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Mouse and human spermatozoa can be freeze-dried without damaging their chromosomes

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BACKGROUND: Although mouse spermatozoa can be freeze-dried without losing their reproductive capacity, the technique needs further improvements to reduce the incidence of chromosomal damage to spermatozoa. Effects of freeze-drying on human spermatozoa are unknown. METHODS: Mouse spermatozoa were suspended in a Tris-buffered EGTA solution briefly (10 min at 37°C) or for 1–7 days at 4°C before freeze-drying. Freeze-dried spermatozoa were maintained for up to 1 year at 4°C before injection. Sperm chromosomes were examined during the first mitosis (cleavage) of zygotes. The ability of sperm to support embryo development was assessed by examining mid-gestation fetuses (Day 14) after transfer of 2-cell embryos to surrogate mothers. Chromosome integrity of freeze-dried human spermatozoa was examined by injecting individual spermatozoa into mouse oocytes which were previously enucleated. RESULTS: When mouse spermatozoa were freeze-dried immediately after suspension in Tris-buffered EGTA solution, only c.40% had normal chromosomes. When the mouse spermatozoa were kept in the same solution for 3–7 days before freeze-drying, 85–95% had normal chromosomes and they were able to support embryo development better than those which were in the solution briefly ($P < 0.05$). Freeze-dried human spermatozoa well maintained their chromosomes regardless of the duration of pre-freeze-drying incubation of spermatozoa in the Tris-buffered EGTA solution. CONCLUSIONS: Prior incubation of mouse spermatozoa in Tris-buffered EGTA solution for several days makes sperm chromosomes more resistant to freeze-drying. As the consequence, spermatozoa freeze-dried this way support embryo development better than those exposed to Tris-buffered EGTA solution only briefly. Freeze-dried human spermatozoa well maintained their chromosomes without pre-freeze-drying incubation in Tris-buffered EGTA solution.

Keywords: ICSI; freeze-drying; human sperm; mouse sperm; chromosome

Introduction

Long-term preservation of mammalian spermatozoa is now possible by various methods including: (i) deep freezing with cryoprotection (Polge *et al*., 1949; Nakagata, 2000); (ii) deep freezing without cryoprotection (Wakayama *et al*., 1998b; Ward *et al*., 2003); (iii) freeze-drying (Wakayama and Yanagimachi, 1998; Kusakabe *et al*., 2001); (iv) drying without freezing (Bhowmick *et al*., 2003; McGinnis *et al*., 2005; Li *et al*., 2007) and (v) freezing of whole animal body or of isolated testes (Ogonuki *et al*., 2006). In all but (i), spermatozoa must be injected microsurgically into oocytes because they are motionless or "dead" in the conventional sense. An advantage of freeze-drying or drying spermatozoa is obvious: neither liquid nitrogen nor dry ice is needed for the storage and shipment of preserved spermatozoa. Someday, it could be possible to store spermatozoa indefinitely at ambient temperature and ship them to any parts of the world without dry ice or liquid nitrogen.

Ward *et al*. (2003) compared chromosome integrity and reproductive potential of freeze-dried mouse spermatozoa with those frozen in liquid nitrogen without any cryoprotection. When examined 12 months after storage of freeze-dried spermatozoa at 4°C, the frozen spermatozoa stored in liquid nitrogen supported embryo development better than the freeze-dried spermatozoa stored at 4°C. Structural chromosome aberrations were less common in frozen spermatozoa than in freeze-dried spermatozoa. We report here that chromosome damage in freeze-dried mouse spermatozoa can be alleviated when spermatozoa are kept in an EGTA solution for several days (at 4°C) before freeze-drying. To our surprise, human spermatozoa do not require this extra step to maintain their chromosome integrity.

Materials and Methods

Animals

Oocytes and spermatozoa were collected from hybrid mice ($B6D2F_1$) at 7–12 weeks of age. Fully mature albino females [Crlj:cD-1(ICR)] were used as surrogate mothers after mating with vasectomized albino males [Crlj:cD-1(ICR)] the day before embryo transfer. The females were

maintained under 14-h light/10-h dark cycles at 22–24°C. Food and water were given *ad libitum*. All animals were handled and euthanized according to the animal study protocol approved by the Laboratory Animal Committee, Asahikawa Medical College.

