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Regular Article

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Abstract

To evaluate cytogenetic validity of a simple vitrification technique for embryo cryopreservation, mouse 1-cell embryos were vitrified 4, 6, and 8 h after *in vitro* fertilization (IVF). In addition, chromosomal damage of spermatozoa treated with methyl methanesulfonate (MMS) was estimated using vitrified 1-cell embryos. More than 90% of embryos survived vitrification regardless of the time after IVF. In the 4-h and 6-h groups, some of the surviving embryos swelled after recovery. The incidence of structural chromosome aberrations and aneuploidy in embryos with morphologically normal features did not significantly increase in any group. The vitrification technique preserved 1-cell embryos derived from MMS-treated spermatozoa without alteration of chromosome damage. This technique will enable us to manage the optimal time for chromosome preparation of mouse 1-cell embryos.

Keywords: cryopreservation, vitrification, chromosome analysis, mouse embryos

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Introduction

Chromosome analysis of 1-cell embryos is fundamental to estimating primary chromosome aberrations in gametes and assessing heritable risk of chromosome aberrations. In mice, artificial reproductive technologies, such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), have been used to systematically and efficiently produce 1-cell embryos. However, time management is crucial when preparing embryo chromosome slides (Matsuda *et al.* 1985; Yoshizawa *et al.* 1993; Tateno and Kamiguchi 2007). In our protocol (Tateno and Kamiguchi 2007), when IVF or ICSI was performed one morning (10:00), chromosome slides of 1-cell embryos at the first cleavage metaphase must be prepared the next morning (04:00–05:00) (See Figure 1). If IVF/ICSI was planned for 14:00 to prepare chromosome slides the next morning (08:00–09:00), gonadotropins must be injected into females at 22:00 on days 1 and 3 prior to superovulation. Moreover, embryos must be treated with vinblastine at 22:00 on day 4.

Cryopreservation has been widely used for both preservation and transportation of mouse embryos (Suzuki *et al.* 1996; Landel 2005). Vitrification is the ultra-rapid freezing method to avoid intracellular ice crystallization that causes damage to the cells. Embryo vitrification is a very simple and efficient technique (Nakao *et al.* 1997) because a number of embryos can be handled easily during the cooling and warming procedures. However, effect of vitrification on embryo chromosomes remains unknown. If the vitrification technique can be introduced to cytogenetic research of 1-cell embryos, the time management of chromosome preparation will be easier. Furthermore, the chances for collaborative work may increase.

To evaluate cytogenetic validity of the vitrification technique, we analyzed chromosomes of mouse 1-cell embryos vitrified at various times after IVF. Furthermore, an attempt was made to confirm that sperm DNA damage induced by the clastogenic agent methyl methanesulfonate (MMS) is neither qualitatively nor quantitatively altered by vitrification.

Materials and methods

In this study, hybrid (C57BL/6Cr×DBA/2Cr) F1 mice (B6D2F1), 7–12 weeks of age, were used because their oocytes are successfully fertilized *in vitro*, and incidence of

spontaneous chromosome aberrations in 1-cell embryos is low (Tateno and Kamiguchi 2007; Yamagata *et al.* 2009; Tateno 2010). Mice were maintained in a temperature- and light-controlled room (23 ± 2°C, 14 h light from 05:00 to 19:00) with access to food and water *ad libitum*. All experiments were performed according to the Guidelines for Animal Experiments of Asahikawa Medical University. All chemical reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan) unless otherwise stated. Toyoda-Yokoyama-Hosi (TYH) medium was used for sperm capacitation and IVF (Toyoda *et al.* 1971). Vitrification of 1-cell embryos was performed using modified phosphate-buffered saline (PB1) (Whittingham 1974).

Oocytes were obtained from the oviducts of hormonally stimulated females and put in a droplet (200 µl) of TYH medium at 37°C under 5% CO_2 in air. Prior to oocyte collection, spermatozoa were retrieved from the caudal epididymides, and incubated in TYH medium for 2 h at 37°C under 5% CO_2 in air to induce capacitation. A small amount of capacitated spermatozoa were added to the oocyte droplets. The final sperm concentration at the time of insemination was 150–200 cells/µl. Fertilized ova (1-cell embryos) were vitrified at different times following IVF.

