Characterization of chromosomal damage accumulated in freeze-dried mouse spermatozoa preserved under ambient and heat stress conditions

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Structural chromosome aberrations and DNA damage generated in freeze-dried mouse spermatozoa were investigated. Freeze-dried sperm samples were preserved at 4, 25 and 50°C for short duration (1 day to 2 months) and at 25°C for long duration (2 years). The spermatozoa were injected into mouse oocytes to analyse the chromosomal abnormalities of the zygotes at the first cleavage metaphase. Chromosome break of the chromosome-type aberrations was the most common type of structural chromosome aberrations observed in all freeze-dried samples. The frequency of chromatid exchanges rapidly increased in freeze-dried spermatozoa preserved at 50°C for 1–5 days. The frequency of chromatid-type aberrations (break and exchange) gradually increased in freeze-dried spermatozoa preserved at 25°C for up to 2 months. Alkaline comet assay revealed significant migration of damaged DNA accumulated in freeze-dried spermatozoa preserved at 50°C for 3 days and 25°C for 2 years. However, no DNA damage was detected using the same sperm samples by neutral comet assay, which can detect mostly DNA double-strand breaks in cellular DNA. These results suggest that DNA single-strand breaks were accumulated in freeze-dried spermatozoa preserved under ambient or heat conditions, and then chromatid-type aberrations, especially the chromatid exchanges, were formed via post-replication repair system in zygotes.

Introduction
Freeze drying of mammalian spermatozoa has great potential as a safe and powerful preservation tool since freeze-dried and vacuum-packed sperm samples possess strong resistance to environmental factors such as gamma-ray irradiation (1). Furthermore, preservation of freeze-dried spermatozoa may prove to be an economical way to maintain cellular genomes without cryostorage.

Full-term development of mammalian oocytes injected with freeze-dried spermatozoa was first reported using laboratory strain mice (2). Developmental competence or chromosomal integrity of zygotes derived from freeze-dried spermatozoa has also been noted in cattle (3), dog (4), hamster (5), human (5–8), pig (9), rabbit (6,10) and rat (11–14). Since the freezing and drying processes are prone to chromosomal damage in mouse spermatozoa, a medium for freeze drying, ethylene glycol tetraacetic acid (EGTA)- and Tris–HCl-buffered solution (ETBS), has been developed to prevent the induction of chromosomal damage (15–17). In addition, mice derived from the spermatozoa freeze dried in ETBS were reported to be stable genomically in subsequent two generations (18).

Recently, we have reported modified ETBS (50-mM EGTA and 100-mM Tris–HCl) as a medium for freeze-drying mouse spermatozoa (7). The protocol for freeze drying involves an incubation step to suspend the sperm in modified ETBS prior to freeze drying (pre-freeze-drying incubation). The pre-freeze-drying incubation plays an important role in preventing chromosomal damage of the spermatozoa. We determined the optimal conditions for the pre-freeze-drying incubation to be 3–7 days at 4°C.

However, we have not yet established a successful method for preserving semi-permanently mammalian spermatozoa at ambient temperatures. DNA damage is accumulated in freeze-dried spermatozoa preserved at room temperature (19,20). In fact, mouse oocytes injected with freeze dried or evaporated mouse spermatozoa preserved at room temperature have lower developmental competency than those preserved at 4°C (2,20–22).

Our aim in the present study was to characterize the chromosomal damage accumulated in freeze-dried spermatozoa preserved at ambient (25°C) or heat (50°C) temperature, the latter being the rather unphysiological condition for fresh spermatozoa. Freeze-dried spermatozoa are also expected to be transported everywhere without any refrigerants such as dry ice. During the transportation, it is preferable that the freeze-dried spermatozoa can withstand the temperature rise up to 50°C that is the approximate maximum temperature in the world. On the basis of accelerated degradation kinetics, long-term stability of freeze-dried samples can be extrapolated using freeze-dried samples preserved for short time at extremely high temperatures (20).

In the present study, the freeze-dried spermatozoa were injected into oocytes to analyse chromosomal aberrations of the zygotes at the first cleavage metaphase. Alkaline and neutral comet assays were also performed to examine whether the heat stress directly targeted DNA in the freeze-dried spermatozoa. Alkaline comet assay can detect alkali-labile sites, single-strand breaks (SSBs) and double-strand breaks (DSBs) in the cellular DNAs, while the neutral comet assay is known to mostly reveal DSBs (23).