Media

All chemicals were obtained from Nacalai Tesque, Inc. (Kyoto, Japan) unless otherwise stated. The medium for oocyte collection and sperm injection was a modified CZB medium (Chatot *et al*., 1989; Chatot *et al*., 1990) with 20 mM HEPES, 5 mM $NaHCO_3$, and 0.1 mg/ml polyvinyl alcohol (cold water soluble; molecular weight: 30 000–70 000, Sigma Chemical Co. Ltd., St. Louis, MO, USA) instead of bovine serum albumin (BSA) (termed HEPES-CZB) (Kimura and Yanagimachi, 1995). EGTA medium previously used (Kusakabe *et al*., 2001) was modified by eliminating NaCl, keeping EGTA constant at 50 mM and increasing the concentration of Tris–HCl: it consisted of 50 mM EGTA and 100 mM Tris–HCl. This medium, called Tris-buffered EGTA, was prepared by mixing 1 ml of 0.5 M EGTA (pH 8.0, adjusted with NaOH) and 1 ml of 1 M Tris–HCl (Trizma®-HCl, pH 7.4, Sigma-Aldrich) and diluting the mixture with water (Water for Embryo Transfer, Sigma-Aldrich) up to 10 ml. Its final pH was 7.7–7.9. This medium was used within 30 days after preparation.

Collection of mouse spermatozoa

Dense masses of spermatozoa were collected from the cauda epididymis and placed at the bottom of a 1.5 ml polypropylene microcentrifuge tube (SARSTEDT, Aktiengesellschaft & Co., Numbrecht, Germany) containing 1.2 ml of Tris-buffered EGTA that had been warmed to 37°C. The tube was left standing for 10 min at 37°C to allow spermatozoa to swim into the solution. The upper 1 ml of the sperm suspension was transferred into another tube and was kept in a refrigerator (4°C) for up to 16 days before freeze-drying. Live/dead staining of spermatozoa was performed using FertiLight (Molecular Probes, Inc., Eugene, OR, USA).

Collection of human spermatozoa

Semen sample collected from two healthy men (donors A and B) was allowed to liquefy at 37°C for 30 min. A 0.5 ml aliquot was gently placed at the bottom of a small test tube containing 2 ml of Tris-buffered EGTA that had been pre-warmed to 37°C. The tube was left standing for 10 min at 37°C to allow spermatozoa to disperse into the medium. One ml of the upper layer containing the spermatozoa was transferred into another test tube.

Freeze-drying

One-hundred μl of each sperm suspension as prepared above was put in a 2 ml glass ampoule (Wheaton Scientific, Millville, NJ, USA). Each ampoule was plunged into liquid nitrogen and kept there for 1 min before connection to a lyophilizer (type: fZ2.5, Labconco, Kansas City, MO, USA). After vacuuming for 4 h, each ampoule was flame-sealed and preserved at c.4°C (range of temperature: 3–7°C) in a refrigerator. The inside pressure of ampoules at the time of sealing was $22–42 \times 10^{-3}$ mbar.

Preparation of oocytes

Females were each injected with 10 IU of pregnant mare's serum gonadotrophin (Teikokuzohki, Tokyo, Japan). Forty-eight hours later, they were injected with 10 IU of HCG (Mochida, Tokyo, Japan). Mature metaphase II oocytes were collected from oviducts between 15 and 17 h after HCG injection. Oocytes were freed from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (1000 units/mg, Sigma-Aldrich) in the HEPES-CZB medium, then rinsed and kept in modified CZB medium at 37°C for <60 min before sperm injection.

ICSI

ICSI was carried out according to Kimura and Yanagimachi (1995) except that it was performed at 18–25°C. Freeze-dried spermatozoa in each ampoule were rehydrated by adding 100 μl of water (Water for Embryo Transfer, Sigma-Aldrich) immediately after opening the ampoule. All rehydrated spermatozoa were immotile. They often had broken or missing tails. Those without tails (heads only) were individually injected into each oocyte. Those with broken or "intact" tails were freed from tails by applying a single or a few Piezo pulses to the neck region of the spermatozoon and only the head was injected into each oocyte (Kuretake *et al*., 1996). Freeze-dried and rehydrated human spermatozoa had grossly "intact" tails. As human spermatozoon was much smaller than mouse spermatozoon, the entire body of human spermatozoon was injected into each oocyte. Prior to sperm injection, mouse oocytes were enucleated after 10 min treatment with 5 μM cytochalasin B (Wakayama *et al*., 1998a). Enucleation was done to avoid ethical criticism of creation of an embryo with human and mouse genomes. Theoretically, enucleation is unnecessary because human and mouse chromosomes are markedly different from each other in their structures and therefore mouse oocytes injected with human spermatozoa would not develop beyond the two-cell stage. ICSI was completed within 1 h after rehydration of freeze-dried mouse and human spermatozoa.

