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

Kusakabe, Hirokazu ; Tateno, Hiroyuki

Title:

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

Authors:

Hirokazu Kusakabe, Hiroyuki Tateno

Affiliation:

Department of Biological Sciences

Asahikawa Medical University

2-1-1-1 Midorigaoka-higashi, Asahikawa

Asahikawa 078-8510, Japan

Correspondence:

Hirokazu Kusakabe, Ph.D.

Department of Biological Sciences

Asahikawa Medical University

2-1-1-1 Midorigaoka-higashi, Asahikawa

Asahikawa 078-8510, Japan

TEL: +81-166-68-2730

FAX: +81-166-68-2783

E-mail: hkusa55@asahikawa-med.ac.jp

Abstract

Structural chromosome aberrations and DNA damage generated in freeze-dried mouse spermatozoa were investigated. Freeze-dried sperm samples were preserved at 4°C, 25°C 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 analyze the chromosomes 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 to 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.

Keywords

Chromosome aberration, comet assay, freeze-dry, sperm preservation, heat stress, intracytoplasmic sperm injection

49 **1. Introduction**

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

55 Full-term development of mammalian oocytes injected with freeze-dried
56 spermatozoa was first reported using laboratory strain mice (2). Developmental
57 competence or chromosomal integrity of zygotes derived from freeze-dried spermatozoa
58 has also been noted in cattle (3), dog (4), hamster (5), human (5-8), pig (9), rabbit (6,10)
59 and rat (11-14). Since the freezing and drying processes are prone to chromosomal
60 damage in mouse spermatozoa, a medium for freeze-drying, EGTA Tris-HCl buffered
61 solution (ETBS), has been developed to prevent the induction of chromosomal damage
62 (15-17). In addition, mice derived from the spermatozoa freeze-dried in ETBS were
63 reported to be stable genomically in subsequent two generations (18).

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

70 However, we have not yet established a successful method for preserving
71 semi-permanently mammalian spermatozoa at ambient temperatures. DNA damage is
72 accumulated in freeze-dried spermatozoa preserved at room temperature (19,20). In fact,

73 mouse oocytes injected with freeze-dried or evaporated mouse spermatozoa preserved at
74 room temperature have lower developmental competency than those preserved at 4°C (2,
75 20-22).

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

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

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

94

95

96

97 **2. Methods**

98 *2.1. Animals*

99 Hybrid (B6D2F_1) male and female mice (six weeks of age) were purchased from
100 Sankyo Labo Service (Sapporo, Japan). The mice were maintained on the bedding for
101 laboratory animal (Japan SLC, Hamamatsu, Japan) for 1 to 6 weeks under a 14-h
102 light/10-h dark photoperiod at a temperature of 22°C to 24°C. Food (MF, solid type,
103 Oriental Yeast, Tokyo, Japan) and water were given *ad libitum*. The mice were
104 euthanized by cervical dislocation just before use under the animal study protocol
105 approved by the Laboratory Animal Committee, Asahikawa Medical University, Japan.

106

107 *2.2. Media for culture and freeze-drying*

108 All chemicals were obtained from Nacalai Tesque (Kyoto, Japan), unless otherwise
109 stated. The medium for oocyte collection and sperm injection was a modified CZB
110 medium (24,25) with 20 mM HEPES, 5 mM $NaHCO_3$, and 0.1 mg/ml polyvinyl alcohol
111 (PVA; cold water soluble; molecular weight: 30000-70000, Sigma Chemical, St. Louis,
112 MO, USA) (HEPES-CZB) (26). Tris-buffered EGTA solution (modified
113 EGTA/Tris-HCl buffered solution: modified ETBS) used for suspending spermatozoa
114 for freeze-drying consisted of 50 mM EGTA and 100 mM Tris-HCl buffer. To prepare
115 the 0.5 M EGTA stock solution, EGTA (Sigma-Aldrich, St. Louis, MO, USA) was
116 dissolved with water and adjusted to pH 8.0 with NaOH solution. For working solutions,
117 1 ml of 0.5 M EGTA and 1 ml of 1 M Trisma®-HCl, pH 7.4 (DNase-, RNase- and
118 protease-free, purchased as liquid form, Sigma-Aldrich, St. Louis, MO, USA) were
119 diluted with 8 ml water at a final concentration of 50 mM and 100 mM, respectively.