From the results of chromosome analysis and comet assays, we discuss the relationship between types of chromosome aberrations and DNA damage accumulated in freeze-dried spermatozoa preserved under ambient or heat condition.

Methods

Animals

Hybrid (B6D2F1) male and female mice (6 weeks of age) were purchased from Sankyo Labo Service (Sapporo, Japan).
mice were maintained on the bedding for laboratory animal (Japan SLC, Hamamatsu, Japan) for 1–6 weeks under a 14-h light/10-h dark photoperiod at a temperature of 22–24°C. Food (MF, solid type, Oriental Yeast, Tokyo, Japan) and water were given *ad libitum*. The mice were euthanized by cervical dislocation just before use under the animal study protocol approved by the Laboratory Animal Committee, Asahikawa Medical University, Japan.

**Media for culture and freeze drying**

All chemicals were obtained from Nacalai Tesque (Kyoto, Japan), unless otherwise stated. The medium for oocyte collection and sperm injection was a modified CZB medium (24,25) with 20-mM HEPES, 5-mM NaHCO3, and 0.1-mg/ml polyvinyl alcohol (cold water soluble; molecular weight 30 000–70 000, Sigma Chemical, St Louis, MO, USA) (HEPES–CZB) (26). Tris-buffered EGTA solution (modified EGTA/Tris–HCl-buffered solution: modified ETBS) used for suspending spermatozoa for freeze drying consisted of 50-mM EGTA and 100-mM Tris–HCl buffer. To prepare the 0.5-M EGTA stock solution, EGTA (Sigma-Aldrich, St Louis, MO, USA) was dissolved with water and adjusted to pH 8.0 with NaOH solution. For working solutions, 1 ml of 0.5-M EGTA and 1 ml of 1-M Tris–HCl, pH 7.4 (DNase-, RNase- and protease-free, purchased as liquid form, Sigma-Aldrich), were diluted with 8-ml water at a final concentration of 50 and 100 mM, respectively.

**Sperm collection and freeze drying**

Freeze drying involved the pre-freeze-drying incubation step (7). Two cauda epididymides of a male were removed and punctured with a sharply forcep. The dense sperm mass was collected from the epididymis and placed in the bottom of a 1.5-ml polypropylene microcentrifuge tube containing 1.2-ml modified ETBS (37°C). The tube was left standing for 10 min at 37°C to allow sperm to disperse by swimming into the solution. The upper 1 ml of the sperm suspension was transferred into another tube. Suspended sperm were incubated for 3–7 days at 4°C in a refrigerator or 1–7 days at 25°C in an incubator (Compact Cool Incubator, ICI-1, As One, Osaka, Japan) prior to freeze drying. After the pre-freeze-drying incubation, 100-µl aliquots were put in 2-ml glass ampoules (Wheaton Scientific, Millville, NJ, USA).

The glass ampoules containing the sperm suspensions were plunged into liquid nitrogen for 1 min, and then connected to a cryovialy (FZ2-5, Labconco, Kansas City, MO, USA). After vacuuming for 4 h, each ampoule was flame sealed and kept in the shade at 25°C in an incubator (Compact Cool Incubator, ICI-1, As One) or 50°C in an incubator (Mini Incubator, IC-150MA, As One). The inside pressure of the glass ampoules at the time of sealing was around 22 mbar.

To prepare positive control samples, the spermatozoa suspended in modified ETBS were treated with an alkylating agent, methyl methanesulfonate (MMS) (Nacalai Tesque, Kyoto, Japan) and an anti-tumour antibiotic, neocarzinostatin (NCS) (Sigma-Aldrich), for 2 h at 37°C prior to freeze drying. For chromosome analysis, the final concentrations of MMS and NCS were set at 100 and 1.0 µg/ml, respectively. For comet assay, those were set at 200 µg/ml (MMS) and 2.0 µg/ml (NCS). NCS directly induces both SSBs and DSBs in plasmid DNAs (27), and mainly chromosome-type aberrations in human spermatozoa (28). MMS induces mainly chromosome breaks and chromatid exchanges in human spermatozoa (29), although it does not directly induce DSBs (30,31).