Culture and examination of mouse oocytes injected with spermatozoa

Sperm-injected mouse oocytes were transferred into droplets (50–100 μl) of the modified CZB medium supplemented with 5.56 mM glucose and 5 mg/ml BSA (fraction V, Sigma) under paraffin oil (Merck KGaA, Darmstadt, Germany) in a Petri dish. The dish was maintained under a humidified atmosphere of 5% CO_2 in air. Eggs with the second polar body and two pronuclei 5–6 h after ICSI were considered normally activated. Eggs at the 2-cell and blastocyst stages were examined 24 and 96 h after ICSI, respectively. When an enucleated mouse oocyte was injected with a human spermatozoon, only one large pronucleus appeared in the oocyte. This was the pronucleus of sperm origin. Thus, an oocyte with one pronucleus and one first polar body was considered activated.

Examination of post-implantation development of mouse embryos

Two-cell mouse embryos were transferred into oviducts of pseudo-pregnant females that had mated with vasectomized males during the previous night. Because chromosome damage induced in male germ cells contributes to early post-implantation death (Singer *et al*., 2006), the number of normally developing fetuses and implantation sites were determined in all but one (mouse freeze-dried experiment at 1 year) case on Day 14 of gestation. In practice, this provides information on the extent of early embryonic loss after implantation, and normal fetuses at Day 14 of pregnancy very rarely fail to develop to full term (Kusakabe *et al*., 2001).

Chromosome analysis

Between 5 and 6 h after ICSI, sperm-injected oocytes were transferred to a modified CZB medium containing 0.01 μg/ml vinblastine sulfate to arrest them at the metaphases of the first cleavage. Between 20 and 22 h after ICSI, the oocytes were freed from the zonae pellucidae by treatment with 0.5% protease (Actinase E, 1000 tyrosine unit/mg, Kaken Pharmaceuticals, Tokyo, Japan) followed by 4–10 min treatment with a hypotonic solution composed of a 1:1 mixture of 30% fetal bovine

serum and 1% sodium citrate (Kishikawa *et al*., 1999; Tateno *et al*., 2000). The oocytes were fixed by the gradual fixation method (Kamiguchi and Mikamo, 1986), air-dried on glass slides, and stained with Giemsa's solution (Merck KGaA, Darmstadt, Germany) for chromosome examination. When mouse chromosomes were examined, the structural chromosome aberrations were recorded without discriminating between paternal and maternal origins. Because mouse oocytes seldom had chromosome aberrations at the first mitotic metaphase after normal fertilization and parthenogenetic activation (Tateno and Kamiguchi, 2002), the chromosome groups with aberrant constitution were most likely those of sperm origin. Thus, chromosome integrity of mouse spermatozoa could be expressed by the frequency (%) of zygotes with normal chromosome composition (40 acrocentric or telocentric chromosomes) without structural aberrations.

Chromosome integrity of human spermatozoa was expressed by the frequency (%) of the spermatozoa with normal chromosome composition (23 chromosomes) without any structural chromosome aberrations. Of all Giemsa-stained chromosome spreads with normal chromosome composition (semen donor A), 20 were randomly selected and further examined after Q-band staining (Caspersson *et al*., 1970).

Statistical analysis

Comparisons of chromosomal integrity of spermatozoa, *in vitro* development and post-implantation development of embryos were made by chi-square analysis using Yate's correction for continuity. Significant differences were accepted with $P < 0.05$.

Results

Ability of mouse spermatozoa to activate oocytes after freeze-drying in Tris-buffered EGTA

The majority of mouse spermatozoa in Tris-buffered EGTA became immotile by 10 min, but they were "alive" (had intact plasma membranes) according to the Live/Dead staining (Fig. 1a). No spermatozoa were alive when they were kept in Tris-buffered EGTA at 4°C for 6 days (Fig. 1b). The spermatozoa which were freeze-dried after 4–6 days storage in Tris-buffered EGTA (Fig. 1c) activated oocytes very well, but those kept in the Tris-buffered EGTA for 7 days or more had inferior ability to activate oocytes (Fig. 1d).