When spermatozoa were treated with MMS, they were kept in a droplet (100 µl) of TYH medium containing 100 µg/ml MMS for 2 h at 37°C under 5% CO_2 in air. The spermatozoa were washed twice with MMS-free TYH medium by centrifugation at 350×g for 5 min, and used for IVF.

The vitrification of 1-cell embryos was carried out according to the procedure of Nakao *et al.* (1997). Briefly, the embryos were transferred to a droplet (50 µl) of PB1 medium containing 1 M dimethyl sulfoxide (1 M-DMSO solution) at room temperature. Thirty to sixty embryos were transferred into a 2-ml cryotube (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) together with 5 µl of 1 M-DMSO solution. The cryotube was placed in ice water and kept for 10 min. PB1 medium (95 µl) containing 2 M DMSO, 1 M acetamide, and 3 M propylene glycol (DAP213), pre-cooled in ice water, was gently added to the cryotube. After 5 min, the cryotube was directly plunged into liquid nitrogen and stored for at least 5 days. The embryos derived from intact spermatozoa were vitrified 4, 6 and 8 h after IVF. The embryos derived from MMS-treated spermatozoa were vitrified 8 h after IVF.

To recover the vitrified embryos, the cryotube was removed from liquid nitrogen and kept at room temperature for 60 sec. A 0.9-ml aliquot of PB1 medium containing 0.25 M sucrose, pre-warmed to 37°C, was added to the cryotube and immediately mixed. The mixture containing embryos was transferred to a 35-mm culture dish. The viable embryos were collected, thoroughly washed with PB1 medium, and transferred to a droplet (100 µl) of TYH medium for cultivation.

One-cell embryos were exposed to 0.02 µg/ml vinblastine sulfate 7–9 h after IVF and cultured until they reached the first cleavage metaphase. Between 17 and 19 h after IVF, they were treated with 0.5% protease to digest the zona pellucida and then placed in a hypotonic solution (1:1 mixture of 1% sodium citrate and 30% FBS) for 8–10 min at room temperature. Chromosome slides of the embryos were made by the gradual fixation-air drying method (Mikamo and Kamiguchi 1983). The slides were stained with 2% Giemsa (Merck Japan, Tokyo, Japan) in phosphate-buffered solution (pH 6.8) for 8 min for conventional chromosome analysis. Subsequently, centromeric heterochromatin was detected by C-banding to identify dicentric chromosomes and acentric fragments, as described elsewhere (Tateno *et al.* 2000). Polyspermic and parthenogenetic eggs were eliminated from the chromosome analysis.

All percentage data were transformed into arcsine values for statistical analysis. When data were compared between two different groups, one-way ANOVA followed by Student's *t*-test or the Aspin-Welch method was used. For multiple comparisons, one-way ANOVA and the Tukey-Kramer method were used. Differences were considered to be statistically significant at $P < 0.05$.

Results and discussion

More than 90% of 1-cell embryos survived vitrification regardless of the time after IVF (Table 1). Among viable embryos, cytoplasmic swelling was found in 10.5% in the 4-h group and 20.9% in the 6-h group (Figure 2), while there were no swollen embryos in the 8-h group. Although

Table 1. Survivability of mouse 1-cell embryos vitrified at various times after IVF

Time (h) after IVF	No. of embryos vitrified	No. of embryos recovered	No. (%) of embryos survived	Morphology	
				Normal	Swelling
4	308	307	285 (92.8)	255	30
6	263	258	244 (94.6)	193	51
8	231	225	211 (93.8)	211	0

