

Full-term Development of Hamster Embryos Produced by Injecting Freeze-dried Spermatozoa into Oocytes

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Abstract: Although injected freeze-dried hamster spermatozoa can develop into male pronuclei even after 12 months of storage at 4°C, the developmental competence of hamster pronuclear oocytes is not well understood. Furthermore, production of live offspring from freeze-dried spermatozoa is limited in some animals, such as mice, rabbits and rats. Here, we report the birth of hamster offspring following intracytoplasmic injection with freeze-dried spermatozoa. The integrity of the sperm DNA after freeze-drying and rehydration is very important for the developmental competence of hamster embryos produced by injecting freeze-dried spermatozoa into oocytes. This study used the TUNEL method to examine DNA fragmentation and the chromosomal integrity of spermatozoa after freeze-drying using two freezing media: M2 medium, and a Tris-HCl buffered solution containing 50 mM EGTA (EGTA solution). The rate of DNA fragmentation when hamster spermatozoa freeze-dried in EGTA solution was used was significantly ($P < 0.05$) lower than that in M2 medium (4.3% vs. 41.4%), and the chromosomal integrity in EGTA solution was higher in EGTA solution than in M2 medium (81.1% vs. 41.0%). The percentage of morulae/blastocysts derived from hamster spermatozoa freeze-dried in EGTA solution was significantly ($P < 0.05$) higher than that derived from spermatozoa freeze-dried in M2 medium (62.2% vs. 12.5%). After transfer to foster mothers, 3 of 23 morulae/blastocysts developed into live offspring.

Key words: DNA fragmentation, Freeze-drying, Intracytoplasmic sperm injection, Hamster spermatozoa

Introduction

Sperm cryopreservation is widely used in the assisted reproduction of livestock, especially cattle; it is also used for laboratory animals as well as in humans [1, 2]. The most common way to preserve spermatozoa for long-term storage is freezing in liquid nitrogen (–196°C), a method that requires the continuous replenishment of liquid nitrogen for long-term storage. In addition, many liquid nitrogen tanks are needed to store the various types of spermatozoa used for genetic resources. Freeze-drying sperm is a new preservation method, developed using mice, which is used in conjunction with intracytoplasmic sperm injection (ICSI) with a piezo-micromanipulator [3–5]. Freeze-dried spermatozoa do not require a continuous supply of liquid nitrogen for storage, plus the freeze-dried spermatozoa can be stored in ampoules in a small space, such as in a small conventional refrigerator. In addition, freeze-dried spermatozoa are easy to transport.

The first report of the injection of freeze-dried sperm was by Uehara and Yanagimachi [6]. When freeze-dried human spermatozoa were injected into hamster oocytes, the nuclei developed into male pronuclei. Katayose *et al.* [7] confirmed that freeze-dried hamster and human sperm nuclei remained capable of developing into pronuclei, even after 12 months of storage at 4°C. Wakayama and Yanagimachi [4] subsequently reported the birth of normal mouse offspring from oocytes injected with freeze-dried spermatozoa. The birth of live offspring has been obtained by ICSI with freeze-dried spermatozoa in rabbits [8] and rats [9]; however, the only newborn rabbit died soon after birth. At present, the routine production of live offspring using freeze-dried sperm injection is limited mainly to mice [4, 5, 10–17].

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The chromosomal stability of the freeze-dried mouse spermatozoa is affected by the freeze-drying medium. Kusakabe *et al.* [5] reported that the use of Tris-HCl buffer containing 50 mM EGTA increased the percentage of freeze-dried spermatozoa with normal karyotypes. That is, the EGTA solution successfully maintained the genetic integrity of freeze-dried mouse spermatozoa.

The objective of the present study was to examine the effect of the freeze-drying medium on the integrity of freeze-dried hamster sperm DNA, and on the developmental competence of hamster oocytes fertilized by ICSI with the freeze-dried spermatozoa. We further report the birth of live offspring following intracytoplasmic injection of freeze-dried hamster spermatozoa.

