Development of feline embryos produced using freeze-dried sperm

1

Development of feline embryos produced using freeze-dried sperm

Yasunori Tsujimoto[a], Takehito Kaneko[b], Takumi Yoshida[a], Kazuto Kimura[a], Toshio Inaba[a], Kikuya Sugiura[a], Shingo Hatoya[a*]

[a]Department of Advanced Pathobiology, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Izumisano, Osaka 598-8531, Japan

[b]Division of Science and Engineering, Graduate School of Arts and Science, Iwate University, Morioka, Iwate 020-8551, Japan

*Corresponding author: Shingo Hatoya, Department of Advanced Pathobiology, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Izumisano, Osaka 598-8531, Japan. Tel./fax: +81 72 463 5379; E-mail address: hatoya@vet.osakafu-u.ac.jp

1   **Abstract**

2   Freeze drying has been developed as a new sperm preservation method that

3   eliminates the necessity of using liquid nitrogen. An advantage of freeze-dried sperm is

4   that it can be stored at 4°C and transported at room temperature. To develop assisted

5   reproductive techniques (ARTs) for domestic cats, we evaluated the effect of the freeze-

6   dry procedure on cat sperm DNA by analyzing DNA integrity (experiment 1) and by

7   generating cat embryos using freeze-dried sperm that had been preserved for several

8   months (experiment 2). In experiment 1, the rate of DNA damage to freeze-dried sperm

9   was not significantly different than that of sperm cryopreserved with liquid nitrogen ($P >$

10   0.05). In experiment 2, the proportions of cleaved embryos, morulae, and blastocysts and

11   the cell number of blastocysts did not differ between experimental groups in which fresh

12   sperm and freeze-dried sperm were used ($P > 0.05$). In addition, we generated feline

13   blastocysts using freeze-dried sperm stored for 1–5 months. These results support an

14   expansion of the repertoire of ARTs that are potentially applicable to both domestic and

15   endangered species of cats.

16

17   **Keywords:** freeze-dry, sperm, cat, blastocyst, assisted reproductive technique

18

1. Introduction

Assisted reproductive techniques (ARTs) such as gamete preservation and artificial insemination are important for increasing the contribution of genetically valuable individuals in specific populations, whether they are highly pedigreed purebred cats, medically important laboratory cats, or endangered non-domestic cats. The domestic cat is used as a model species to develop ARTs for wild feline families that face extinction [1]. In addition, since the domestic cat is a popular companion animal and also used as a laboratory animal, there is a need to improve ARTs for domestic cats in order to efficiently maintain highly pedigreed purebreds and medically important laboratory cats [2].

Sperm preservation is an important tool for preserving cats as future genetic resources. The cryopreservation of sperm has been used for decades in humans [3], mice [4,5], cattle [6], and cats [7]. With the cryopreservation methods of storage at -196℃, it is thought that sperm can be stored semi-permanently. However, this method has the need of periodic replenishment of liquid nitrogen and the risk of temperature increase due to depletion of the liquid nitrogen. In addition, when transporting cryopreserved sperm, it is necessary to use a liquid nitrogen container or dry shipper that conforms to international standards and there are disadvantages pertaining to the associated labor and costs.

Freeze drying is expected to become a new preservation method for sperm

3

1   because it does not necessitate the use of liquid nitrogen. An advantage of freeze-dried

2   sperm is that it can be stored at 4°C [8–10] and stored and transported for short periods

3   of time at room temperature without the use of liquid nitrogen and/or dry ice as cooling

4   agents [10]. However, since freeze-dried sperm lose their motility after rehydration, an

5   essential requirement for completing physiological fertilization, a relatively difficult and

6   specialized technique such as intracytoplasmic sperm injection (ICSI) must be applied

7   when using freeze-dried sperm. Attempts to freeze-dry sperm from several species of

8   mammals have been reported [11–15], including sperm from cats [16]. However, it is

9   unclear whether freeze-dried cat sperm maintains embryonic potential since blastocysts

10   did not develop when the freeze-dried sperm was used.

11   In addition, sperm DNA can be damaged during freeze drying and especially

12   during storage if adequate protection is not provided [17]. It has been suggested that DNA

13   damage can be induced by mechanical stress throughout the freeze-drying process or by

14   oxidative stress, which occurs after rehydration [18,19]. Studies using mice and rats have

15   reported that a solution containing 10 mM Tris and 1 mM EDTA (TE buffer) adjusted to

16   pH 8.0 protect sperm DNA from physical damage by the freeze-drying process and from

17   endogenous nuclease activity during storage [20,21]. When damaged DNA sperm is used

18   for ICSI, fertilization occurs but the rates of embryo development and implantation

1 decrease [22]. Therefore, it is important to evaluate the damage rate of freeze-dried sperm

2 DNA.

3   We performed two experiments in this study. First, we evaluated the level of

4 DNA damage that occurred to cat sperm during the freeze-drying procedure by analyzing

5 DNA integrity (experiment 1). We then generated cat embryos using freeze-dried sperm

6 that had been stored for several months (experiment 2).