120

121 *2.3. Sperm collection and freeze-drying*

122 Freeze-drying involved the pre-freeze-drying incubation step (7). Two caudae
123 epididymides of a male were removed and punctured with a sharply forcep. The dense
124 sperm mass was collected from the epididymis and placed in the bottom of a 1.5-ml
125 polypropylene microcentrifuge tube containing 1.2 ml modified ETBS (37°C). The tube
126 was left standing for 10 min at 37°C to allow sperm to disperse by swimming into the
127 solution. The upper 1 ml of the sperm suspension was transferred into another tube.
128 Suspended sperm were incubated for 3 to 7 days at 4°C in a refrigerator or 1 to 7 days at
129 25°C in an incubator (Compact Cool Incubator, ICI-1, As One, Osaka, Japan) prior to
130 freeze-drying. After the pre-freeze-drying incubation, 100-μl aliquots were put in 2-ml
131 glass ampoules (Wheaton Scientific, Millville, NJ, USA).

132 The glass ampoules containing the sperm suspensions were plunged into liquid
133 nitrogen for 1 min, and then connected to a lyophylizer (FZ2.5, Labconco, Kansas City,
134 MO, USA). After vacuuming for 4 h, each ampoule was flame-sealed and kept in the
135 shade at 25°C in an incubator (Compact Cool Incubator, ICI-1, As One, Osaka, Japan)
136 or 50°C in an incubator (Mini Incubator, IC-150MA, As One, Osaka, Japan). The inside
137 pressure of the ampoules at the time of sealing was around 22×10^{-3} to 42×10^{-3} mbar.

138 To prepare positive control samples, the spermatozoa suspended in modified ETBS
139 were treated with an alkylating agent, methyl methanesulfonate (MMS) (Nacalai Tesque,
140 Kyoto, Japan), and an antitumor antibiotic, neocarzinostatin (NCS) (Sigma Aldrich, St.
141 Louis, MO, USA), for 2 h at 37°C prior to freeze-drying. For chromosome analysis, the
142 final concentrations of MMS and NCS were set at 100 and 1.0 μg/ml, respectively. For
143 comet assay, those were set at 200 μg/ml (MMS) and 2.0 μg/ml (NCS). NCS directly

144 induces both SSBs and DSBs in plasmid DNAs (27), and mainly chromosome-type
145 aberrations in human spermatozoa (28). MMS induces mainly chromosome breaks and
146 chromatid exchanges in human spermatozoa (29), although it does not directly induce
147 DSBs (30,31).

148

149 *2.4. Oocyte preparation*

150 Female mice were injected with 10 units of pregnant mare's serum gonadotrophin
151 (PMSG, Asuka Pharmaceutical, Tokyo, Japan). After 48 h, the mice were injected with
152 10 units of human chorionic gonadotrophin (hCG, Mochida, Tokyo, Japan). Oocytes
153 were collected from oviducts between 15 and 17 h after hCG injection. They were freed
154 from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (999
155 units/mg solid, Sigma-Aldrich, St. Louis, MO, USA) in HEPES-CZB medium, then
156 rinsed and kept in HEPES-CZB medium at 37°C before sperm injection.

157

158 *2.5. Intracytoplasmic sperm injection*

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

168 *2.6. Culture of oocytes*

169 Modified CZB medium was used for culture of sperm-injected oocytes. In the case of
170 pre-freeze-drying incubation at 25°C, most of the freeze-dried spermatozoa lost their
171 ability to activate oocytes. Therefore, sperm-injected oocytes were transferred to
172 droplets (50-100 μl) of a modified CZB medium supplemented with 10 mM strontium
173 dichloride ($SrCl_2$), instead of $CaCl_2$, to activate the oocytes artificially. After culturing
174 for 1 h, the oocytes were transferred to droplets (50-100 μl) of modified CZB medium.
175 The oocytes were then cultured at 37°C under a paraffin oil (Merck KGaA, Darmstadt,
176 Germany) overlaid in a humidified atmosphere of 5% CO_2 in air.

