (93.9, 81.8%) or exposed group (94.0, 78.8%).

This result showed that exposure to the vitrification solution (EFS40) for 30 sec. at zygote stage had no detrimental effect on subsequent development to blastocyst and/or hatching blastocyst. But the development of the vitrified embryos was significantly reduced compared with that of control or exposed group ($p<0.05$). This injury might be have been caused by cooling or warming shock. Other factors, contributing to the decreased survival of early preimplantation stages of embryos, are cryoprotectant toxicity and osmotic injury reported by many investigators. (Kasai *et al.*, 1990; Ali and Shelton, 1993). Also, Bouquet *et al.* (1993) demonstrated that disruption of the cytoskeleton was caused by cooling, warming or exposure to cryoprotectants.

2. SCE frequency of embryos exposed to or vitrified in EFS40

To examine the effect of cytogenetic damage

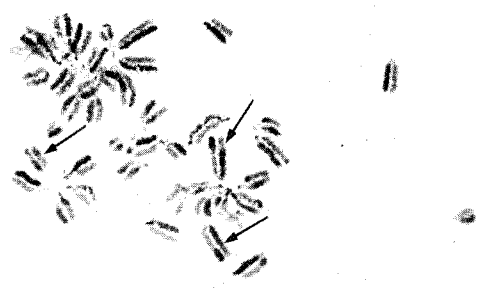

Fig. 1. Preimplantation embryo blastomere at the second division following *in vitro* incubation in 3×10^{-5} μM BrdU. SCEs indicated by arrows.

Table 1. *In vitro* development of zygotes exposed to or vitrified in EFS40

Treatment	No. of Zygotes	Development (%)		
		2-cell (D1)	≥BL (D4)	≥Hing (D5)
Control	66	66 (100.0)	62[a] (93.9)	54[a] (81.8)
Exposed	67	66 (98.5)	63[a] (94.0)	52[a] (78.8)
Vitrification	63	60 (95.2)	40[b] (66.7)	30[b] (50.0)

[a,b] Different superscripts within column were significantly different ($p<0.05$).

Table 2. SEC frequency of embryos exposed to or vitrified in EFS40

Treatment	No. of embryos		SCE/ Metaphase
	Examined	Scored of SCE (%)	
Control	94	21 (22.3)	16.8±1.5[a]
Exposed	91	27 (29.7)	20.2±2.1[b]
Vitrification	61	19 (31.3)	21.4±3.2[b]

[a,b] Different superscripts within column were significantly different ($p<0.05$).

of embryos exposed to or vitrified in EFS40, the SCE frequencies were observed in control, exposed to cryoprotectant and vitrified embryos, respectively. One blastomere derived from a morula embryo is shown in Fig. 1. SCEs can be observed. The rates of embryos scored for SCE were 22.3, 29.7 and 31.1%, respectively, from the control, exposed and vitrified group rates of embryos scored fored for SCEwere very low in all groups. As indicated in Table 2, the significant incerase in average SCE was observed in the vitrified or exposed to EFS40 embryos compared with control embryos. This result suggested that cryopreservation and cryoprotectant exposure might cause an alteration either in the DNA structure or of embryo components implicated in repair mechanisms.

SCEs are considered to be a sensitive indirect indicator of DNA lesion due to various conditions and a more sensitive cytogenetic test (Saito *et al.*, 1984). This is detected by incorporating a thymidine analogue, BrdU into replication DNA for two successive cell cycles and subsequently subjecting it to photodegradation. SCEs are seen in most normal eukaryotic cells, including human cells (Carrano *et al.*, 1980). Besides, it has been known that BrdU can provoke chromosome aberration in mammalian cells (Latt, 1981; Ishii and Bender, 1978). Therefore, some SCEs could be expected from the presence alone of this substance. However, since in our study the treated and control groups were submitted to the same method of exposure and the same concentration of BrdU, the observed differences between the SCE frequencies cannot be due to the effects of BrdU alone.

The consistently higher frequency of SCEs in vitrified embryos requires further investigation about the development of embryos after embryos transfer and the production of descendants. Indeed, the chromosome analysis of produced descendants is necessary. In addition, the incidence of polyploidy is high in mouse and human embryos obtained by the fertilization of frozen-thawed oocytes in vitro (Al-Hasani *et al.*, 1987; Mandelbaum *et al.*, 1988; Bouquet *et al.*, 1993). The consequences of increased rates of SCE will also have to be studied and the possible negative effects on conceptus induced by embryo freezing will be considered.

In conclusion, this study shows that development of embryos were not decreased by exposure to EFS40 at 25℃ without freezing, but by vitrification. However, the SCE frequency was significantly increase by vitrification or only exposure to cryoprotectant.

SUMMARY

This study was undertaken to investigate the

sister chromatid exchange (SCE) frequency and embryonic development after exposure to cryoprotectants and vitrification of mouse zygotes. Mouse IVF zygotes were cryopreserved by vitrification using vitrification solution, EFS40 (40% ethylene glycol, 30% Ficoll and 0.3 M sucrose in phosphate buffer saline containing 10% FBS). After mouse zygotes were exposed to EFS40 at 25°C for 30 sec., they were immediately plunged into LN_2 or cultured for cryoprotectant toxicity test without freezing. The results obtained in these experiments were summarized as follows:

After thawing, survival rates to the 2-cell stage of zygotes exposed to or vitrified in EFS 40 (98.5%, 95.2%) were not significantly difference compared with that of control (100%). However, the developmental rates upto blastocyst and hatching blastocyst in vitrified groups (66.7, 50.0%) were lower than those of control (93.9, 81.8%) or exposed group (94.0, 78.8%) ($p<0.05$). When the influence of vitrification and exposure to cryoprotectant on the *in vitro* development of mouse zygotes was assessed by the SCE frequency, the SCE frequency in exposed (20.2 ± 2.1) to or vitrified embryos (21.4 ± 3.2) was higher than that in control embryos (16.8 ± 1.5).

These results suggest that the frequency of SCE was increased after cryoprotectant cxposure or Vitrification although developmental rates of zygotes upto blastocysts and /or hatching blastocysts were not afected by cryoprotectant.

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