7

8 **2. Material and methods**

9 *2.1. Animals included in the study*

10   The ovaries and testes with epididymides were collected from domestic short-

11 haired cats at a local veterinary clinic following routine ovariohysterectomy and

12 castration, respectively. The male cats were between 8 mo and 6 y of age and the female

13 cats were between 6 mo and 7 y of age. We do not have any informations regarding estrous

14 cycle stage of ovary donors. All the animals were clinically healthy. The collected ovaries

15 and testes were maintained in 0.9% (w/v) saline solution and transported at room

16 temperature (24ºC) to our laboratory within 6 h of collection. The animals did not receive

17 surgery nor were they euthanized specifically for the study. All cats were privately owned

18 and the owners provided consent for collection and use of the tissues.

1

2 *2.2. Sperm freeze drying*

3 Freeze drying of the sperm was carried out using a partial modification of the

4 procedure suggested by Kaneko *et al.* [23]. Briefly, after removing the external tissues

5 from the testes, the epididymides were separated and cut into small pieces in Tris-EDTA

6 buffer (TE buffer), pH 8.0 (Ambion, Austin, TX, USA). The processed epididymis tissues

7 were then cultured for 10 min at 38.5°C under 5% CO_2 in humidified air. After filtering

8 through a 20-µm filter (Nipro, Osaka, Japan), the semen was centrifuged for 5 min at 500

9 $\times g$ and the supernatant was removed by aspiration. The pelleted sperm were resuspended

10 in 1 mL TE buffer and 100 µL aliquots were transferred into long-necked glass ampoules

11 for freeze drying (Wheaton, Millville, NJ, USA). The ampoules were plunged into liquid

12 nitrogen for 20 s and were then connected to the manifold of an FZ-compact freeze-drying

13 machine (Labconco, Kansas City, MO, USA). Sperm suspensions were dried for 4 h at a

14 pressure of 0.04 hPa at -50°C. The ampoules containing the freeze-dried sperm were

15 flame-sealed and stored at 4°C until use.

16

17 *2.3. Sperm collection and cryopreservation*

18 Sperm collection and cryopreservation were carried out as previously described

1 [24]. Epididymides prepared from cat testes were cut into small pieces in Dulbecco's

2 phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS (−); Nacalai, Kyoto, Japan) and

3 cultured at 38.5°C under 5% CO_2 in humidified air for 10 min. After filtering through a

4 20-µm filter (Nipro), the semen was centrifuged for 5 min at $500 \times g$ and the supernatant

5 was removed by aspiration. To prepare a sperm suspension, the pelleted sperm were

6 resuspended in 1 ml modified Human Tubal Fluid (m-HTF) medium (Nippon Medical &

7 Chemical Instruments Co., Ltd., Osaka, Japan).

8   The sperm suspension was mixed with egg yolk Tris-fructose citrate (EYT-FC)

9 solution, which was comprised of 20% (v/v) egg yolk supplemented with 13 µg/mL citric

10 acid (Nacalai), 10 µg/mL d-fructose (Nacalai), 24 µg/mL Tris aminomethane (Nacalai),

11 1,000 IU/mL penicillin (Sigma–Aldrich, St. Louis, MO, USA), and 1 mg/mL

12 streptomycin (Sigma–Aldrich). The mixture was incubated at 4°C for 1 h and then EYT-

13 FC solution containing 14% (v/v) glycerol (Nacalai) was added to obtain a final

14 concentration of 12.5×10^6 cells/mL with a final glycerol concentration of 7%. This

15 solution was then loaded into 0.25-mL straws (Fujihira, Tokyo, Japan). After sealing, the

16 straws were laid horizontally on a rack 4 cm above liquid nitrogen vapor for 5 min,

17 plunged into liquid nitrogen, and then stored in a liquid nitrogen storage tank until use.

18

1 *2.4. Thawing of cryopreserved sperm*

2   Straws containing the cryopreserved sperm were thawed in a 37ºC water bath for

3 30 s. The sperm were then transferred into 10 ml m-HTF medium and the solution was

4 centrifuged for 5 min at $500 \times g$. The pelleted sperm were resuspended in 5 ml Human

5 Tubal Fluid medium (Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan)

6 and centrifuged again for 5 min at $500 \times g$ with the final sperm pellet being resuspended

7 in 1 ml m-HTF medium (1×10^6 cells/mL).

8

9 *2.5. DNA integrity*

10   DNA integrity was evaluated using acridine orange (AO) following a slight

11 modification of the method described by Tejada *et al.* [25]. The staining solution was

12 prepared by adding 10 mL of 10 mg/mL AO (Sigma-Aldrich, St. Louis, MO, USA) in

13 distilled water to 40 mL of 0.1 M citric acid (Sigma–Aldrich) and 2.5 mL of 0.3 M

14 $Na_2HPO_4 \cdot 7H_2O$ (Nacalai) at pH 2.5. The freeze-dried sperm were rehydrated by adding

15 100 µL of distilled water. Air-dried smears on microscope slides that were prepared using

16 10 µL of rehydrated sperm were fixed overnight in ethanol-acetic acid (3:1, v:v) at room

17 temperature. Thereafter, the smears were air dried, stained with AO solution for 5 min,

18 and rinsed with distill water. Two hundred spermatozoa were evaluated under confocal

1 laser microscopy (TE2000E; Nikon, Tokyo, Japan) and characterized as having normal

2 DNA integrity (green fluorescence), or denatured or single stranded DNA (yellow, orange,

3 or red fluorescence). Fresh sperm and cryopreserved sperm were stained with AO in the

4 same manner and also evaluated under confocal laser microscopy.