177

178 *2.7. Chromosome analysis*

179 At 5 to 6 h from completion of ICSI, sperm-injected oocytes were transferred to
180 modified CZB medium containing 0.01 μg/ml vinblastine sulfate to arrest the
181 metaphases of the first cleavage. At 20 to 22 h from the completion of ICSI, the zygotes
182 were freed from the zonae pellucidae by treatment with 0.5% protease (Actinase E,
183 1000 tyrosine unit/mg, Kaken Pharmaceuticals, Tokyo, Japan) followed by treatment
184 with a hypotonic solution composed of a 1:1 mixture of 30% fetal bovine serum and 1%
185 sodium citrate (32,33) for 4 to 10 min. The zygotes were fixed and air-dried on glass
186 slides, and then stained by Giemsa's solution (Merck KGaA, Darmstadt, Germany)
187 diluted to 4% (v/v) for chromosome analysis (34). Structural chromosome aberrations
188 were recorded without discriminating between paternal and maternal origins. Because
189 mouse oocytes seldom had chromosome aberrations at the first mitotic metaphase after
190 normal fertilization and parthenogenetic activation (35), chromosome aberrations
191 observed in zygotes derived from freeze-dried spermatozoa were most likely those of

192 the sperm origin. Types of structural chromosome aberrations were classified into break
193 and exchange of chromatid and chromosome types. Moreover, zygotes with
194 chromosome fragmentation or pulverization were scored as multiple aberrations.
195
196 *2.8. Comet assay*
197 *2.8.1. Alkaline comet assay*
198 Instead of standard alkaline comet assay, alkaline comet assay with "A/N protocol"
199 (i.e., alkaline DNA unwinding followed by electrophoresis under neutral condition) (30)
200 was carried out according to the procedure as described previously (36). Freeze-dried
201 sperm samples were rehydrated by adding 50 to 70 µl distilled water to each glass
202 ampoule immediately after breaking the ampoule neck. For comet assay, we used the
203 normal melting point agarose (Agarose L03, gelling temperature: 35 to 37°C, Takara
204 Bio, Otsu, Japan) because it could be held tightly on glass slides. The agarose was
205 dissolved in phosphate buffered saline (without Ca^{2+} and Mg^{2+}, pH 6.8) heated by a
206 microwave oven at the concentration of 1% (w/v). The 1% agarose solution was
207 incubated at 50°C for 1 h or more to lower its temperature. Surface on each glass slide
208 was pre-smeared with the 1% agarose solution on a hot plate heated at 70°C. The sperm
209 suspension was mixed with the 1% agarose solution to the final concentration of 0.7%.
210 The mixture (100 µl) was applied on each pre-smeared glass slide warmed at 50°C.
211 Cover slips were put on the slides and then stored at 4°C for 5 to 10 min. All comet
212 slides were coded in each freeze-dried sample.
213 After removing the cover slips, the slides were incubated at 4°C for 2 h, and then
214 further 1 h at 37°C in lysis buffer composed of 2.5 M NaCl, 50 mM EDTA-Na, 10 mM

215 Tris-HCl (pH 10), 1% Triton X-100 and 10 mM DL-dithiothreitol (Sigma-Aldrich,
216 Buchs, Switzerland).

217 The slides were washed three times (3 min each) with cold water (4°C). The slides
218 were immersed for exactly 1 min in 300 mM NaOH supplemented with 1 mM
219 EDTA-Na (4°C), and then transferred to TAE buffer (Tris acetate-EDTA, Sigma-Aldrich,
220 St. Louis, MO, USA) for neutralization. The slides were subjected to electrophoresis for
221 10 min (12 V, 10 mA, 0.5 V/cm) at room temperature in TAE buffer. After
222 electrophoresis, the slides were fixed with ethanol (100%), and then the air-dried slides
223 were stained by YOYO iodide (Invitrogen, Eugene, OR, USA).