Chromosomes of freeze-dried mouse spermatozoa

Fig. 2 shows two typical examples of chromosomes seen on metaphase plates of the first mitotic division (cleavage). Either two groups (Fig. 2a) or one group (Fig. 2b) had normal haploid set (20) of chromosomes. As oocyte chromosomes seldom show abnormalities during normal fertilization and parthenogenetic activation (Tateno and Kamiguchi, 2002), the chromosome group with abnormal constitutions is most likely of sperm origin.

Analysis of chromosomes that appeared on the metaphase plates of the first mitotic division after injection of freeze-dried mouse spermatozoa revealed that the incidence of zygotes with normal chromosomes increased (from 41 to 95%) as the spermatozoa were kept in Tris-buffered EGTA for more than 2 days before

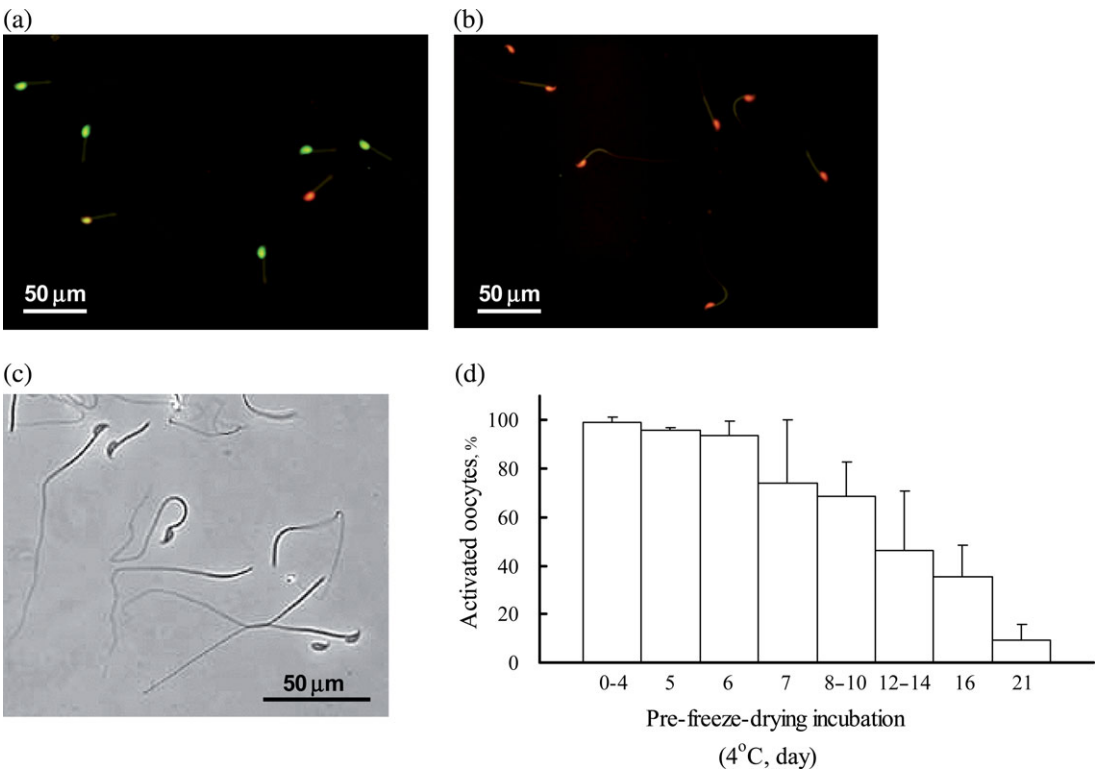

Figure 1: Freeze-dried mouse spermatozoa after Live/Dead staining
(**a**) Most spermatozoa immediately after suspension in Tris-buffered EGTA were stained green (alive), (**b**) whereas those kept in the same solution for 6 days at 4°C were stained red (dead). (**c**) Freeze-dried spermatozoa after rehydration; most had broken tails; heads of some spermatozoa were completely separated from tails. (**d**) A diagram showing that the ability of freeze-dried mouse spermatozoa to activate oocytes by ICSI decreases when incubation of spermatozoa prior to freeze-drying is for longer than 6 days. The values are expressed as mean ± SD