5

6 *2.6. Collection and in vitro maturation (IVM) of oocytes*

7 Oocyte collection and IVM were conducted as previously described [26]. Briefly,

8 cumulus–oocyte complexes (COCs) were collected after mincing the ovaries in Medium

9 199 (Thermo Fisher Scientific, Waltham, MA, USA). Oocytes completely surrounded

10 with more than four layers of compacted cumulus cells were used. The COCs were

11 collected and rinsed three times in IVM medium, which was comprised of Medium 199

12 supplemented with 0.4% (w/v) bovine serum albumin (BSA; Sigma–Aldrich), 10 IU/mL

13 17β-estradiol (Sigma–Aldrich), 100 µg/mL gentamycin (Sigma–Aldrich), 137 µg/mL

14 sodium pyruvate (Sigma–Aldrich), 0.02 IU/mL human recombinant follicle-stimulating

15 hormone (Follistim®; MSD, Tokyo, Japan), and 25 ng/mL epidermal growth factor

16 (Sigma–Aldrich). The COCs were then incubated for 28 h in 100 µL IVM medium

17 droplets covered with mineral oil (Sigma–Aldrich) in an incubator at 38.5°C with 5%

18 CO_2.

1

2.7. Piezo-ICSI of freeze-dried sperm and fresh epididymal sperm

The freeze-dried sperm were rehydrated with 100 µL distilled water and

resuspended in 10% polyvinylpyrrolidone (molecular weight: 36,000; Kanto Kagaku,

Tokyo, Japan). Piezo-ICSI was carried out as previously described [24]. Briefly,

microinjection was performed using an IX71 inverted microscope (Olympus, Tokyo,

Japan) equipped with a piezo micromanipulator (MB-U, Prime Tech, Ibaraki, Japan). The

COCs incubated in IVM medium were gently pipetted several times to remove the

cumulus layers. Denuded oocytes demonstrating a first polar body under a

stereomicroscope were considered to be in metaphase II (M II) and were the only oocytes

used for microinjection. Two droplets of freeze-dried sperm–polyvinylpyrrolidone

suspension and six 5-µL droplets of m-HTF medium containing the denuded oocytes were

placed in the chamber under mineral oil. The chamber was comprised of a 40-mm × 50-

mm cover glass (Matsunami Glass, Osaka, Japan) bonded to a 50-mm × 50-mm slide

glass (Matsunami Glass) that had a 30-mm diameter hole in the center. Morphologically

normal freeze-dried sperm were used. To avoid damaging the oocytes, the M II oocytes

were positioned on the holding pipette with the polar body at the 12 o'clock or 6 o'clock

position and the injection pipette at the 3 o'clock position. The zona pellucida of each

1 oocyte was pierced by applying several piezo pulses to the sperm-injection pipette. After

2 the sperm was pushed forward to the point where its head was near the tip of injection

3 pipette, the pipette was advanced until its tip almost reached the opposite side of the

4 oocyte cortex. The oolemma was broken by applying a single piezo pulse and the entire

5 sperm was expelled into the ooplasm. The pipette was then gently withdrawn. All Piezo-

6 ICSI procedures were performed at room temperature. The injected oocytes were

7 maintained at room temperature for approximately 10 min.

8   Fresh epididymal sperm were injected into oocytes using the same procedure

9 described above for the freeze-dried sperm. Only morphologically normal and

10 progressively motile fresh epididymal sperm were used.

11

12 *2.8. Embryo cultures*

13   Embryo cultures were maintained as previously described [27]. After Piezo-ICSI,

14 oocytes were cultured in Only-One medium (Nippon Medical & Chemical Instruments

15 Co., Ltd.) supplemented with 0.3% (w/v) BSA for 2 d at 38.5ºC with 5% O_2, 5% CO_2,

16 and 90% N_2. Subsequently, cleaved embryos were washed and cultured for 5 d in Only-

17 One medium containing 5% (w/v) fetal bovine serum (Biosolutions International,

18 Melbourne, Australia) at 38.5ºC with 5% O_2, 5% CO_2, and 90% N_2. The percentages of

1 cleaved embryos, morulae, and blastocysts relative to the number of injected oocytes were

2 determined on days 2, 5, and 7 after microinjection, respectively. All blastocysts were

3 rinsed twice with PBS (−). The blastocysts were then fixed and stained with 25 µg/mL

4 bisbenzimide (Hoechst 33342, Sigma–Aldrich) in 100% ethanol for 2 to 3 h at 4°C. The

5 stained blastocysts were examined under a fluorescence microscope to determine the

6 numbers of cells.

7

8 *2.9. Statistical analyses*

9   Experiment 1 was performed nine times. Experiment 2 was independently

10 performed seven times. Results are expressed as the mean $\pm$ standard error of the mean

11 (SEM). Statistical differences were evaluated by ANOVA and the Tukey–Kramer test

12 using StatCell 3 (OMC Publishing Inc., Saitama, Japan). For all cases, differences were

13 considered statistically significant when *P* values were < 0.05.