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

228

229 *2.8.2. Neutral comet assay*

230 Slide preparation, fixation, staining and analysis of comets for neutral comet assay
231 were performed according to the protocol described above unless otherwise stated. After
232 removing the cover slips, the slides were incubated at 4°C for 2 h, and then further 1 h
233 at 37°C in the lysis buffer supplemented with 100 μg/ml proteinase K (Sigma-Aldrich,
234 St. Louis, MO, USA). The slides were washed three times (3 min each) with cold water
235 (4°C), then subjected to electrophoresis for 10 min (12 V, 10 mA, 0.5 V/cm) and/or 5
236 min (25 V, 10 mA, 1 V/cm) at room temperature in TAE buffer.

237

238

239 *2.9. Statistical analysis*

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

244

245 **3. Results**

246 The results of chromosome analysis and sample codes (A to I) of the freeze-dried
247 spermatozoa injected into oocytes are summarized in Table 1. Most of spermatozoa
248 freeze-dried after pre-freeze-drying incubation at 25°C for 3 to 7 days (samples C, D, E
249 and G) lost their ability to activate oocytes. Oocytes injected with the spermatozoa were
250 activated artificially by the treatment with $SrCl_2$. In contrast, spermatozoa freeze-dried
251 after 1-day incubation at 25^oC (sample B) could activate oocytes without the treatment.
252 The total frequencies of zygotes with structural chromosome aberrations showed no
253 significant difference between zygotes derived from sample B (23%) and sample C
254 (24%), both of which were preserved for the short duration (within 7 days). Thus, there
255 was no effect of the $SrCl_2$ treatment to induce *de novo* chromosome aberrations in the
256 sperm-injected oocytes.

257 Chromosome break was the main type of structural chromosome aberration
258 observed in all samples including positive control samples (Table 1 and Fig. 1a). The
259 frequency of chromatid-type aberrations showed a gradual increase during the
260 post-freeze-drying preservation at 25°C up to 2 months as shown in samples C, D and E
261 (Fig. 1b). The frequency of chromatid exchanges became higher in samples F and G
262 preserved at 50°C than any other samples (Fig. 1b and Fig. 2).

263 Induction of chromosome damage in spermatozoa freeze-dried without
264 pre-freeze-drying incubation was examined using samples H (preserved at 4°C) and I
265 (preserved at 25°C). The total frequencies of zygotes with structural chromosome
266 aberrations increased considerably in those samples (P< 0.05, vs. samples A, B and C)
267 (Table 1). However, zero and a low incidence of chromatid exchanges were shown in
268 samples H and I, respectively (Fig. 1b). Chromosome and chromatid exchanges
269 increased specifically in zygotes derived from spermatozoa freeze-dried after treatment
270 with NCS and MMS, respectively (Table 1).

271 Alkaline comet assay screened clear difference of the DNA damage levels between
272 freeze-dried samples preserved at 4°C and 50°C (Fig. 3). The spermatozoa preserved at
273 50°C had more intense comet tails than those preserved at 4°C (Fig. 4a, b). Positive
274 control samples freeze-dried after treatment with MMS had extensive DNA migration
275 (Fig. 4c).

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

281 DNA damage accumulated in freeze-dried spermatozoa preserved for a long
282 duration (2 years) was also evaluated by alkaline and neutral comet assays (Fig. 6).
283 Alkaline comet assay revealed significant DNA migration in the spermatozoa
284 freeze-dried without pre-freeze-drying incubation and those preserved at 25°C after
285 freeze-drying (Fig. 4d, Fig. 6a). However, the neutral comet assay could not detect the
286 DNA damage (Fig. 6b).

287 **4. Discussion**

288 The present results show that chromatid-type aberrations were accumulated in
289 freeze-dried spermatozoa preserved under ambient or heat conditions. It is still unclear,
290 however, why the marked chromatid exchanges occurred in freeze-dried spermatozoa
291 preserved under the heat condition. Structural chromosome aberrations were known to
292 be induced in mouse spermatozoa suspended in culture medium heated at 56°C for 30
293 min (37). To our knowledge, however, there has been no report on the marked incidence
294 of chromatid exchanges in mammalian spermatozoa exposed to heat conditions.