**Oocyte preparation**

Female mice were injected with 10 units of pregnant mare’s serum gonadotrophin (Asuka Pharmaceutical, Tokyo, Japan). After 48 h, the mice were injected with 10 units of human chorionic gonadotrophin (hCG, Mochida, Tokyo, Japan). Oocytes were collected from oviducts between 15 and 17 h after hCG injection. They were freed from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (999 units/ml solid, Sigma-Aldrich) in HEPES–CZB medium, then rinsed and kept in HEPES–CZB medium at 37°C before sperm injection.

**Intracytoplasmic sperm injection**

Intracytoplasmic sperm injection (ICSI) was carried out as previously described (26) with some modifications. Briefly, the freeze-dried sperm samples were rehydrated by adding 50-µl water to each glass ampoule immediately after breaking off the ampoule neck. All operations were performed at room temperature (18–25°C). For intact sperm, a single spermatozo–ion was picked up with an injection pipette attached to a piezo impact drive unit (Prime Tech, Tsuchiura, Japan). The sperm head was separated from the midpiece and tail by applying one or more piezo pulses. The midpiece and tail were discarded, and the head was injected into an oocyte. ICSI was completed within 1 h after rehydration of freeze-dried spermatozoa.

**Culture of oocytes**

Modified CZB medium was used for culture of sperm-injected oocytes. In the case of pre-freeze-drying incubation at 25°C, most of the freeze-dried spermatozoa lost their ability to activate oocytes. Therefore, sperm-injected oocytes were transferred to droplets (50–100 µl) of a modified CZB medium supplemented with 10-mM strontium dichloride (SrCl2), instead of CaCl2, to activate the oocytes artificially. After culturing for 1 h, the oocytes were transferred to droplets (50–100 µl) of modified CZB medium. The oocytes were then cultured at 37°C under a paraffin oil (Merck KGaA, Darmstadt, Germany) overlaid in a humidified atmosphere of 5% CO2 in air.

**Chromosome analysis**

At 5–6 h from completion of ICSI, sperm-injected oocytes were transferred to modified CZB medium containing 0.01-µg/ml vinblastine sulphate to arrest the metaphases of the first cleavage. At 20–22 h from the completion of ICSI, the zygotes were fixed and air dried on glass slides, and then stained by Giemsa’s solution (Merck KGaA, Darmstadt, Germany) diluted to 4% (v/v) for chromosome analysis (34). 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 partheno-genetic activation (35), chromosome aberrations observed in zygotes derived from freeze-dried spermatozoa were most likely those of the sperm origin. Types of structural

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chromosome aberrations were classified into break and exchange of chromatid and chromosome types. Moreover, zygotes with chromosome fragmentation or pulverization were scored as multiple aberrations.

**Comet assay**

**Alkaline comet assay.** Instead of standard alkaline comet assay, alkaline comet assay with ‘A/N protocol’ (i.e. alkaline DNA unwinding followed by electrophoresis under neutral condition) (30) was carried out according to the procedure as described previously (36). Freeze-dried sperm samples were rehydrated by adding 50- to 70-µl distilled water to each glass ampoule immediately after breaking the ampoule neck. For comet assay, we used the normal melting point agarose (Agarose L03, gelling temperature: 35–37°C, Takara Bio, Otsu, Japan) because it could be held tightly on glass slides. The agarose was dissolved in phosphate-buffered saline (without Ca2+ and Mg2+, pH 6.8) heated by a microwave oven at the concentration of 1% (w/v). The 1% agarose solution was incubated at 50°C for ≥1 h to lower its temperature. Surface on each glass slide was pre-smeared with the 1% agarose solution on a hot plate heated at 70°C. The sperm suspension was mixed with the 1% agarose solution to the final concentration of 0.7%. The mixture (100 µl) was applied on each pre-smeared glass slide warmed at 50°C. Cover slips were put on the slides and then stored at 4°C for 5–10 min. All comet slides were coded in each freeze-dried sample.

After removing the cover slips, the slides were incubated at 4°C for 2 h, and then further 1 h at 37°C in lysis buffer composed of 2.5 M NaCl, 50-mM EDTA–Na, 10-mM Tris–HCl (pH 10), 1% Triton X-100 and 10-mM DL-dithiothreitol (Sigma-Aldrich, Buchs, Switzerland).