14

15 *2.10. Experiment design*

16 *2.10.1. Experiment 1: DNA integrity*

17   In experiment 1, samples obtained from nine male cats were used. Semen from

18 the each individual animal was divided into the following three experimental groups:

1   fresh sperm (n = 9), cryopreserved sperm stored in liquid nitrogen for 1 mo (n = 9), and

2   freeze-dried sperm stored at 4°C for at 1 mo (n = 9). To evaluate the effect of the freeze-

3   drying procedure on the DNA integrity of cat sperm, the above three groups were stained

4   with AO solution. The fresh sperm group was used as the control.

5

6   *2.10.2. Experiment 2: in vitro embryo development after Piezo-ICSI using freeze-dried*

7   *sperm*

8   In experiment 2, morphologically normal freeze-dried sperm and

9   morphologically normal and progressively motile fresh sperm were used. A total of 455

10   M II oocytes from 37 female cats were divided among the following three treatment

11   groups: Piezo-ICSI using freeze-dried sperm (n = 260), Piezo-ICSI using fresh sperm (n

12   = 93), and sham-treated oocytes that were injection without sperm (n = 102). The sham-

13   treated oocytes served as the control group. The freeze-dried sperm used in this

14   experiment was stored at 4°C for at least 1 mo and for a maximum of 5 mo. The

15   developmental competence of the microinjected oocytes was assessed on days 2, 5, and

16   7 of *in vitro* culturing (day 0 = day of ICSI) in terms of the percentages of all the oocytes

17   that underwent cleavage, morula formation, and blastocyst formation, respectively.

18   Embryos with ≥ 16 cells without a blastocoel were classified as morulae. Only embryos

1   with ≥ 32 cells and a visible blastocoel cavity were classified as blastocysts.

2

3   **3. Results**

4   *3.1. DNA integrity*

5   DNA integrity of the sperm is shown in Fig. 1. As shown in Fig. 2, based on

6   percentage of DNA fragmentation there was no difference between the freeze-dried sperm

7   and cryopreserved sperm samples ($P > 0.05$). However, the DNA damage rate of the fresh

8   sperm was lower than that of the freeze-dried and cryopreserved sperm samples ($P < 0.05$).

9

10 *3.2. In vitro embryo development after Piezo-ICSI using freeze-dried sperm*

11 As shown in Table 1, there were no differences in the proportions of cleaved

12 embryos, morulae, or blastocysts comparing the fresh sperm and freeze-dried sperm

13 groups ($P > 0.05$). Blastocysts generated using fresh sperm and blastocysts generated

14 using freeze-dried sperm were not morphologically different. In addition, the blastocyst

15 quality as measured by the number of cells in each blastocyst were not different in the

16 fresh sperm group relative to that in the freeze-dried sperm group (Fig.3A and 3B). No

17 blastocyst development was observed in the sham group. The proportions of cleaved

18 embryos, morulae, and blastocysts to the number of cells in each blastocyst using freeze-

14

1 dried sperm stored for 5 mo are shown in Supplementary table (Table S1).

2

3 **4. Discussion**

4 In this study, we succeeded in preserving freeze-dried cat sperm for 1-5 mo at

5 4°C. When preserving sperm, motility, cell membrane integrity, and the rate of DNA

6 damage are primary indicators used in utility evaluation of the preservation method [28].

7 When DNA-damaged sperm is used for ICSI, fertilization may occur but embryo

8 development and the implantation rate both decrease [22]. Therefore, the DNA damage

9 rate of sperm may serve as an important index when long-term stored sperm is used for

10 ICSI.

11 The DNA damage rate for cryopreserved cat spermatozoa has been previously

12 reported to be 12-15% [28,29] with Prochowska et al. reporting it to be 3% [30]. The

13 DNA damage rate is affected by the cryopreservation methods and by the individual

14 sperm condition [28-32]. In Experiment 1 of the current study, to avoid the effect of

15 individual differences in DNA damage rates, we used nine male cats and divided the

16 semen from individual animals into three groups (fresh, cryopreserved, and freeze-dried).

17 As a result, the DNA damage rate of freeze-dried sperm was 9.6% while the DNA damage

18 rate of sperm cryopreserved in liquid nitrogen was 6.6%. Our results suggest that

1 preservation of cat sperm by freeze-drying is equivalent to conventional cryopreservation

2 methods with respect to damaging DNA. The cryopreservation of sperm using

3 conventional methods results in damage to the sperm cell membranes during the

4 cryopreservation treatment and the thawing process [28]. Freeze drying physically

5 damages sperm DNA at the time of preparation, in addition to calcium-dependent

6 endonucleases being present in sperm during storage that also damage sperm DNA upon

7 freeze drying [23,33]. To prevent damage to sperm DNA, TE buffer containing EDTA, a

8 calcium chelating agent, was used based on the reports of Kaneko *et al.* [23,34]. In boars,

9 rabbits, and dogs, the rate of DNA damage for freeze-dried sperm changes depending on

10 the type of calcium chelating agent and the addition of substances that protect cells from

11 physical damage during freezing [35-37]. We used TE buffer, which has been used for

12 preparing freeze-dried sperm of mice and rats. We expect that damage to DNA may be

13 further reduced in the future by identifying the most appropriate solvent for use with

14 freeze-dried cat sperm.