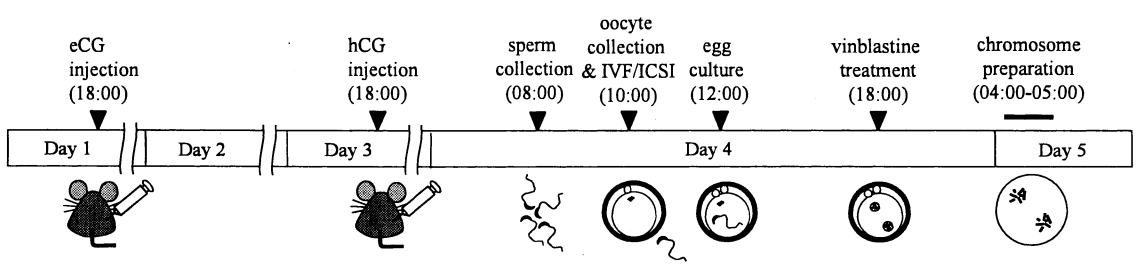

Figure 1 Schematic view of chromosome preparation of mouse 1-cell embryos. eCG: equine chorionic gonadotropin, hCG: human chorionic gonadotropin.

Table 2. Chromosome analysis of mouse 1-cell embryos vitrified at different times after IVF

Time (h) after IVF	No. of embryos fixed [Exp.]	No.(%) of embryos at metaphase	No. of embryos analyzed	Aneuploidy (%)	Structural chromosome aberrations (%)
Control (fresh)	154 [3]	153 (99.4)	150	2 (1.3)	3 (2.0)
4	166 [4]	147 (88.6)	141	3 (2.1)	6 (4.3)
6	155 [3]	136 (87.7)	134	4 (3.0)	6 (4.5)
8	162 [3]	150 (92.6)	146	2 (1.4)	1 (0.7)

There are no statistically significant differences among any groups.

Table 3. Chromosome analysis of mouse 1-cell embryos derived from spermatozoa following MMS exposure

Embryos	No. of embryos analyzed [Exp.]	Aneuploidy (%)	Structural chromosome aberrations (%)	No. of structurally aberrant chromosomes							
				Chromosome-type					Chromatid-type		
				Break	Gap	Dicentric	Translocation	Ring	Break	Gap	Exchange
Fresh	172 [4]	1 (0.6)	92 (53.5)*	76	4	25	2	2	8	1	11
Vitrified	129 [4]	2 (1.6)	78 (60.5)*	65	1	25	3	1	4	0	17

* Values are significantly ($P < 0.001$) high compared to the matched controls (fresh embryos and embryos vitrified 8 h after IVF, respectively) shown in Table 2.

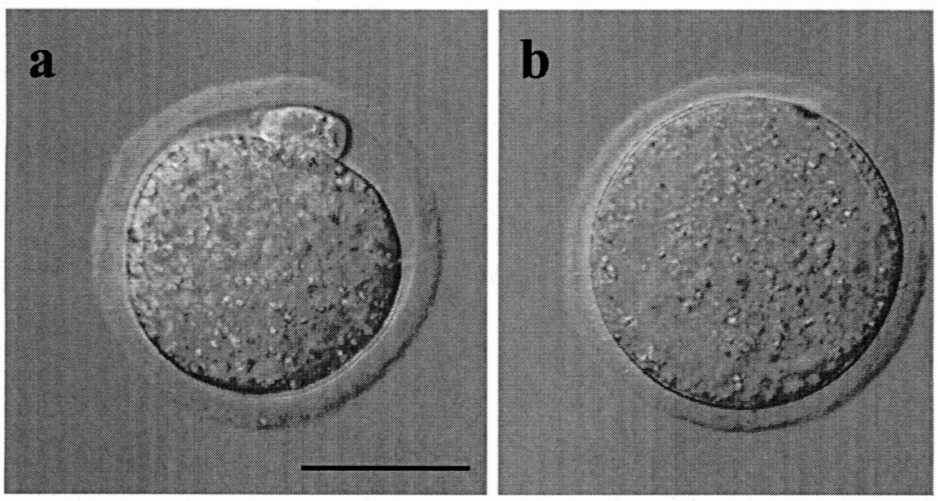

Figure 2 Morphologically normal (a) and swollen (b) 1-cell embryos vitrified 4 h after IVF. Bar = 50 μm.

the swollen embryos seemingly restored their normal figuration by 1 h after incubation, none of them reached the first cleavage metaphase between 17 and 19 h after IVF. The cytoplasmic swelling of embryos found in the 4-h and 6-h groups may be caused by osmotic shock during vitrification and rehydration because the organization of cytoplasmic microtubules is immature in the early pronuclear stage (Schatten *et al.* 1985).