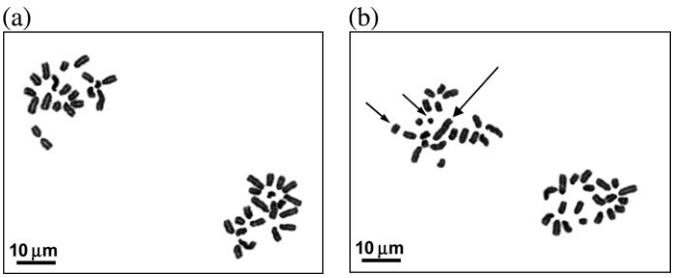

Figure 2: Chromosome spreads of oocytes injected with freeze-dried mouse spermatozoa
(**a**) Chromosomes from two pronuclei (of sperm and oocyte origin) have normal chromosomes. (**b**) The group of chromosomes with abnormal constitution is most likely of sperm origin. Small arrows indicate chromosome fragments derived from chromosome break or chromosome exchange. A large arrow indicates chromosome exchange

freeze-drying (Table I). In other words, spermatozoa became more resistant to freeze-drying as they were maintained in Tris-buffered EGTA longer. Chromosome aberrations most commonly found after brief incubation of spermatozoa in Tris-buffered EGTA were chromosome-type breaks or exchanges (Fig. 2b).

Development of oocytes injected with freeze-dried mouse spermatozoa

Development of mouse oocytes injected with freeze-dried and fresh (non-frozen) spermatozoa was compared. Freeze-dried spermatozoa used in this study were those kept in Tris-buffered EGTA solution for 5–7 days before freeze-drying; the majority of these spermatozoa had normal chromosomes (Table I). Regardless of the period of storage at 4°C, freeze-dried spermatozoa were as good as fresh (non-frozen) spermatozoa in supporting preimplantation development and even better than fresh (non-frozen) spermatozoa in supporting post-implantation development to mid-term fetuses (Table II, Fig. 3a and b).

Chromosomes of freeze-dried human spermatozoa

When freeze-dried human spermatozoa were rehydrated, their gross morphology was normal (Fig. 4a). Many spermatozoa freeze-dried immediately after the sperm collection and those freeze-dried after incubation in buffer had normal chromosomes (Fig. 4b, Table III). Most commonly observed chromosome aberrations were breaks (Table III). Q-banded 20 chromosome spreads of freeze-dried human spermatozoa did not show any structural chromosome aberrations (Fig. 4c and d).

Table I. Chromosome integrity of mouse zygotes produced by injection of oocytes with frozen and freeze-dried spermatozoa.

Mouse spermatozoa injected	Pre-freeze-drying incubation in Tris-buffered EGTA solution (4°C)	Storage period of freeze-dried spermatozoa (4°C)	No. zygotes analyzed (Exp. no.)	No. (%) zygotes with normal chromosome composition [range]
Fresh (non-frozen)[a]	–	–	80 (3)	72 (90)[d] [84–92]
Frozen-thawed[b]	–	–	75 (3)	65 (87)[d,e] [81–100]
Freeze-dried[c]	0 min	1–3 days	78 (4)	32 (41)[f] [36–53]
Freeze-dried	1 days	1–3 days	75 (3)	33 (44)[f] [35–50]
Freeze-dried	2 days	2–5 days	82 (3)	63 (77)[e] [75–78]
Freeze-dried	3–4 days	4–5 days	121 (6)	107 (88)[d] [79–95]
Freeze-dried	5–7 days	2–14 days	93 (4)	79 (85)[d] [81–89]
Freeze-dried	5–7 days	2–4 months	93 (5)	88 (95)[d] [85–100]

[a]Spermatozoa were suspended in (allowed to swim into) Tris-buffered EGTA solution for 10 min at 37°C before injection into oocytes; [b]Spermatozoa, suspended in Tris-buffered EGTA solution for 10 min at 37°C, was snap-frozen with liquid nitrogen, then thawed before injection into oocytes; [c]Spermatozoa were suspended in Tris-buffered EGTA solution for 10 min at 37°C before freeze-drying; [d–f]Significantly different values ($P < 0.05$) between different superscripts.

Table II. Development of mouse oocytes injected with spermatozoa which were freeze-dried after 5–7 days of incubation in Tris-buffered EGTA (4°C).