15   In experiment 2, we succeeded in obtaining blastocysts from freeze-dried cat

16 sperm that had been preserved for several months. There are species-specific differences

17 in the fertilizing ability of freeze-dried sperm [21,38]. While freeze-dried rat sperm have

18 equivalent fertilizing abilities relative to that of fresh sperm [21], the rate of blastocyst

16

1   stage embryogenesis is lower for freeze-dried boar sperm relative to fresh boar sperm

2   [38]. The proportions of cleaved embryos, morulae, and blastocysts to the cell number of

3   blastocysts were not significantly different between groups using fresh sperm and freeze-

4   dried sperm in experiment 2 of our study. The proportion of blastocysts was 14.1% with

5   fresh sperm and 8.2% with freeze-dried sperm. Since approximately 9% of M II oocytes

6   developed into blastocysts after Piezo-ICSI with cryopreserved sperm in our previous

7   report [24], we suggest that freeze-dried cat sperm has the same level of fertilizing

8   potential as do cryopreserved and fresh sperm. Therefore, we suggest the feasibility of

9   preserving cat sperm through freeze-drying. Freeze-dried mouse sperm may be stored for

10   at least 3 y at 4ºC and up to 3 mo at room temperature and still be successfully used [34].

11   In phase 2 of the current study, cat blastocysts were successfully produced using freeze-

12   dried cat sperm that had been stored at 4ºC for 1-5 mo. In the future, if freeze-dried

13   spermatozoa can be stored at room temperature or at 4ºC for extended time, it may

14   become unnecessary to purchase/supplement liquid nitrogen at the time of preservation,

15   which would make the preservation of spermatozoa considerably easier. Furthermore, by

16   applying this technique to the sperm of wild cats, it would be possible to transport sperm

17   internationally at room temperature and to use it for breeding rare wild cats around the

18   world.

1 In summary, we found that cat sperm freeze dried using TE buffer had no

2 significantly different rate of DNA damage compared to that of cryopreserved sperm. In

3 addition, we successfully generated cat blastocysts using freeze-dried sperm that had been

4 stored for 1-5 mo after being freeze dried. The techniques described in our report

5 represent an expansion of the repertoire of available ARTs that have potential application

6 for enhancing the propagation of both domestic and endangered wild cats.

7

8 **Supplementary material**

9 Table S1. Proportions of cleaved embryos, morulae and blastocysts, and the number of

10 cells in each blastocyst obtained using freeze-dried sperm stored for 5 months.

11

12 **Acknowledgments**

17

18 **Author contributions**

Y.T.: Conception and design, collection and/or assembly of data, data analysis

and interpretation, and manuscript writing; T.K.: Collection and/or assembly of data, data

analysis and interpretation, and manuscript writing; T.Y.: Collection and/or assembly of

data, data analysis and interpretation, and manuscript writing; K.K.: Collection and/or

assembly of data, data analysis and interpretation, and manuscript writing; T.I.: Data

analysis and interpretation, and manuscript writing; K.S.: Data analysis and interpretation,

and manuscript writing; S.H.: Financial support, conception and design, data analysis and

interpretation, manuscript writing, and final manuscript approval.

Declarations of interest: none.

References

[1] Pope CE. Embryo technology in conservation efforts for endangered felids.
Theriogenology 2000;53:163–74.

[2] Axnér E, Linde-Forsberg C. Semen collection and assessment, and artificial
insemination in the cat. In: Concannon PW, England G, Verstegen J, Linde-Forsberg
C, editors. Recent advances in small animal reproduction, International Veterinary
Information Service (www.ivis.org); 2002, p. A1228.0702.

1   [3] Sherman JK. Low temperature research on spermatozoa and eggs. Cryobiology

2   1964;1:103–29.

3   [4] Tada N, Sato M, Yamamoto J, Mizorogi J. Kasai K, Ogawa H. Cryopreservation of

4   mouse spermatozoa in the presence of raffinose and glycerol. J Reprod Fertil

5   1990;89:511–6.

6   [5] Nakagata N, Takeshima T. High fertilizing ability of mouse spermatozoa diluted

7   slowly after cryopreservation. Theriogenology 1992;37:1283–91.

8   [6] Polge C. Fertilizing capacity of bull spermatozoa after freezing at -79 ℃. Nature

9   1952;169:626–7.

10   [7] Stachecki JJ, Ginsburg KA, Armant DR. Stimulation of cryopreserved epididymal

11   spermatozoa of the domestic cat using the motility stimulants caffeine, pentoxifylline,

12   and 2'-deoxyadenosine. J Androl 1994;15:157–64.

13   [8] Wakayama T, Yanagimachi R. Development of normal mice from oocytes injected

14   with freeze-dried spermatozoa. Nat Biotechnol 1998;16:639–41.

15   [9] Ward MA, Kaneko T, Kusakabe H, Biggers JD, Whittingham DG, Yanagimachi R.

16   Long-term preservation of mouse spermatozoa after freeze-drying and freezing

17   without cryoprotection. Biol Reprod 2003;69:2100–08.