295 Freeze-drying of mouse spermatozoa is likely the cause of two kinds of injurious
296 effects on the sperm genome. One of these effects, primary chromosome damage, is
297 induced during the freeze-drying process involving both freezing and vacuum-drying.
298 Net incidence of the damage may be obtained from samples H and I that were
299 freeze-dried without pre-freeze-drying incubation. As to sample A, induction of the
300 damage was controlled to the background level in fresh spermatozoa by the inclusion of
301 pre-freeze-drying incubation at 4°C (7). Neutral comet assay using spermatozoa
302 freeze-dried without pre-freeze-drying incubation could not detect DNA damage to lead
303 to the primary chromosome damage (Fig. 6b). Therefore, the DNA migration revealed
304 by alkaline comet assay might have resulted from mostly SSBs and/or alkali-labile sites
305 (Fig. 6a). However, the chromatid exchanges formed theoretically from the SSBs were
306 seldom seen in zygotes derived from the samples H and I (Fig. 1a).

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

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

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

328 Alternatively, frequent incidence of chromatid exchanges in sperm chromosomes
329 has also reportedly been concomitant with a sperm chromatin remodeling disorder (39).
330 The sperm chromatin remodeling (i.e., decondensation and recondensation of the sperm
331 chromatin occurred after fertilization) was adversely affected by ICSI delayed at long
332 intervals after parthenogenetic activation of oocytes. In addition, steric alterations in
333 chromosomal DNA may interfere with the binding of specific proteins that are required
334 for chromosome condensation (40). In bull spermatozoa, susceptibility of the sperm

335 DNA to *in situ* denaturation at low pH increased with increasing time of sperm
336 incubation at 38.5°C within 180 min *in vitro* (41). In mice, heat-stress (40°C) exposure
337 of the scrotal region also reportedly induces the chromatin abnormality in cauda
338 epididymal spermatozoa (42). In the present study, heat stress may induce steric
339 alterations of the chromosomal DNAs and/or denaturation of chromosome-associated
340 proteins in freeze-dried spermatozoa, resulting in the disorder of sperm chromatin
341 remodeling followed by the induction of chromatid exchanges. Thus, the pathway
342 involving chromatid exchange formed by the disorder is still unclear.

343 Little information is known about the denaturation kinetics of DNA in dried cells
344 preserved in vacuum glass ampoules. Further studies using freeze-dried spermatozoa, as
345 well as non-frozen spermatozoa suspended in solution, are necessary to deduce the
346 induction mechanism of the chromatid exchanges accumulated in freeze-dried
347 spermatozoa.

348

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497 **Figure Legends**

498 Figure 1

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

503

504 Figure 2

505 Heat-induced chromatid exchanges (large arrows) observed in zygotes derived from
506 freeze-dried spermatozoa at the first cleavage metaphase. The freeze-dried spermatozoa
507 were preserved at 50°C for 3 days. Chromosome breaks were also induced (small
508 arrows).

509

510 Figure 3

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

517

518 Figure 4

519 Images of comets in alkaline comet assay. Freeze-dried samples preserved for 3 days at

520 4°C (a), treated with MMS at 200 μg/ml (b), preserved for 3 days at 50°C (c) and

521 preserved for 2 years at 25°C (d). Scale bars, 50 μm.

522

523 Figure 5

524 Neutral comet assay. (a) Electrophoresis was performed at 12 V, 10 mA for 10 min

525 (Black bar) and 25V, 10 mA for 5 min (white bar). Freeze-dried spermatozoa preserved

526 at 4°C and 50°C for 3 days (pre-freeze-drying incubation, 3 days at 4°C) were assayed

527 concurrently with positive control samples: spermatozoa freeze-dried after treatment

528 with 2.0 μg/ml neocarzinostatin (NCS) for 2 h at 37°C. Data are expressed as mean ±

529 SD derived from three separate experiments. *Significantly different ($P < 0.05$) from

530 the negative control sample preserved at 4°C for 3 days. (b) Images of the comets. Scale

531 bars, 50 μm.