Mouse spermatozoa injected	Storage period of freeze-dried spermatozoa (4°C)	No. oocytes survived and activated after sperm injection (No. exp.)	No. (%) oocytes developed into		No. 2-cell embryos transferred (No. recipients)	Examination on Day 14 of gestation	
			2-Cell embryos	Blastocysts		No. (%) implants[a]	No. (%) normal fetuses [range, %]
Fresh (non-frozen)[b]	–	111 (5)	111 (100)[c]	90 (81)[c]	–	–	–
Fresh (non-frozen)[b]	–	85 (4)	82 (96)[c,d]	–	76 (5)	56 (74)[c]	33 (43)[c] [13–69]
Freeze-dried	2–6 weeks	119 (4)	116 (97)[c,d]	97 (82)[c]	–	–	–
Freeze-dried	4 days to 3 weeks	66 (3)	64 (97)[c,d]	–	63 (4)	56 (89)[d]	38 (60)[c,d] [44–77]
Freeze-dried	3 months	101 (4)	97 (96)[c,d]	85 (84)[c]	–	–	–
Freeze-dried	3 months	76 (4)	71 (93)[d]	–	71 (4)	62 (87)[c,d]	41 (58)[c,d] [40–76]
Freeze-dried	1 year	88 (3)	84 (95)[c,d]	–	84 (6)	74 (88)[d]	54 (64)[d] [50–77][e]

[a]Absorbed fetuses plus normal fetuses; [b]Spermatozoa were suspended in (allowed to swim into) Tris-buffered EGTA solution for 10 min at 37°C; [c,d]Significantly different values ($P < 0.05$) between different superscripts within each column; [e]Examined on Day 18 of gestation.

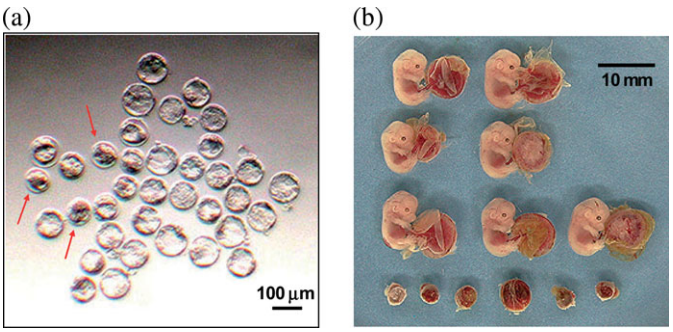

Figure 3: Embryos developed from oocytes injected with freeze-dried mouse spermatozoa
(**a**) Blastocysts developed from the oocytes injected with spermatozoa freeze-dried after incubation in Tris-buffered EGTA for 6 days. Arrows indicate the degenerated embryos. (**b**) Seven normal fetuses and six implantation sites at day 14 of gestation, developed from thirteen 2-cell embryos transplanted into a surrogate mother. These 2-cell embryos were developed from oocytes injected with mouse spermatozoa freeze-dried after incubation in Tris-buffered EGTA for 6 days

Discussion

We previously reported that the incidence of mouse spermatozoa with normal chromosomes was lower when they were freeze-dried (68%) than when were frozen-thawed without cryoprotection (89%) (Ward *et al*., 2003). This was confirmed in the present study. The majority (87–90%) of fresh (non-frozen) or frozen-thawed spermatozoa had normal chromosomes, whereas less than half (41%) of spermatozoa had normal chromosomes when they were freeze-dried after brief suspension in Tris-buffered EGTA (Table I). Interestingly, the proportion of freeze-dried spermatozoa with normal chromosomes increased (77–95%) when spermatozoa were kept in Tris-buffered EGTA for more than 2 days before freeze-drying (Table I). This was quite unexpected because we previously thought that nuclei (chromosomes) of spermatozoa and spermatids became degraded quickly in artificial media when plasma membranes were disrupted or removed (Suzuki *et al*., 1998; Yanagimachi, 2005). We did not expect that spermatozoa retained their plasma membranes intact for many days in such an unphysiological solution (Fig. 1b). The reason why prolonged immersion of spermatozoa in Tris-buffered EGTA makes sperm nuclei more resistant to freeze-drying is not clear. EGTA may slowly penetrate into sperm nuclei and chelate intra-nuclear Ca^{2+}. During rehydration of freeze-dried spermatozoa, sperm nuclear topoisomerase that cleaves DNA in the presence of Ca^{2+} (Shaman *et al*., 2006) may be silenced