18   [10] Kaneko T, Nakagata N. Relation between storage temperature and fertilizing ability

1 of freeze-dried mouse spermatozoa. Comp Med 2005;55:140–4.

2 [11] Keskintepe L, Pacholczyk G, Machnicka A, Norris K, Curuk MA, Khan I, Brackett

3 BG. Bovine blastocyst development from oocytes injected with freeze-dried

4 spermatozoa. Biol Reprod 2002;67:409–15.

5 [12] Kwon IK, Park KE, Niwa K. Activation, pronuclear formation, and development in

6 vitro of pig oocytes following intracytoplasmic injection of freeze-dried spermatozoa.

7 Biol Reprod 2004;71:1430–6.

8 [13] Liu JL, Kusakabe H, Chang CC, Suzuki H, Schmidt DW, Julian M, Preffer R,

9 Bormann CL, Tian C, Yanagimachi R, Yang X. Freeze-dried sperm fertilization leads

10 to full-term development in rabbits. Biol Reprod 2004;70:1776–81.

11 [14] Hirabayashi M, Kato M, Ito J, Hochi S. Viable rat offspring derived from oocytes

12 intracytoplasmically injected with freeze-dried sperm heads. Zygote 2005;13:79–85.

13 [15] Sa´nchez-Partida LG, Simerly CR, Ramalho-Santos J. Freeze-dried primate sperm

14 retains early reproductive potential after intracytoplasmic sperm injection. Fertil

15 Steril 2007;89:742–5.

16 [16] Ringleb J. Waurich R, Wibbelt G, Streich WJ, Jewgenow K. Prolonged storage of

17 epididymal spermatozoa does not affect their capacity to fertilise in vitro-matured

18 domestic cat (Felis catus) oocytes when using ICSI. Reprod Fertil Dev 2011;23:818–

1   25.

2   [17]   Martins CF, Bao SN, Dodea MN, Correa GA, Rumpf R. Effects of freeze-drying on

3   cytology, ultrastructure, DNA fragmentation, and fertilizing ability of bovine 384

4   sperm. Theriogenology 2007;67:1307–17.

5   [18]   Kusakabe H, Szczygiel MA, Whittingham DG, Yanagimachi R. Maintenance of

6   genetic integrity in frozen and freeze-dried mouse spermatozoa. PNAS

7   2001;98:13501–6.

8   [19]   Kusakabe H, Yanagimachi R, Kamiguchi Y. Mouse and human spermatozoa can be

9   freeze-dried without damaging their chromosomes. Hum Reprod 2008;23:233–9.

10   [20]   Kaneko T, Nakagata N. Improvement in the long-term stability of freeze-dried

11   mouse spermatozoa by adding of a chelating agent. Cryobiology 2006;53:279–82.

12   [21]   Kaneko T, Kimura S, Nakagata N. Offspring derived from oocytes injected with rat

13   sperm, frozen or freeze-dried without cryoprotection. Theriogenology

14   2007;68:1017–21.

15   [22]   Sakkas D, Urner F, Bizzaro D, Manicardi G, Bianchi PG, Shoukir Y, Campana A.

16   Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization

17   and embryo development. Hum Reprod 1998;13:11–9.

18   [23]   Kaneko T, Serikawa T. Successful long-term preservation of rat sperm by freeze-

1   drying. PLoS One 2012;7:e35043.

2 [24] Tsujimoto Y, Fujiki K, Emtiaj MD, Tsukamoto M, Azuma R, Kanegi R, Anzai M,

3   Inaba T, Sugiura K, Hatoya S. Development of feline embryos produced by Piezo-

4   actuated intracytoplasmic sperm injection of elongated spermatids. J Reprod Dev

5   2019;65:245-250.

6 [25] Tejada RI, Mitchell JC, Norman A, Marik JJ, Friedman S. A test for the practical

7   evaluation of male fertility by acridine orange (AO) fluorescence. Fertil Steril

8   1984;42:87–91.

9 [26] Kanegi R, Hatoya S, Tsujimoto Y, Takenaka S, Nishimura T, Wijewardana V,

10   Sugiura K, Takahashi M, Kawate N, Tamada H, Inaba T. Production of feline

11   leukemia inhibitory factor with biological activity in *Escherichia coli*.

12   Theriogenology 2016;86:604–11.

13 [27] Alam ME, Iwata J, Fujiki K, Tsujimoto Y, Kanegi R, Kawate N, Tamada H, Inaba

14   T, Sugiura K, Hatoya S. Feline embryo development in commercially available

15   human media supplemented with fetal bovine serum. J Vet Med Sci 2019;81:629–35.

16 [28] Thuwanut P, Chatdarong K, Techakumphu M, Axnér E. The effect of antioxidants

17   on motility, viability, acrosome integrity and DNA integrity of frozen-thawed

18   epididymal cat spermatozoa. Theriogenology 2008;70:233–40.

1 [29] Vick MM, Bateman HL, Lambo CA, Swanson WF. Improved cryopreservation of

2   domestic cat sperm in a chemically defined medium. Theriogenology 2012;78:2120–

3   8.