The slides were washed three times (3 min each) with cold water (4°C). The slides were immersed for exactly 1 min in 300-mM NaOH supplemented with 1-mM EDTA–Na (4°C), and then transferred to Tris acetic–EDTA (TAE) buffer (Sigma-Aldrich) for neutralization. The slides were subjected to electrophoresis for 10 min (12 V, 10 mA, 0.5 V/cm) at room temperature in TAE buffer. After electrophoresis, the slides were fixed with ethanol (100%), and then air-dried slides were stained with YOYO iodide (Invitrogen, Eugene, OR, USA).

In each assay, 50 comets per slide were analysed by a fluorescent microscope (Olympus, Tokyo, Japan). Percent of DNA in the comet (% tail DNA), i.e. [(tail intensity)/(head intensity + tail intensity)] × 100, was measured using the software CometScore Freeware version 1.5 (TriTek, Sumerduck, VA, USA).

**Neutral comet assay.** Slide preparation, fixation, staining and analysis of comets for neutral comet assay were performed according to the protocol described above unless otherwise stated. After removing the cover slips, the slides were incubated at 4°C for 2 h, and then further 1 h at 37°C in the lysis buffer supplemented with 100 µg/ml proteinase K (Sigma-Aldrich). The slides were washed three times (3 min each) with cold water (4°C), then subjected to electrophoresis for 10 min (12 V, 10 mA, 0.5 V/cm) and/or 5 min (25 V, 10 mA, 1 V/cm) at room temperature in TAE buffer.

**Statistical analysis**

Comparisons of data on the number of zygotes with structural chromosome aberrations were made by chi-square analysis using Yate’s correction for continuity. For comet assay, the mean % tail DNA was compared using one-tailed Mann–Whitney test. Significant differences were determined at P < 0.05.

**Results**

The results of chromosome analysis and sample codes (A–I) of the freeze-dried spermatozoa injected into oocytes are summarized in Table 1. Most of spermatozoa freeze-dried after pre-freeze-drying incubation at 25°C for 3–7 days (samples C, D, E and G) lost their ability to activate oocytes. Oocytes injected with the spermatozoa were activated artificially by the treatment with SrCl2. In contrast, spermatozoa freeze dried after 1-day incubation at 25°C (sample B) could activate oocytes without the treatment. The total frequencies of zygotes with structural chromosome aberrations showed no significant difference between zygotes derived from sample B (23%) and sample C (24%), both of which were preserved for the short duration (within 7 days). Thus, there was no effect of the SrCl2 treatment to induce de novo chromosome aberrations in the sperm-injected oocytes.

Chromosome break was the main type of structural chromosome aberration observed in all samples including positive control samples (Table I and Figure 1a). The frequency of chromatid-type aberrations showed a gradual increase during the post-freeze-drying preservation at 25°C up to 2 months as shown in samples C, D and E (Figure 1b). The frequency of chromatid exchanges became higher in samples F and G preserved at 50°C than any other samples (Figures 1b and 2). Induction of chromosome damage in spermatozoa freeze dried without pre-freeze-drying incubation was examined using samples H (preserved at 4°C) and I (preserved at 25°C). The total frequencies of zygotes with structural chromosome aberrations increased considerably in those samples (P < 0.05, versus samples A, B and C) (Table I). However, zero and a low incidence of chromatid exchanges were shown in samples H and I, respectively (Figure 1b). Chromosome and chromatid exchanges increased specifically in zygotes derived from spermatozoa freeze dried after treatment with NCS and MMS, respectively (Table I).

Alkaline comet assay screened clear difference of the DNA damage levels between freeze-dried samples preserved at 4 and 50°C (Figure 3). The spermatozoa preserved at 50°C had more intense comet tails than those preserved at 4°C (Figure 4a and b). Positive control samples freeze dried after treatment with MMS had extensive DNA migration (Figure 4c).

In neutral comet assay (Figure 5), electrophoresis was carried out for 10 min (12V, 10 mA) and 5 min (25V, 10 mA) to detect DNA damage at the low and high background damage levels, respectively. The neutral comet assay could not detect the DNA damage accumulated at 50°C at the both electrophoresis conditions, but revealed DNA damage induced in the spermatozoa freeze dried after treatment with NCS.