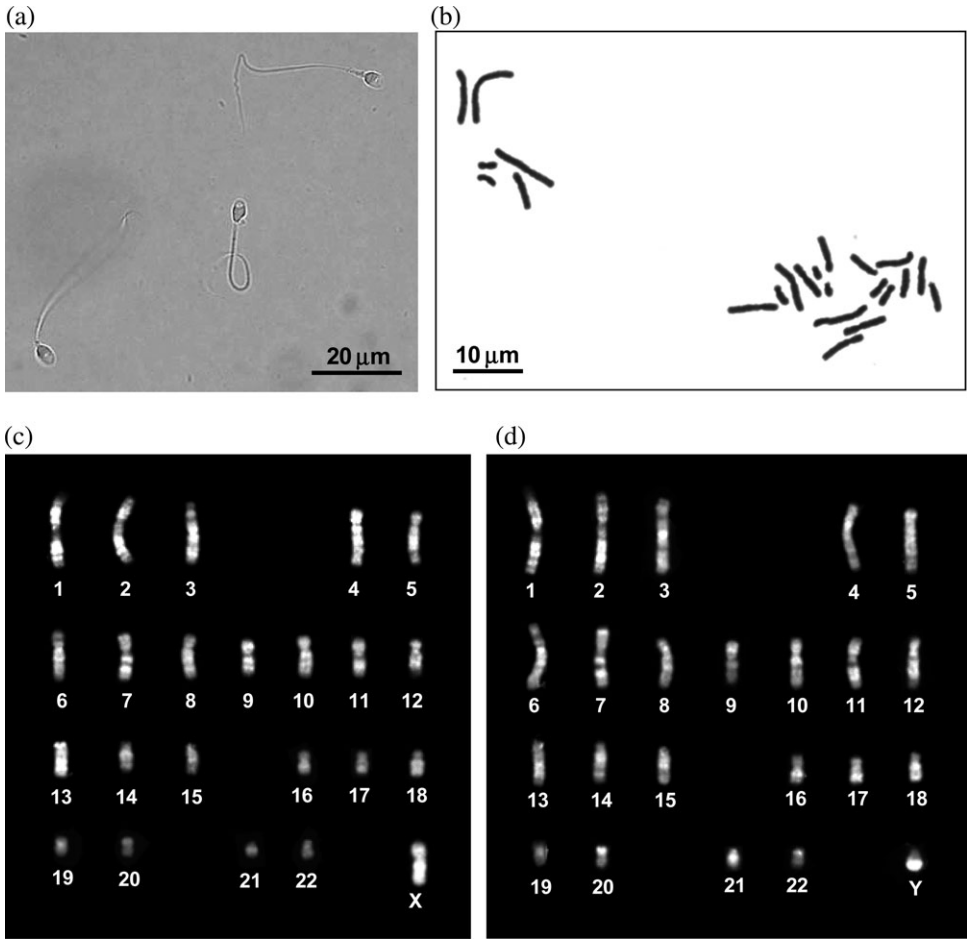

Figure 4: Freeze-dried human spermatozoa and their chromosomes
(**a**) Freeze-dried spermatozoa rehydrated. (**b**) Human sperm chromosomes with normal constitution that appeared at the first mitotic metaphase after injection of a single freeze-dried spermatozoon into an enucleated mouse oocyte. (**c**) and (**d**), Normal Q-banded chromosomes of a freeze-dried X-bearing spermatozoon (c) and a Y-bearing spermatozoon (d)

Table III. Chromosome analysis of freeze-dried human spermatozoa after injection into enucleated mouse oocytes.

Semen donor	Pre-freeze-drying sperm incubation in Tris-buffered EGTA (4°C)	Storage period of freeze-dried spermatozoa (4°C)	No. of oocytes injected with spermatozoa (No. exp)	No. (%) oocytes activated and reached metaphase of first mitosis	No. (%) oocytes with normal sperm chromosome	No. spermatozoa with chromosome aberrations						
						Structural aberrations					Aneuploidy	
						ctb	cte	csb	cse	mul	hyper	hypo
A	0 min[a]	1 day	71 (4)	63 (89)[b]	56 (89)[d]	0	0	7	0	0	0	0
A	0 min[a]	3–7 days	71 (5)	61 (86)[b,c]	58 (95)[d]	0	0	3	0	0	0	0
A	0 min[a]	4 weeks	74 (5)	67 (91)[b]	63 (94)[d]	1	0	2	1	0	0	0
A	6–7 days	3 weeks	78 (5)	57 (73)[c]	50 (88)[d]	0	0	6	1	0	0	0
B	0 min[a]	3 days to 4 weeks	62 (4)	57 (92)[b]	49 (86)[d]	0	0	6	1	1	0	0
B	4 h	4 days to 4 weeks	66 (4)	58 (88)[b,c]	49 (84)[d]	1	2	5	1	0	0	0
B	2 days	17 days to 4 weeks	56 (4)	41 (73)[c]	33 (80)[d]	0	1	5	0	2	0	0

