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Chromosomal integrity of freeze-dried mouse spermatozoa after Cs-137 gamma-ray irradiation

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Abstract

This study demonstrated that freeze-dried mouse spermatozoa possess strong resistance to ^{137}Cs γ-ray irradiation at doses of up to 8 Gy. Freeze-dried mouse spermatozoa were rehydrated and injected into mouse oocytes with an intracytoplasmic sperm injection (ICSI) technique. Most oocytes can be activated after ICSI by using spermatozoa irradiated with γ-rays before and after freeze-drying. Sperm chromosome complements were analyzed at the first cleavage metaphase. Chromosome aberrations increased in a dose-dependent manner in the spermatozoa irradiated before freeze-drying. However, no increase in oocytes with chromosome aberrations was observed when fertilized by spermatozoa that had been irradiated after freeze-drying, as compared with freeze-dried spermatozoa that had not been irradiated. These results suggest that both the chromosomal integrity of freeze-dried spermatozoa, as well as their ability to activate oocytes, were protected from γ-ray irradiation at doses at which chromosomal damage is found to be strongly induced in spermatozoa suspended in solution.

1. Introduction

Freeze-drying technology is commonly introduced for the preservation of bioactive molecules, e.g., DNA, enzymes, albumins, antibiotics, and other types of molecules. Furthermore, human red blood cells have been reported to maintain their bioactivity after freeze-drying [1]. Mouse spermatozoa lose their motility after freeze-drying and are judged cytologically to be “dead sperm”, as based on an analysis using a “sperm Live/Dead staining” technique [2]. However, when mouse oocytes are injected with freeze-dried mouse spermatozoa using an intracytoplasmic sperm injection (ICSI) [3] technique, the oocytes are normally activated and can develop into live offspring [4].

Mouse spermatozoa that are freeze-dried after suspension in EGTA Tris-HCl buffered solution (ETBS) tend to retain their chromosomal integrity, resulting in an increase in normal fetal development [2,5]. The effects of ETBS on the maintenance of chromosomal integrity have been studied using mouse spermatozoa treated with detergent [6] and antioxidants [7].

Ionizing radiation induces damage to bioactive molecules, not only via direct effects (e.g., an ionization track through DNA), but also due to indirect effects, e.g., the production of water-derived radicals, such as the hydroxyl radical, which injure to DNA molecules in the presence of water molecules. Dry DNA and DNA solution containing hydroxyl radical scavengers are damaged only by the direct effects of ionizing radiation [8]. Therefore, freeze-dried sperm DNA may not be damaged by indirect effects, but can be damaged by direct effects. However, no report to date has examined chromosomal damage to mammalian spermatozoa induced by ionizing radiation without indirect effects.

In this study, we determined the frequencies of chromosomal aberrations in mouse (B6D2F1) oocytes injected with γ-ray-irradiated spermatozoa before and after freeze-drying. Our results are discussed here with respect to the resistance of freeze-dried spermatozoa to

radiation and the relevance of the direct effects of radiation in inducing chromosomal damage.

2. Materials and Methods

2.1. Animals

B6D2F1 hybrid male and female mice, 7-12 weeks of age, were used in this study. The animals were maintained under a 14-h light/10-h dark photoperiod at a temperature of 22-24^{o}C. Food and water were provided *ad libitum*.

2.2. Chemicals

All chemicals were obtained from Nakalai Tesque unless otherwise stated. The medium used for the preparation of oocytes and sperm injection was Hepes-CZB medium [3], which was a modified CZB medium [9] with 20 mM Hepes, 5 mM $NaHCO_3$, and 0.1 mg/ml polyvinyl alcohol (PVA; cold water soluble; molecular weight: 30,000-50,000; Sigma) instead of BSA.

2.3. Sperm collection and freeze-drying

Two cauda epididymes were removed from a male and punctured with sharply pointed forceps. A dense mass of spermatozoa was squeezed out of the cauda region and placed in a 1.5 ml polypropylene centrifugation tube containing 0.5 to 1 ml of EGTA Tris-HCl buffered solution (ETBS) that was prepared according to a previously described method [2]. The tube was left standing for 10 min at 37^{o}C to allow the sperm to swim up. The sperm suspension (0.1 ml) was transferred into 2-ml glass ampoules (Wheaton Scientific). The ampoules were plunged into liquid nitrogen and then were connected to a freeze-drying machine (type: FZ2.5, Labconco, Kansas City, MO, USA). The pressure inside of the ampoules at the time of flame-sealing was 22-38 x 10^{-3} mbar. The sealed ampoules were preserved in a refrigerator at 4^{o}C for 1 to 44 days.