DNA damage accumulated in freeze-dried spermatozoa preserved for a long duration (2 years) was also evaluated by alkaline and neutral comet assays (Figure 6). Alkaline comet assay revealed significant DNA migration in the spermatozoa freeze dried without pre-freeze-drying incubation and those preserved at 25°C after freeze drying (Figures 4d and 6a). However, the neutral comet assay could not detect the DNA damage (Figure 6b).
The present results show that chromatid-type aberrations were accumulated in freeze-dried spermatozoa preserved under ambient or heat conditions. It is still unclear, however, why the marked chromatid exchanges occurred in freeze-dried spermatozoa preserved under the heat condition. Structural chromosome aberrations were known to be induced in mouse spermatozoa suspended in culture medium heated at 56°C for 30 min (37). To our knowledge, however, there has been no report on the marked incidence of chromatid exchanges in mammalian spermatozoa exposed to heat conditions.

Freeze drying of mouse spermatozoa is likely the cause of two kinds of injurious effects on the sperm genome. One of these effects, primary chromosome damage, is induced during the freeze-drying process involving both freezing and vacuum drying. Net incidence of the damage may be obtained from samples H and I that were freeze dried without pre-freeze-drying incubation. As to sample A, induction of the damage was controlled to the background level in fresh spermatozoa by the inclusion of pre-freeze-drying incubation at 4°C (7).

Neutral comet assay using spermatozoa freeze dried without pre-freeze-drying incubation could not detect DNA damage to spermatozoa preserved under ambient or heat conditions. It is still unclear, however, why the marked chromatid exchanges occurred in freeze-dried spermatozoa preserved under the heat condition. Structural chromosome aberrations were known to be induced in mouse spermatozoa suspended in culture medium heated at 56°C for 30 min (37). To our knowledge, however, there has been no report on the marked incidence of chromatid exchanges in mammalian spermatozoa exposed to heat conditions.

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**Table I.** Chromosome analysis of mouse zygotes produced by injection of freeze-dried spermatozoa into oocytes

<table>
<thead>
<tr>
<th>Sample codes</th>
<th>Pre-freeze-drying incubation*</th>
<th>Post-freeze-drying preservation</th>
<th>SrCl&lt;sub&gt;2&lt;/sub&gt; (10 mM)</th>
<th>No. of zygotes analysed</th>
<th>No. of structural chromosome aberrations in each aberration type</th>
<th>No. of zygotes with structural chromosome aberrations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>°C</td>
<td>°C Duration</td>
<td></td>
<td></td>
<td>ctb</td>
<td>cte</td>
</tr>
<tr>
<td>A'</td>
<td>4</td>
<td>4 2-14 days</td>
<td>–</td>
<td>214</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4 1-7 days</td>
<td>–</td>
<td>83</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>25</td>
<td>25 1-6 days</td>
<td>+</td>
<td>139</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>25</td>
<td>25 1 month</td>
<td>+</td>
<td>132</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>E</td>
<td>25</td>
<td>25 2 month</td>
<td>+</td>
<td>81</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>F</td>
<td>4</td>
<td>50 3 days</td>
<td>–</td>
<td>45</td>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>G</td>
<td>25</td>
<td>50 1-5 days</td>
<td>+</td>
<td>166</td>
<td>12</td>
<td>44</td>
</tr>
<tr>
<td>H&lt;sup&gt;c&lt;/sup&gt;</td>
<td>—</td>
<td>4 1-3 days</td>
<td>–</td>
<td>78</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>I</td>
<td>—</td>
<td>25 1-5 days</td>
<td>–</td>
<td>72</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>NCS, 1.0 μg/ml&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4</td>
<td>1-4 days</td>
<td>–</td>
<td>38</td>
<td>68</td>
<td>6</td>
</tr>
<tr>
<td>MMS, 100 μg/ml&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4</td>
<td>2-8 days</td>
<td>–</td>
<td>30</td>
<td>44</td>
<td>62</td>
</tr>
</tbody>
</table>

Numbers 1–7 in superscript denote significantly different values (P < 0.05) between different superscript numbers. ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange; mul, number of zygotes with multiple aberrations, i.e. severely damaged chromosomes resulting chromosome fragmentation.

* Sperm suspension was incubated in modified ETBS for 3–7 days before freeze drying.

<sup>a</sup> Sperm-injected oocytes were artificially activated by treatment with 10-mM strontium dichlorides (SrCl<sub>2</sub>).

<sup>c</sup> Data reported in part previously by Kusakabe et al. (7).

<sup*e</sup> Sperm were incubated for 1 day in modified ETBS before freeze drying.

<sup>e</sup> Sperm were treated with NCS or MMS for 2 h (37°C) before freeze drying.