532

533 Figure 6

534 (a) Alkaline comet assay and (b) neutral comet assay (25V, 10 mA, 5 min) using

535 freeze-dried spermatozoa preserved at 4°C and 25°C for long duration (2 years). Data

536 are expressed as mean ± SD derived from three separate experiments. *Significantly

537 different ($P < 0.05$) from the freeze-dried spermatozoa preserved at 4°C for 2 years

538 (pre-freeze-drying incubation, 4°C). PFI: pre-freeze-drying incubation; +: with PFI; －:

539 without PFI.

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543 Figure 1

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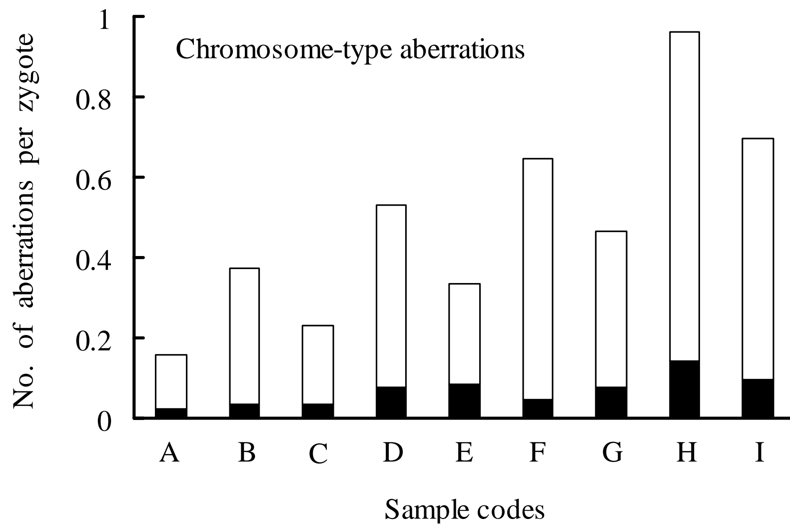

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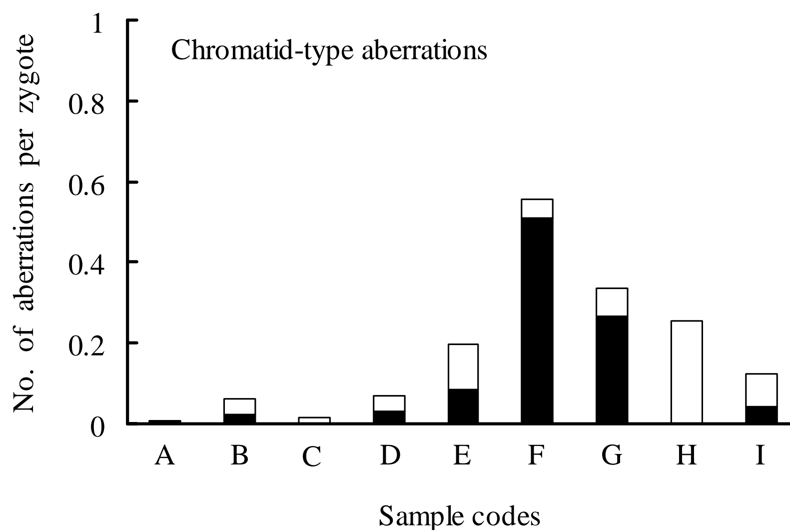