4 [30] Prochowska S, Niżański W, Partyka A. Comparative analysis of in vitro

5   characteristics of fresh and frozen-thawed urethral and epididymal spermatozoa from

6   cats (Felis domesticus). Theriogenology 2016;86:2063–72.

7 [31] Villaverde AI, Fioratti EG, Penitenti M, Ikoma MR, Tsunemi MH, Papa FO, Lopes

8   MD. Cryoprotective effect of different glycerol concentrations on domestic cat

9   spermatozoa. Theriogenology 2013;80:730–7.

10 [32] Penfold LM, Jost L, Evenson DP, Wildt DE. Normospermic versus teratospermic

11   domestic cat sperm chromatin integrity evaluated by flow cytometry and

12   intracytoplasmic sperm injection. Biol Reprod 2003;69:1730–5.

13 [33] Maione B, Pittoggi C, Achene L, Lorenzini R, Spadafora C. Activation of

14   endogenous nucleases in mature sperm cells upon interaction with exogenous DNA.

15   DNA Cell Biol 1997;16:1087–97.

16 [34] Kaneko T, Serikawa T. Long-term preservation of freeze-dried mouse spermatozoa.

17   Cryobiology 2012;64:211–4.

18 [35] Nakai M, Kashiwazaki N, Takizawa A, Maedomari N, Ozawa M, Noguchi J,

1   Kaneko H, Shino M, Kikuchi K. Effects of chelating agents during freeze-drying of

2   boar spermatozoa on DNA fragmentation and on developmental ability in vitro and

3   in vivo after intracytoplasmic sperm head injection. Zygote 2007;15:15–24.

4   [36] Domingo P, Olaciregui M, González N, De Blas I, Gil L. Long-term preservation of

5   freeze-dried rabbit sperm by adding rosmarinic acid and different chelating agents.

6   Cryobiology 2018;81:174–77.

7   [37] Olaciregui M, Luño V, Gonzalez N, De Blas I, Gil L. Freeze-dried dog sperm:

8   Dynamics of DNA integrity. Cryobiology 2015;71:286–90.

9   [38] Men NT, Kikuchi K, Nakai M, Fukuda A, Tanihara F, Noguchi J, Kaneko H, Linh

10   NV, Nguyen BX, Nagai T, Tajima A. Effect of trehalose on DNA integrity of freeze-

11   dried boar sperm, fertilization, and embryo development after intracytoplasmic

12   sperm injection. Theriogenology 2013;80:1033–44.

13

1 **Figure legends**

2 Fig. 1. Acridine orange (AO) staining of cat sperm evaluated under confocal laser

3 microscopy (magnification, 200x). (A) Fresh sperm. (B) Cryopreserved sperm. (C)

4 Freeze-dried sperm. The scale bar represents 40 µm. Arrow head indicates normal DNA

5 integrity. Arrow indicates fragmented DNA.
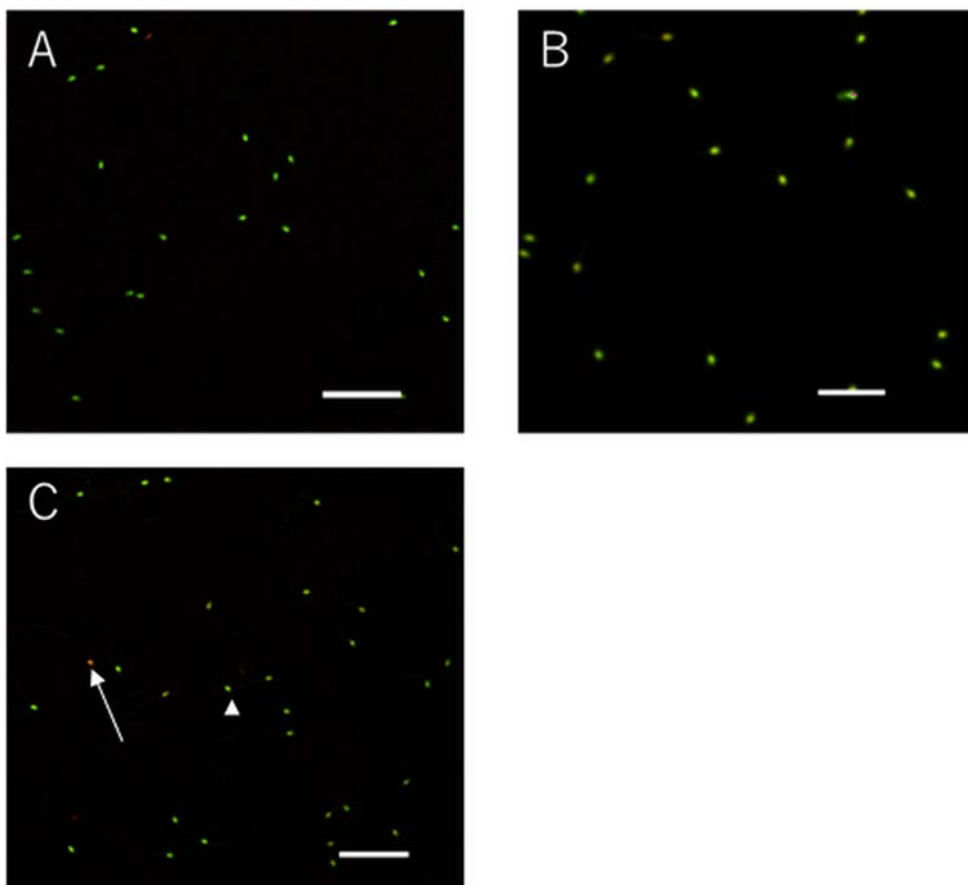
6