The ampoules containing the freeze-dried spermatozoa (Fig. 1a) were irradiated with a

single dose (4 Gy and 8 Gy) of ^{137}Cs γ-ray (Gamma Cell 40, 0.89 Gy/min). Some ampoules were not freeze-dried, but were irradiated. The doses of irradiation were regarded as sufficient or in excess of that required to induce chromosomal damage in murine [10] and human spermatozoa [11].

2.4. Intracytoplasmic sperm injection (ICSI)

ICSI was carried out according to previously described procedures [3], with some modifications [2]. To collect the mouse oocytes, female mice were superovulated with an i.p. injection of 10 units of pregnant mare's serum gonadotrophin (Teikokuzouki, Japan) and 10 units of human chorionic gonadotrophin (Mochida, Japan) given 48 h apart. Cumulus-free oocytes were placed in droplets of Hepes-CZB medium.

A small amount (0.05 to 0.1 ml) of distilled water was added to glass ampoules containing freeze-dried spermatozoa. As soon as the spermatozoa were rehydrated, the sperm suspension was applied into a small drop (less than 10 μl) of Hepes-CZB medium supplemented with PVP under a paraffin oil (Merck) overlay. A single spermatozoon was drawn tail first into an injection pipet. At the opening of the pipet, the sperm head was separated from the tail by applying one or more piezoelectric pulses [3]. A sperm head was injected into an oocyte that was drawn by a holding pipet. In most cases, sperm heads and tails of many freeze-dried spermatozoa were separated, therefore, a sperm head without tail was just picked up with an injection pipet and injected into an oocyte. ICSI was carried out within 1 h after rehydration.

2.5. Culture of sperm-injected oocytes

Sperm-injected oocytes were transferred into droplets (50-100 μl) of modified CZB medium [9] supplemented with 5.56 mM glucose and 5 mg/ml BSA (fraction V, Sigma) under a paraffin

oil (Merck) overlay in a humidified atmosphere of 5% CO_2 in air.

2.6. Chromosome analysis

Chromosome specimens of mouse oocytes injected with mouse spermatozoa were prepared at the first cleavage metaphase [12-14] (Fig. 1b). At 5-6 h after ICSI, oocytes were placed into a modified CZB medium containing 0.006 μg/ml vinblastine to arrest the metaphases of the first cleavage. After treatment with vinblastine for 16-18 h, the zona pellucida was removed with 0.5% pronase (1000 units/mg, Kaken Pharmaceuticals, Tokyo) before placing the oocytes in a hypotonic citrate solution (1:1 mixture of 30% FBS and 1% sodium citrate)[12,13]. Fixation of oocytes and spreading of chromosomes onto glass slides were performed according to the methods described previously [14]. The chromosomes on slide specimens were stained with 2% (v/v) Giemsa solution (Merck) for 8 min. Structural chromosome aberrations, i.e., chromatid and chromosome breaks and exchanges, were scored as previously described [15]. Uncountable numbers of structural aberrations such as chromosome fragmentation and multiple exchanges were arbitrarily assigned as 10 aberrations per oocyte. The number of aberrations per oocyte was recorded without discriminating between paternal and maternal chromosome sets, because it was difficult to determine the paternal origin in both chromosome sets.

2.7. Analysis of data

The number of ICSI oocytes with normal metaphase plates was counted before and after γ-ray irradiation, and the results were compared using the chi-square test and Yates' correction for continuity.

Results

Most of the γ–irradiated spermatozoa were able to activate the mouse oocytes before and after freeze-drying (activation rate: 95% or more, Table 1). In non-irradiated spermatozoa, the frequency of normal metaphase plates (chromosomal integrity) was lower in the freeze-dried spermatozoa (73%, Table 2) than it was in fresh spermatozoa before freeze-drying (98%, Table 2). No. of structural chromosome aberrations per oocyte also shows extremely higher (more than a factor of 20) in freeze-dried spermatozoa than in the spermatozoa before freeze-drying (Table 2). In addition, chromosomally damaged oocytes after injection with freeze-dried spermatozoa tend to have a large number of chromosome aberrations such as multiple aberrations (Table 2).