Materials and Methods

Chemicals

Inorganic salts were purchased from either Sigma-Aldrich (St. Louis, MO, USA) or Nacalai Tesque Inc. (Kyoto, Japan). All organic reagents were purchased from Sigma-Aldrich unless otherwise stated.

Animals

Golden hamsters (*Mesocricetus auratus*) were fed a standard diet *ad libitum* and were maintained in a temperature- and light-controlled room at 25°C, with a 14 h light/10 h dark cycle (the light from 0600 h). All hamsters were purchased from Japan SLC Inc. (Shizuoka, Japan). Mature females, 2–4 months of age, and mature male hamsters, 4–5 months of age, were used for the collection of unfertilized oocytes and motile spermatozoa, respectively. The experiments were approved by the Committee for Ethics on Animal Experiments of the Prefectural University of Hiroshima, Japan.

Culture medium and buffered solution for sperm freezing or freeze-drying

The medium used for oocyte collection, sperm injection and embryo culture from the one-cell to the two-cell stage was TCM 199 containing 26 mM sodium bicarbonate and 25 mM HEPES buffer (Gibco BRL, Grand Island, NY, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS, ICN Biomedicals Inc., Ohio, USA), 5 mM taurine, and 25 μ M EDTA (ethylenediaminetetraacetic acid) as reported previously [18, 19]. This medium was called M199TE. For the first 24 h after sperm injection, oocytes were

cultured in the above medium under 10% CO₂, 10% O₂ and 80% N₂. They were subsequently cultured for another 2 days in Hamster Embryo Culture Medium-9 (HECM-9) [20] supplemented with 0.5 mg/ml human serum albumin (HSA, Cohn Fraction V, A1653) under the same atmosphere.

For sperm freezing and freeze-drying, M2 medium [21] or the following buffered solution were used: 10 mM Tris-HCl buffer with 50 mM NaCl and 50 mM EGTA [ethylene glycol-bis(-aminoethyl ether)-N,N,N,N-tetraacetic acid] [5] adjusted to pH 8.0 by adding 1 M HCl [11–14]. This is termed 'EGTA solution'.

Sperm collection and sperm freezing or freeze-drying

In each experiment, two caudae epididymides were removed from a male hamster and punctured with sharply pointed forceps. A drop of sperm mass was placed at the bottom of a 1.5 ml centrifuge tube containing 1 ml of M2 medium [21] or EGTA solution, and the tube was incubated at 37°C for 10 min to allow the spermatozoa to disperse into the solution. The upper 800 μ l of the sperm suspension in the M2 medium or EGTA solution was removed, and each 100 μ l aliquots were transferred to 1.5 ml polypropylene microcentrifuge tubes for freezing or to 2 ml long-necked glass ampoules for freeze-drying (Wheaton Scientific, Millville, NJ, USA). For freezing, the 100 μ l sperm suspension was placed in a polypropylene microcentrifuge tube that was plunged into liquid nitrogen for 1 min. The tube was then thawed in a water bath (37°C). Alternatively, for freeze-drying, samples in ampoules were plunged into liquid nitrogen for 1 min and then connected to a freeze-drying machine (EYELA-FD 1000, Tokyo, Japan). Six hours later, ampoules containing the freeze-dried spermatozoa were flame-sealed. The inside pressure of the ampoules was 35–40 \times 10³ hPa at the time of sealing. The ampoules were stored in a refrigerator at 4°C for up to 1 week. For microinjection of the freeze-dried spermatozoa, an ampoule was opened and 100 μ l of sterile distilled water was added to rehydrate the spermatozoa. Both freezing and freeze-drying caused the spermatozoa to become immobile and to lose their acrosomes.