![Chromosome-type aberrations](https://example.com/chromosome.png)

**Fig. 1.** Number of chromosome-type (a) and chromatid-type (b) observed in zygotes derived from freeze-dried spermatozoa in the respective sample codes (A–I) shown in Table I. White and black portions of a bar represent the frequencies of break and exchanges, respectively.

![Heat-induced chromatid exchanges](https://example.com/heat.png)

**Fig. 2.** Heat-induced chromatid exchanges (large arrows) observed in zygotes derived from freeze-dried spermatozoa at the first cleavage metaphase. The freeze-dried spermatozoa were preserved at 50°C for 3 days. Chromosome breaks were also induced (small arrows).
lead to the primary chromosome damage (Figure 6b). Therefore, the DNA migration revealed by alkaline comet assay might have resulted from mostly SSBs and/or alkali-labile sites (Figure 6a). However, the chromatid exchanges formed theoretically from the SSBs were seldom seen in zygotes derived from the samples H and I (Figure 1a).

The other effect, accumulative chromosome damage, is addressed mainly in this study. The incidence of chromatid-type aberrations and/or chromatid exchange may become an indicator for distinguishing the accumulative chromosome damage from the primary chromosome damage.

From a study using Syrian and Chinese hamsters irradiated with ionizing radiation, the frequency of chromatid exchanges observed in the sperm chromosomes is known to show a species-specific (oocyte-specific) pattern depending upon the repair system in oocytes fertilized with spermatozoa (38). An increase in the frequency can be explained in part by the post-replication repair system, which operates predominantly to repair sperm DNA lesions in the oocytes. From the present results of alkaline comet assay, the chromatid exchanges appear to be formed from heat-induced SSBs by post-replication repair in mouse oocytes.

The majority of SSBs induced in mammalian spermatozoa by DNA-damaging compounds are probably converted to DSBs after oocyte fertilization with spermatozoa, leading to the frequent incidence of chromosome-type aberrations (29). A possible mechanism on the conversion may be due to the enzymatic action capable of converting SSBs to DSBs, such as the well-known single-strand nuclease. Results of the neutral comet assay performed in the present study showed that few DSBs are induced directly in freeze-dried spermatozoa by the heat stress. If the SSBs accumulated in heat-stressed spermatozoa fail to be converted to DSBs, the chromatid exchanges are most likely formed from the SSBs that persisted until the DNA synthetic stage (pronuclear stage).

Alternatively, frequent incidence of chromatid exchanges in sperm chromosomes has also reportedly been concomitant with a sperm chromatin remodelling disorder (39). The sperm chromatin remodelling (i.e. decondensation and recondensation of the sperm chromatin occurred after fertilization) was adversely affected by ICSI delayed at long intervals after parthenogenetic activation of oocytes. In addition, steric alterations in chromosomal DNA may interfere with the binding of specific proteins that are required for chromosome condensation (40). In bull spermatozoa, susceptibility of the sperm DNA to in situ denaturation at low pH increased with increasing time of sperm incubation at 38.5°C within 180 min in vitro (41). In mice, heat-stress (40°C) exposure of the scrotal region also reportedly induces the chromatin abnormality in cauda epididymal spermatozoa (42). In the present study, heat stress may induce steric alterations of the chromosomal DNAs and/or denaturation of chromosome-associated proteins in freeze-dried spermatozoa, resulting in the disorder of sperm chromatin remodelling followed by the induction of chromatid

Fig. 3. Alkaline comet assay. Freeze-dried spermatozoa preserved at 4 and 50°C for 3 days (pre-freeze-drying incubation, 3 days at 4°C) were assayed concurrently with positive control samples: spermatozoa freeze dried after treatment with 200 μg/ml MMS for 2 h at 37°C. Data are expressed as mean ± standard deviation derived from three separate experiments. *Significantly different (P < 0.05) from the negative control sample preserved at 4°C for 3 days.

Fig. 4. Images of comets in alkaline comet assay. Freeze-dried samples preserved for 3 days at 4°C (a), treated with MMS at 200 μg/ml (b), preserved for 3 days at 50°C (c) and preserved for 2 years at 25°C (d). Scale bars, 50 μm.
exchange of DNA in dried cells preserved in vacuum glass ampoules. Further studies using freeze-dried spermatozoa, as well as non-frozen spermatozoa suspended in solution, are necessary to deduce the induction mechanism of the chromatid exchanges accumulated in freeze-dried spermatozoa.

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References