The results of chromosome analysis of viable 1-cell embryos with morphologically normal features are shown in Table 2. Although the percentage of embryos at the metaphase was lower in the vitrification groups than the control group, the difference was not statistically significant ($0.12 < P < 0.87$). Good chromosome slides were prepared from vitrified embryos as with fresh embryos. There was no significant increase in incidences of aneuploidy ($0.89 < P < 1.0$) and structural chromosome aberrations ($0.12 < P < 0.87$) in any vitrification group, indicating that vitrification has no effect on induction of chromosome aberrations.

Chromosome analysis of 1-cell embryos derived from MMS-treated spermatozoa found that there was no significant difference in incidences of aneuploidy ($P = 0.81$) and structural chromosome aberrations ($P = 0.46$) between fresh and vitrified embryos (Table 3), though MMS significantly ($P < 0.001$) induced structural chromosome aberrations. As reported in previous studies with mouse spermatozoa (Matsuda and Tobari 1988) and human spermatozoa (Kamiguchi *et al.* 1995), the major types of MMS-induced structural chromosome aberrations were chromosome break, dicentric and chromatid exchange (Figure 3). The distribution of aberration types was not altered by vitrification.

In this study, the vitrification of 1-cell embryos was carried out at 4, 6 and 8 h after IVF. These times correspond to G1, early S and mid S phases of the first cell cycle of BDF1 mouse embryos used in this study, respectively (Ajduk *et al.* 2006). Because there was no significant increase of chromosome damage in 1-cell embryos vitrified at any stage of the cell cycle, it is concluded that the vitrification at this embryonic stage did not affect DNA

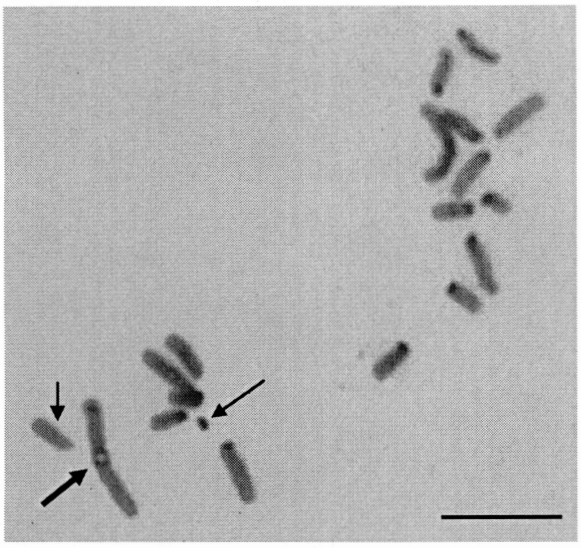

Figure 3 MMS-induced structural chromosome aberrations found in vitrified 1-cell embryos. A long arrow and a short arrow indicate a chromosome break and a derivative acentric fragment, respectively. A thick arrow indicates a chromatid exchange. Bar = 2 μm.

molecules and synthesis.

Mammalian spermatozoa lack the ability to repair DNA damage (Marchetti and Wyrobek 2008). In contrast, mammalian zygotes have the ability to repair DNA damage (Jaroudi and SenGupta 2007), so sperm DNA damage can be repaired in zygotes. When the repair-defective female mice were mated with irradiated males (Marchetti *et al.* 2007) or when mouse zygotes derived from MMS-treated spermatozoa were exposed to repair inhibitors (Matsuda *et al.* 1989), incidence of sperm-derived structural chromosome aberrations evidently increased. In the present result, incidence of structural chromosome aberrations in embryos derived from MMS-treated spermatozoa were not altered by vitrification. This indicates that the capacity of 1-cell embryos to repair DNA damage remains unchanged after vitrification.

The vitrification technique is very simple; it takes approximately 30 min to vitrify a considerable number of 1-cell embryos and 15 min to restore them. The introduction of this technique to the chromosome study of mouse 1-cell embryos will enable us to prepare chromosome slides of them without temporal restraint. In addition, the ease of embryo transport will increase the chance of collaborative work.

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