Detection of sperm DNA fragmentation by the TUNEL assay

DNA fragmentation was detected using an *in situ* cell death detection kit (Roche Diagnostics, East Sussex, UK). Smears from each sperm sample were prepared on glass slides and air-dried. The sperm smears were

fixed with 4% paraformaldehyde for 20 min at room temperature. The cells were then washed in PBS supplemented with 0.1% polyvinylpyrrolidone (PVP) and permeabilized with 1% Triton X-100 in PBS supplemented with 0.1% PVP for 10 min at room temperature. The permeabilized spermatozoa were incubated in the dark at 37°C for 1 h with the TUNEL reaction mixture which contained terminal deoxynucleotidyl transferase (TdT) plus a rhodamine-conjugated dUTP label. After labeling, the cells were washed in PBS with 0.1% PVP-40 and counterstained with 20 $\mu\text{g}/\text{ml}$ Hoechst 33342 to visualize total DNA. For each sample, negative controls in which TdT was omitted from the reaction mixture and positive controls that used only DNase I (1 mg/ml for 20 min at room temperature) were also examined. At least 100 cells in each sample were analyzed using a fluorescence microscope. Each spermatozoon was scored as containing either normal DNA (blue nuclear fluorescence due to Hoechst 33342) or fragmented DNA (red nuclear fluorescence).

Analysis of chromosomes in frozen-thawed or freeze-dried spermatozoa

Analysis of sperm chromosomes was performed by intracytoplasmic injection of hamster spermatozoa into mouse oocytes. The ICSI analysis methods have been described elsewhere [22]. Briefly, about 5 h after sperm injection, oocytes with two well-developed pronuclei and a distinct second polar body about 5 h after sperm injection were transferred to CZB medium containing 0.006 $\mu\text{g}/\text{ml}$ vinblastine. Oocytes arrested at the first cleavage metaphase after 19–21 h of culture in the vinblastine solution were treated with 0.5% pronase (Actinase K, Kaken Pharmaceuticals, Tokyo, Japan) in PBS for a few minutes to remove the zonae pellucidae. The oocytes were then treated with a hypotonic solution (1:1 mixture of 30% fetal bovine serum and 1% sodium citrate) for a few minutes, fixed, and air-spread on a glass slide for chromosome analysis [23]. An egg with 20 chromosomes (mouse) and another 22 chromosomes (hamster) with no structural and numerical abnormalities was recorded as karyotypically normal. The number of aberrations per oocyte in the hamster sperm chromosomes was also recorded.

Collection of hamster oocytes

Golden hamster females (brown coats and black eyes) were induced to superovulate by an i.m. injection of 30 IU eCG (Asuka Pharmaceuticals, Tokyo, Japan) in the morning of the day of postestrous discharge [24].

This was followed by an i.m. injection of 25 IU hCG (Asuka Pharmaceuticals, Tokyo, Japan) 56 h later [25]. Mature unfertilized oocytes were collected from oviducts approximately 15 h after hCG injection and were freed from cumulus cells by a 1 min treatment with 0.1% hyaluronidase in M2 medium [21]. The oocytes were rinsed with TCM199TE, and kept in the fresh medium. All experiments were performed in a dark room with a small incandescent lamp, and red filters were used on the microscope light source, as reported previously [18, 19].

Microinjection of sperm heads into oocytes

Intracytoplasmic injection of sperm heads was carried out according to the method of Yamauchi *et al.* (2002) [18]. Immediately before injection, one drop (6 μl) of frozen-thawed or freeze-dried sperm suspension was mixed thoroughly with a 2x (12 μl) volume of Ca- and Mg-free M2 medium containing 12% (w/v) polyvinylpyrrolidone (PVP; molecular weight, ~ 360,000, MP Biochemicals, OH, USA). The sperm mixture was transferred to the micromanipulation chamber on the microscope stage. A single spermatozoon was drawn tail first into the injection pipette and moved back and forth until the connecting piece at the opening of the injection pipette. The head was separated from the tail by applying one or more piezo pulses. After discarding the tail, the head was redrawn into the pipette and injected into an oocyte.

Embryo culture

Sperm-injected oocytes were incubated in 35 μl droplets of M199TE under mineral oil at 37.5°C under 10% CO₂, 10% O₂, and 80% N₂ for about 5 h, then examined using an inverted microscope (Diaphot TMD; Nikon, Tokyo, Japan) equipped with Hoffman modulation contrast optics. An oocyte with two distinct pronuclei and a clearly visible second polar body was considered to be fertilized normally. All fertilized oocytes were transferred into 35 μl droplets of HECM-9 [20] 24 h after sperm injection and cultured for 52 h at 37.5°C under 10% CO₂, 10% O₂, and 80% N₂.