The chromosomal integrity decreased dose-dependently in oocytes injected with spermatozoa that had been irradiated before freeze-drying (98% to 32%, $p < 0.01$, Table 2). However, no significant difference in chromosomal integrity was observed between freeze-dried spermatozoa that had been exposed to γ–irradiation and those that had not been exposed to the irradiation (60% to 73%, Table 2 and Fig. 1b).

The net incidences of oocytes with chromosome aberrations were calculated by a formula described previously [10,16,17] (Fig. 2). Large differences in the net incidence of oocytes with chromosome aberrations can clearly be seen between γ-irradiated spermatozoa before and after freeze-drying (Fig. 2). The net incidences are 2.8-times (4 Gy) and 5.0-times (8 Gy) higher in the spermatozoa before freeze-drying than in the freeze-dried spermatozoa (Fig. 2). Although the net incidence of oocytes with chromosome aberrations increased slightly among spermatozoa irradiated after freeze-drying, no dose-dependence was observed (Fig. 2).

Discussion

This paper is the first report demonstrating the extent to which freeze-dried mouse spermatozoa are affected by γ-rays. In mouse spermatozoa both before and after freeze-drying, a high rate of activation of oocytes (Table 1) was observed, which suggested that sperm-born oocyte activating factor(s) (SOAF) [18] induced no damage at doses of up to 8 Gy. In addition, the chromosomal integrity of freeze-dried spermatozoa was not found to be affected by ^{137}Cs γ-ray irradiation at up to 8 Gy. The level of chromosomal integrity in γ-irradiated spermatozoa after freeze-drying (60% and 63%, Table 2) is considered to be sufficient to obtain normal fetuses [2,5,19].

Freeze-drying *per se* induces damage to the sperm plasma membrane [2]. Prolonged exposure of membrane-damaged spermatozoa to culture media is detrimental to chromosomal stability [12]. In this study, chromosome aberrations were observed at the high background frequency in oocytes injected with non-irradiated spermatozoa after freeze-drying (Table 2). But the frequency (73%) (Table 2) was found to be similar to those observed in previous reports [2,19]. Therefore, the background frequency of chromosome aberrations is regarded to be held to a minimum. In the case, the net incidence of oocytes with chromosome aberrations is useful to show the actual frequency of γ-ray-induced chromosome aberrations (Fig. 2).

We were unable to identify direct effects (e.g., an ionization track through DNA) of γ-ray irradiation at doses of up to 8 Gy on the freeze-dried mouse spermatozoa. However, the net incidence of oocytes with chromosome aberrations increased slightly in spermatozoa γ-irradiated after freeze-drying (Fig. 2), which indicated the putative direct effect of γ-irradiation. It also remains possible that DNA damage that does not lead to an increase in chromosomal damage may accumulate in freeze-dried sperm DNA exposed to γ-ray irradiation at doses of up to 8 Gy.

Recently, studies of freeze-dried spermatozoa have been reported using not only mouse spermatozoa, but also bull [20] and rabbit [21] spermatozoa. Furthermore, normal mouse fetuses were successfully obtained from oocytes injected with mouse spermatozoa that had been desiccated after suspension in ETBS [22]. Thus, both freeze-drying and the desiccation of mammalian spermatozoa are expected to function as supplementary means of sperm cryopreservation using liquid nitrogen. However, in order to evaluate the stability of freeze-dried spermatozoa during the preservation process, it will be necessary to examine whether or not freeze-dried spermatozoa deteriorate as a result of other physical circumstances such as ultraviolet light and non-ionizing radiation (e.g., visible light, microwaves, and ELF [23], etc.). ICSI using freeze-dried mouse spermatozoa has the potential to be a powerful tool for use in studying the direct mutagenic properties of physical circumstances associated with the absence of water and air.

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Table 1 Intracytoplasmic sperm injection (ICSI) in mouse (B6D2F1) spermatozoa irradiated by ^{137}Cs γ-ray before and after freeze-drying

Sperm[1] irradiated: (Gy)	Dose (Gy)	No. of oocytes injected (No. ICSI)	% of oocytes activated[2]
Before FD	0	125 (7)	98
	4	159 (6)	99
	8	125 (5)	100
After[3] FD	0	200 (7)	97
	4	132 (4)	96
	8	152 (5)	95

[1]Spermatozoa were irradiated by γ-ray before and after freeze-drying (FD).

[2]The incidence of activation in oocytes was judged by the presence of a second polar body and two pronuclei.

[3]Glass ampoules containing freeze-dried spermatozoa were irradiated by γ-ray after preservation at 4^{o}C in a refrigerator for 1 to 44 days.