[a]Spermatozoa were suspended in (allowed to swim into) Tris-buffered EGTA solution for 10 min at 37°C before freeze-drying; [b,c]Significantly different values ($P = 0.05$) between different superscripts within the data from 'A' or 'B'; [d]Not significant difference ($P = 0.05$) within the data from 'A' or 'B'. csb, chrmosome break; cse, chromosome exchange; ctb, chromatid break; cte, chromatid exchange; hype, hyperploidy; hypo, hypoploidy; mul, multiple aberrations.

by EGTA. Why do human spermatozoa withstand freeze-drying without long periods in Tris-buffered EGTA? It is known that human spermatozoa are unusual in that their nuclear chromatin is less intensely crosslinked by disulfide (S-S) bonds than that of spermatozoa of most other mammals (Bedford *et al*., 1973). SS-poor human sperm nuclei may be more readily penetrable by EGTA than SS-rich sperm nuclei of spermatozoa of other species. EGTA we used in this study does chelate Ca^{2+}, but not Zn^{2+}. Since human sperm chromatin contains 8 mM Zn^{2+} (Bjorndahl and Kvist, 2003) and EGTA does not chelate Zn^{2+}, EGTA may protect chromatin SH from oxidizing into superfluous S-S bridges during handling *in vitro* which otherwise could affect sperm chromatin decondenzation and further development into metaphase chromosomes.

In the present study, we examined sperm chromosomes by allowing sperm nuclei to transform into metaphase chromosomes of the first mitosis (cleavage) after injection into oocytes. About 10% of non-frozen spermatozoa from normal, fertile mouse males showed chromosome aberrations (Table I), which was at about the same as previously reported (Tateno *et al*., 2000, Ward *et al*., 2003; Tateno and Kamiguchi, 2004). Similarly, ~10% of non-frozen human spermatozoa from normal men showed chromosome aberrations after injection into mouse oocytes (Table III). This level of sperm chromosome aberration was reported previously after injection of human spermatozoa into mouse oocytes (Lee *et al*., 1996; Rybouchkin *et al*., 1996; Watanabe, 2003) or after IVF of zona-free hamster oocytes with human spermatozoa (Rudak *et al*., 1978; Martin *et al*., 1982, 1983; Brandriff *et al*., 1985; Kamiguchi and Mikamo, 1986; Kusakabe *et al*., 1996). The level of chromosome aberrations in mouse and human spermatozoa *in vivo* may be higher or lower depending on individuals. Aberrations at 10% level are likely inherent to the techniques employed (mechanical injection of sperm into oocytes or fusion of sperm with eggs of foreign species). Therefore, >80% normality of sperm chromosomes after freeze-drying of mouse and human spermatozoa (Tables I and III) indicates that freeze-drying itself is not detrimental to sperm chromosomes, as long as the process is it is carried out properly.

Ability of mouse spermatozoa to activate oocytes decreased after the pre-freeze-drying incubation for more than 7 days in Tris-buffered EGTA (Fig. 1d). This is likely due to deterioration of sperm-born oocyte-acting factors (SOAFs). The nature of mammalian SOAF has been the subject of intense studies. Thus far, strong candidates include phospholipase C zeta (Cox *et al*., 2002; Saunders *et al*., 2002), truncated c-kit thyrosine kinase (Sette *et al*., 1997, 2002) and WW domain-binding protein (Wu *et al*., 2007). At least part of SOAF is localized in the perinuclear theca (Kimura *et al*., 1998; Wu *et al*., 2007). SOAF may not be very stable in EGTA-buffer, but once it is freeze-dried, it seems to retain its biological activity for long time.

Freeze-dried mouse spermatozoa may maintain their chromosome integrity and developmental potential indefinitely at −80°C (Kawase *et al*., 2005) or at −196°C (liquid nitrogen), but constant need of deep freezers or liquid nitrogen is troublesome. According to Kaneko (2006) and Nakagata (2006), mouse spermatozoa freeze-dried in EDTA solution (1 mM EDTA + 10 mM Tris–HCl) well maintained chromosome integrity. Freeze-dried spermatozoa which were kept at 4°C for 1 year were able to produce live offspring at the about the same rate as we reported in the present paper. These investigators did not study whether sperm incubation in EDTA solution renders sperm chromosomes more resistant to freeze-drying. Our ultimate goal is to be able to maintain freeze-dried mammalian spermatozoa at ambient temperature without using refrigerator/freezer or dry ice/liquid nitrogen.

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