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574 Figure 2

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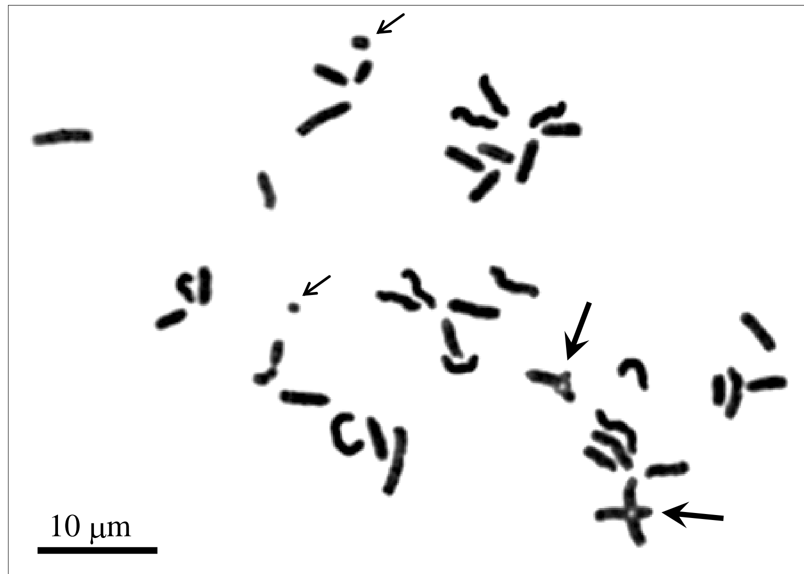

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600 Figure 3

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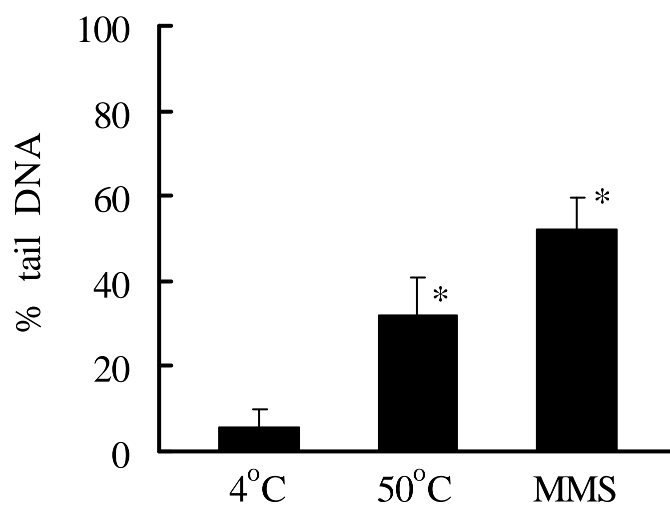

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614 Figure 4

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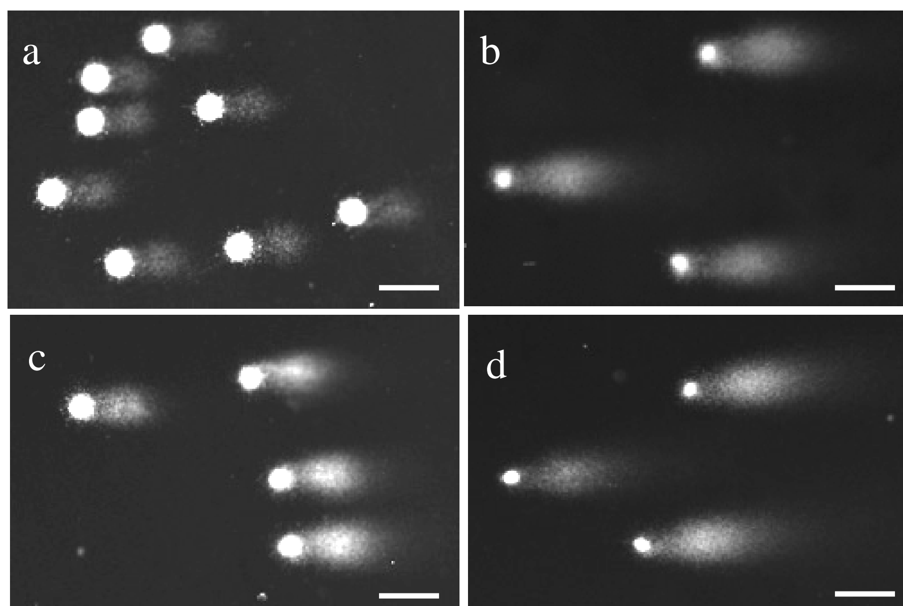