Table 2 Chromosome analysis in mouse (B6D2F1) oocytes injected with spermatozoa irradiated by ^{137}Cs γ-ray before and after freeze-drying

Sperm[1] irradiated:	Dose (Gy)	No. of metaphase plates	No. (%) of normal metaphase plates	No. of structural chromosome aberrations					
				ctb	cte	csb	cse	mul	Total (per oocyte)
Before FD	0	104	102 (98)	0	0	2	0	0	2 (0.019)
	4	102	54 (53)*	8	2	43	5	0	58 (0.57)
	8	94	30 (32)*	10	8	69	12	0	99 (1.1)
After[2] FD	0	158	115 (73)	10	13	30	6	20	79 (0.50)
	4	101	61 (60)	4	9	43	3	0	59 (0.58)
	8	119	75 (63)	14	8	44	3	20	89 (0.75)

Abbreviations, ctb: chromatid break; cte: chromatid exchange; csb: chromosome break; cse: chromosome exchange; mul: multiple aberrations; FD: freeze-drying.

[1]Spermatozoa were irradiated by γ-ray before and after freeze-drying.

[2]Glass ampoules containing freeze-dried spermatozoa were irradiated by γ-ray after preservation at 4^{o}C in a refrigerator for 1 to 44 days.

*Significant difference at $P< 0.01$ (chi-square test using Yates' correction for continuity), compared with control (0 Gy).

Figure 1

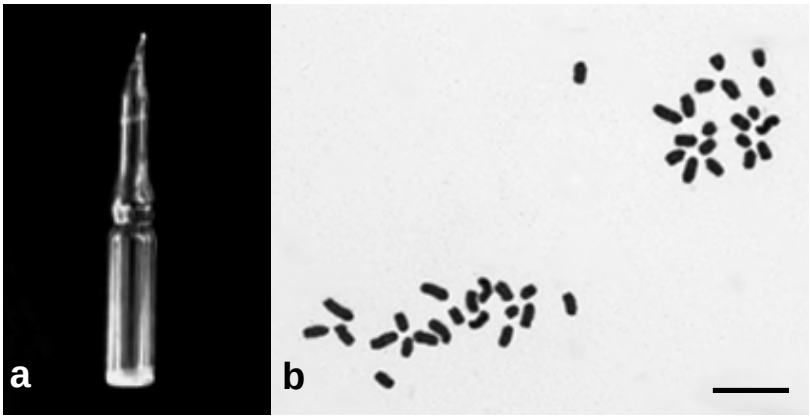

Figure 2

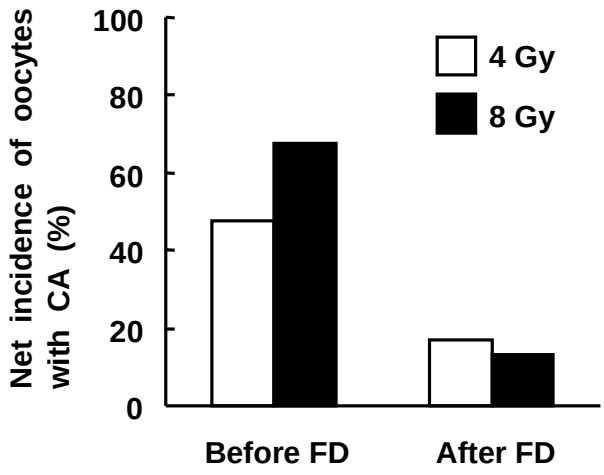
Net incidence of oocytes with CA (%)
100
80
60
40
20
0
4 Gy
8 Gy
Before FD
After FD

Figure Legends

Figure 1

Chromosome analysis of mouse (B6D2F1) oocytes injected with freeze-dried mouse (B6D2F1) spermatozoa. (a) A glass ampoule containing the freeze-dried spermatozoa. The freeze-dried spermatozoa adhered as a thin layer on the bottom of the glass ampoule. (b) A normal metaphase plate of oocytes injected with spermatozoa that had been freeze-dried and then irradiated by ^{137}Cs γ-rays at 8 Gy. The scale bar represents 10 μm.

Figure 2

Net incidence of oocytes with chromosome aberrations (CA) that had been injected with mouse spermatozoa irradiated at 4 Gy and 8 Gy before and after freeze-drying. The net incidence (%) was calculated using the following formula: [1-N(irradiated)/N(control)] x 100, N =(no. normal metaphase plates)/(no. total metaphase plates analyzed).