Embryo transfer

About 76 h after sperm injection, morulae and blastocysts were selected randomly and transferred into the uterus of the recipient albino females that had been mated naturally with albino males 3 days previously. The mothers were allowed to deliver and raise their own pups as well as the foster pups (which had a brown coat and black eyes) [18, 19, 26].

Statistical analysis

The fertilization percentages and embryo development rates were compared using the chi-square test. Differences between means were judged by one-way analysis of variance (ANOVA) using GraphPad PRISM (GraphPad Software, La Jolla, CA, USA). The differences were considered to be significant at $P < 0.05$.

Results

DNA fragmentation in frozen-thawed or freeze-dried spermatozoa

The percentage of hamster spermatozoa with DNA fragmentation was significantly higher in spermatozoa freeze-dried in M2 medium than those freeze-dried in EGTA solution (41.4% vs. 4.3%) or frozen-thawed in M2 medium or EGTA solution (5.0% vs. 2.7%, Fig. 1E). Fluorescence images of fragmented DNA in spermatozoa freeze-dried in M2 medium and in EGTA solution obtained using the TUNEL method are shown in Fig. 1A and B, respectively. As a negative control, none of the freeze-dried spermatozoa incubated in the TUNEL reaction mixture without terminal transferase showed fragmentation (blue sperm heads, Fig. 1C). As a positive control, freeze-dried spermatozoa treated with DNase were stained by the TUNEL method (red sperm heads, Fig. 1D).

Chromosomal analysis of frozen-thawed or freeze-dried spermatozoa

The results of chromosomal analysis of frozen-thawed or freeze-dried hamster spermatozoa by injected into mouse oocytes are shown in Table 1. The proportion of freeze-dried spermatozoa with normal karyotypes was significantly ($P > 0.05$) higher after freeze-drying in EGTA solution than in M2 medium (81% vs. 41%, Table 1 and Fig. 2). In agreement with the DNA fragmentation results obtained using the TUNEL method, the hamster spermatozoa freeze-dried in EGTA solution had greater chromosome integrity than those freeze-dried in the M2 medium. The proportion of normal karyotypes in spermatozoa freeze-dried in EGTA solution was similar to that in spermatozoa frozen-thawed in EGTA solution or M2 medium.

In vitro development of embryos produced by freeze-dried sperm injection

Table 2 summarizes the results of *in vitro* development of embryos produced by injecting frozen-