7

8

9

1   Fig. 2. Percentage of DNA fragmentation in three types of cat sperm specimens. Fresh cat

2   sperm, cryopreserved cat sperm, and freeze-dried cat sperm were analyzed for DNA

3   integrity and the percentage of fragmentation is plotted. Values are presented as mean ±

4   SEM. Bars with different letters (a, b) are significantly different ($P < 0.05$).
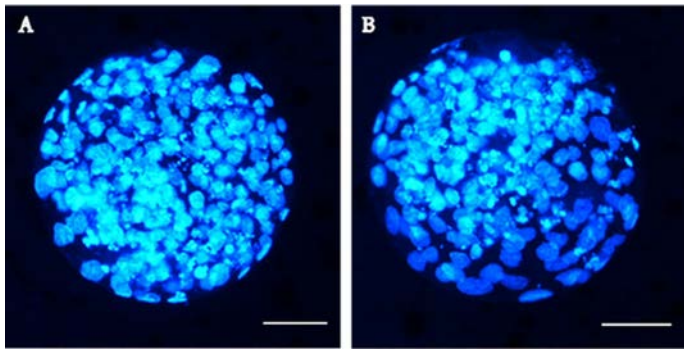
5
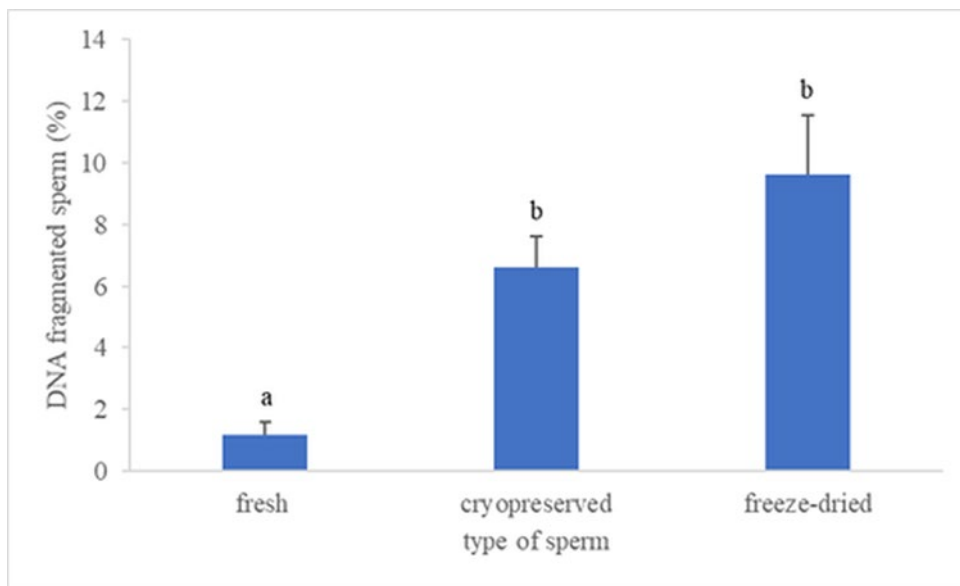
6

7

8

9

10

11

12

13

1   Fig. 3. Cat blastocysts stained with bis-benzimide (Hoechst 33342) were visualized under

2   a fluorescence microscope (magnification, 200x). (A) Blastocyst derived from Piezo-

3   ICSI using freeze-dried sperm. (B) Blastocyst derived from Piezo-ICSI using fresh sperm.

4   The scale bar represents 20 µm.

5

6

1 **Table**

2 Table 1. Developmental competence of cat embryos after Piezo-ICSI using fresh sperm

3 and freeze-dried sperm

Group	Number of oocytes	Stage of development (%)			Blast cell number (Mean ± SEM)
		Cleavage	Morula	Blastocyst	
fresh	93	57.6 ± 3.7[a]	16.3 ± 3.9[a,b]	14.1 ± 3.3[a]	167.4 ± 20.2[a]
freeze-dried	260	56.2 ± 5.3[a]	18.2 ± 2.5[a]	8.2 ± 1.1[a]	141.1 ± 12.2[a]
sham	102	24.8 ± 6.7[b]	5.1 ± 2.1[b]	0	N.D.

4 Data are expressed as the mean ± standard error of the mean (SEM). [a,b]Statistical

5 differences within a column ($P < 0.05$). N.D, not determined.

6

7

8

9

10

11

12

1   Table S1 Proportions of cleaved embryos, morulae and blastocyst, and the number of cells

2   in each blastocyst obtained using freeze-dried sperm stored for 5 month

3

Group	Number of oocytes	Stage of development (%)			Blast cells, N
		Cleavage	Morula	Blastocyst	(Mean)
freeze-dried	24	54.2	16.7	8.3	109

4

5

6   Data are expressed as the mean.