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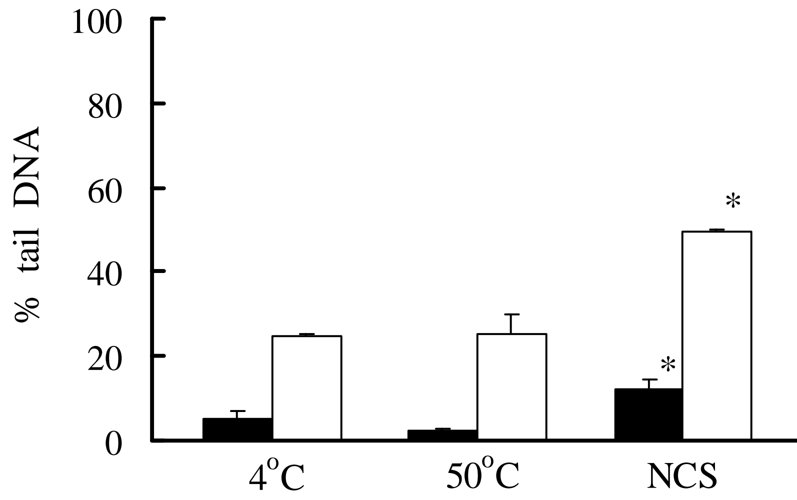

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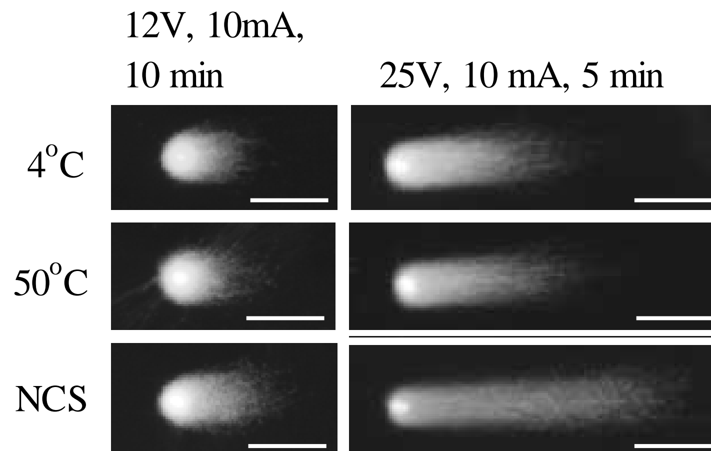

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657 Figure 6

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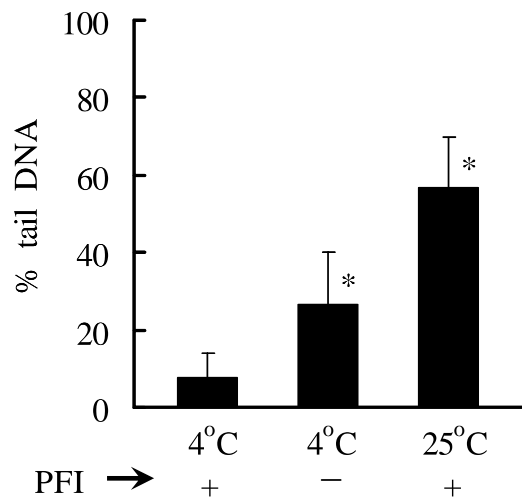

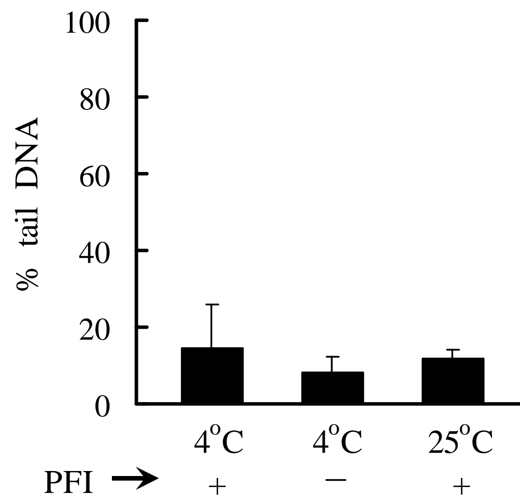

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