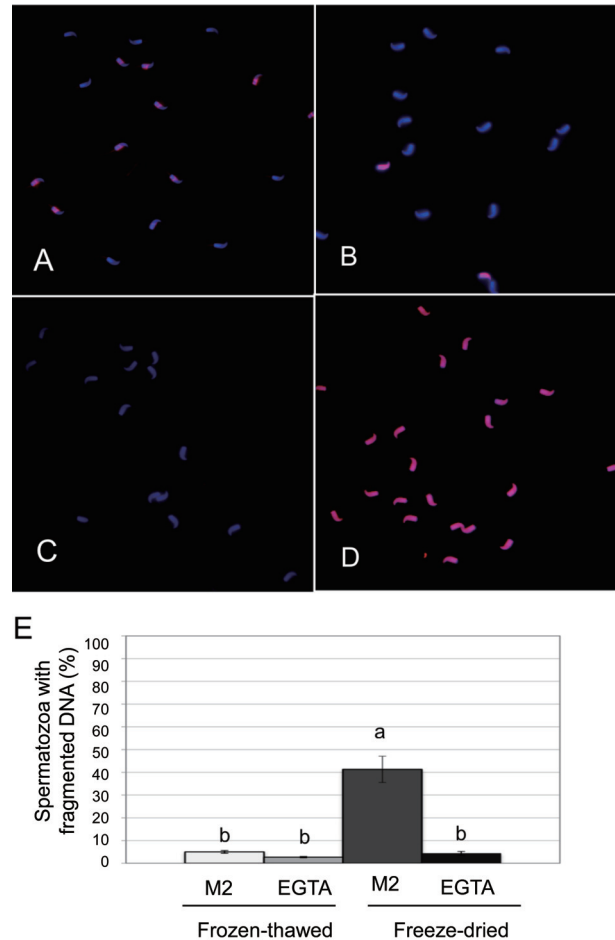


Fig. 1. DNA fragmentation in hamster spermatozoa frozen-thawed and freeze-dried in M2 medium (A) or EGTA solution (B). DNA fragmentation was assayed by the TUNEL method. As a negative control, freeze-dried spermatozoa were incubated in TUNEL reaction mixture without terminal transferase (C). As a positive control, freeze-dried spermatozoa were treated with DNase (D). Sperm DNA was stained by Hoechst dye (blue), and fragmented DNA was labeled using rhodamine (red). The percentage of DNA fragmentation in spermatozoa freeze-dried in M2 medium was higher than that observed in spermatozoa preserved under different conditions (E). The bar labeled 'a' has a value significantly different ($P < 0.05$) value than bars labeled 'b'.

thawed or freeze-dried spermatozoa into hamster oocytes. The proportions of normally fertilized oocytes that developed to the morula and blastocyst stages were significantly higher in ICSI using spermatozoa freeze-dried in EGTA solution than in ICSI using spermatozoa freeze-dried in M2 medium (62.2% vs 12.5%). The percentages of morulae and blastocysts

Table 1. Chromosomal analysis of frozen-thawed or freeze-dried hamster spermatozoa by intracytoplasmic injection into mouse oocytes: Effect of freezing medium on sperm chromosome integrity

Sperm treatment	Sperm freezing/ freeze-drying medium*	No. of oocytes analyzed	No. of sperm chromosome aberrations per oocyte**	No. (%) of oocytes with a normal karyotype
Frozen-thawed	M2	30	0.4 ^a	24 (80.0) ^a
	EGTA	37	0.1 ^a	33 (89.2) ^a
Freeze-dried	M2	39	1.7 ^b	16 (41.0) ^b
	EGTA	37	0.3 ^a	30 (81.1) ^a

*Composition of EGTA medium: 50mM NaCl, 50 mM EGTA, and 10 mM Tris-HCl. **The number of structural chromosome aberrations, such as chromosome break per oocyte was shown with hamster sperm chromosomes. Different letters indicate significant differences (a, b: $P < 0.05$).

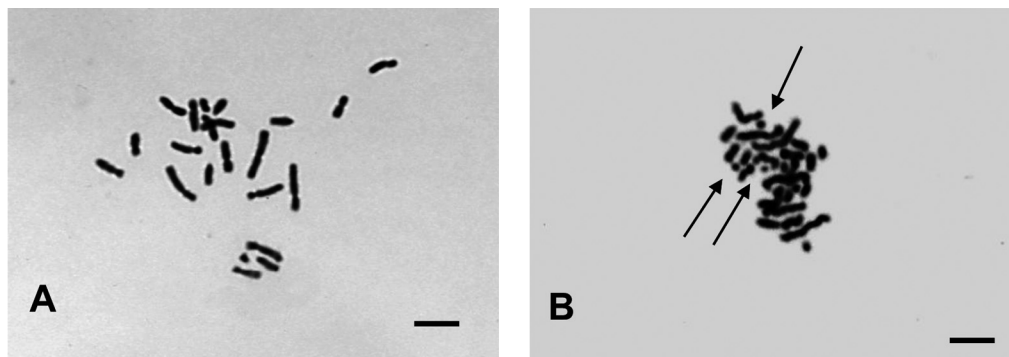


Fig. 2. Chromosomes of hamster spermatozoa freeze-dried in EGTA solution (A) or M2 medium (B) evaluated by injection into mouse oocytes. Representative normal (A, $n = 22$) and abnormal (B, $n > 22$) hamster sperm chromosomes are shown, with arrows indicating chromosome fragments. Bars = 10 μm .

Table 2. *In vitro* development of hamster embryos fertilized by intracytoplasmic injection of hamster spermatozoa that were frozen-thawed or freeze-dried in M2 medium or EGTA solution

Sperm treatment	Sperm freezing/ freeze-drying medium*	No. (%) of oocytes		No. (%) of embryos developed to			
		Injected	Fertilized	≥ 2 -cell	≥ 8 -cell	\geq Morula	Blastocyst
Frozen-thawed	M2	52	43 (82.7)	39 (90.7)	32 (74.4) ^a	20(46.5) ^a	7 (16.3) ^a
	EGTA	52	43 (82.7)	39 (90.7)	35 (81.4) ^a	22 (51.2) ^a	4 (9.3) ^{ab}
Freeze-dried	M2	45	32 (71.1)	30 (93.8)	9 (28.1) ^b	4 (12.5) ^b	0 (0) ^b
	EGTA	52	37 (71.2)	36 (97.3)	33 (89.2) ^a	23 (62.2) ^a	6 (16.2) ^a

*Composition of EGTA solution: 50 mM NaCl, 50 mM EGTA, and 10 mM Tris-HCl. Different letters indicate significant differences (a, b: $P < 0.05$).

obtained with spermatozoa freeze-dried in EGTA solution (Fig. 3) were similar to the percentages obtained with spermatozoa that had been frozen-thawed in EGTA solution or M2 medium (51.2% or 46.5%).

Full-term development of embryos produced by freeze-dried sperm injection

Table 3 summarizes the results of embryo transfer. Out of a total of 23 embryos transferred to recipients, 3 (13%) developed into live young. Figure 4 shows two pups (brown fur) produced by freeze-dried sperm injection and the recipient mother that gave birth to them.

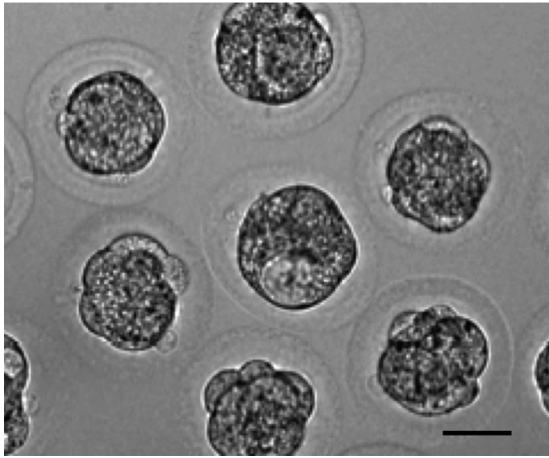


Fig. 3. Morulae and blastocysts developed from hamster oocytes injected with hamster spermatozoa freeze-dried in EGTA solution. Bar = 50 μ m.



Fig. 4. Two pups (brown coats, arrows) conceived from hamster oocytes injected with freeze-dried spermatozoa. The recipient's own pups were albino.

Table 3. *In vivo* development of hamster embryos produced by intracytoplasmic injection of freeze-dried spermatozoa

Recipient I.D.*	No. of embryos transferred			No. of live offspring†		
	Total	Morulae	Blastocysts	Recipient's pups [albino]	Foster (%) [golden]	Sex of foster offspring§
a	5	5	0	7	0	—
b	6	4	2	7	0	—
c	6	4	2	4	2	1M,1F
d	6	4	2	6	1	1F
Total	23	17	6	24	3 (13.0)	1M, 2F

*Transferred embryos derived from freeze-dried spermatozoa in EGTA solution. †The origin of the offspring was determined by eye color (on the day of birth or 1–2 days after birth) and fur color (at a later date). §Babies were sexed between 1 and 4 weeks after birth.

Discussion

This study showed that hamster embryos from oocytes injected with freeze-dried spermatozoa can develop into live offspring after embryo transfer. We also found that the freeze-drying medium affected the integrity of the sperm DNA when freeze-drying sperm for injection into oocytes. This was shown by analyzing sperm DNA integrity using the TUNEL method as well as by injecting hamster spermatozoa to examine their chromosomes into mouse oocytes and examining the sperm chromosomes. There was severe damage to hamster sperm DNA when spermatozoa were freeze-dried using M2 medium but not using 10 mM Tris-HCl buffered solution containing 50 mM EGTA. Thus, the chromosomal integrity of freeze-dried hamster spermatozoa can be maintained by using EGTA solution for freeze-drying. The damage to chromosome

integrity observed when M2 medium was used for freeze-drying suggests that the damage was not induced by physical trauma per se during the freeze-drying process; rather, the damage was most likely due to DNase activation during spermatozoa freeze-drying and rehydration.

The release of endogenous nucleases from spermatozoa with plasma membranes damaged after freeze-drying without cryoprotection is the most likely cause of the observed structural chromosome aberrations. Maione *et al.* [27] reported the existence of a Ca^{2+} -dependent endogenous nuclease in mouse spermatozoa. When EGTA solution was used for freeze-drying spermatozoa, the presence of a chelating agent, EGTA, and the absence of Ca^{2+} during subsequent rehydration probably inhibited the activation of DNase in the sperm heads and contributed to the improvement in chromosome stability. In contrast, M2

medium, which contains Ca^{2+} , was much less effective at maintaining sperm chromosome integrity during freeze-drying/rehydration (Fig. 1 and Table 1). We conclude that EGTA is crucial for stabilizing chromosome structure.

When hamster spermatozoa that had been freeze-dried in EGTA solution was injected into oocytes, the *in vitro* development of the resulting embryos was similar to that of embryos obtained by injecting spermatozoa frozen-thawed in EGTA solution or M2 medium. However, the percentages of morulae and blastocysts developing from injection of hamster spermatozoa freeze-dried in M2 medium were significantly lower than those developing from other methods (i.e. freeze-dried in EGTA solution or frozen-thawed in EGTA solution or M2 medium). These results show that sperm chromosome integrity strongly influences embryo development, since the *in vitro* development of embryos produced using sperm with poor chromosome integrity was extremely low. They further suggest that it is important to examine sperm chromosome integrity to evaluate embryo development. It is simple to evaluate DNA fragmentation using the TUNEL method, and the results of the present study show that this method is as accurate as performing chromosome analysis by injecting spermatozoa into mouse oocytes.

In this study, the freeze-dried hamster spermatozoa were stored at 4°C for up to one week. When freeze-dried mouse spermatozoa are stored long-term, the storage temperature affects sperm chromosome normality and the birth rate of live offspring even when the EGTA solution is used [10, 13–15]. Freeze-dried mouse spermatozoa have been preserved for up to 1.5 years at 4°C [10, 28]. Kawase *et al.* noted that technical advances are needed to successfully preserve freeze-dried mouse spermatozoa for long periods at temperatures higher than –80°C [15, 28]. Little information is available regarding the long-term storage of freeze-dried spermatozoa from animals other than mice. We must examine the effect of the storage temperature on the developmental competence of freeze-dried hamster spermatozoa that are stored long-term, as well as whether long-term freeze-dried hamster spermatozoa stored at temperatures higher than –80°C (especially at 4°C or –20°C in a conventional refrigerator) will remain developmentally competent.

In conclusion, hamster spermatozoa freeze-dried using 10 mM Tris-HCl buffered solution containing 50 mM EGTA maintained their chromosome integrity and developmental potential and produced live offspring after storage for up to one week at 4°C. The use of M2

medium containing the Ca^{2+} ion for freeze-drying the hamster spermatozoa increased the DNA fragmentation of sperm DNA and resulted in decreased morula and blastocyst development rates. This study showed that the selection of freeze-drying medium is important when freeze-drying hamster sperm for storage. This is also the first report of the birth of live hamster offspring obtained by ICSI with freeze-dried hamster spermatozoa. To increase the number of live offspring in the future, the freeze-drying medium needs to be improved.

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