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Abstract

Freeze drying has been developed as a new sperm preservation method that eliminates the necessity of using liquid nitrogen. An advantage of freeze-dried sperm is that it can be stored at 4℃ and transported at room temperature. To develop assisted reproductive techniques (ARTs) for domestic cats, we evaluated the effect of the freeze-dry procedure on cat sperm DNA by analyzing DNA integrity (experiment 1) and by generating cat embryos using freeze-dried sperm that had been preserved for several months (experiment 2). In experiment 1, the rate of DNA damage to freeze-dried sperm was not significantly different than that of sperm cryopreserved with liquid nitrogen ($P > 0.05$). In experiment 2, the proportions of cleaved embryos, morulae, and blastocysts and the cell number of blastocysts did not differ between experimental groups in which fresh sperm and freeze-dried sperm were used ($P > 0.05$). In addition, we generated feline blastocysts using freeze-dried sperm stored for 1–5 months. These results support an expansion of the repertoire of ARTs that are potentially applicable to both domestic and endangered species of cats.

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

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

because it does not necessitate the use of liquid nitrogen. An advantage of freeze-dried sperm is that it can be stored at 4℃ [8–10] and stored and transported for short periods of time at room temperature without the use of liquid nitrogen and/or dry ice as cooling agents [10]. However, since freeze-dried sperm lose their motility after rehydration, an essential requirement for completing physiological fertilization, a relatively difficult and specialized technique such as intracytoplasmic sperm injection (ICSI) must be applied when using freeze-dried sperm. Attempts to freeze-dry sperm from several species of mammals have been reported [11–15], including sperm from cats [16]. However, it is unclear whether freeze-dried cat sperm maintains embryonic potential since blastocysts did not develop when the freeze-dried sperm was used.

In addition, sperm DNA can be damaged during freeze drying and especially during storage if adequate protection is not provided [17]. It has been suggested that DNA damage can be induced by mechanical stress throughout the freeze-drying process or by oxidative stress, which occurs after rehydration [18,19]. Studies using mice and rats have reported that a solution containing 10 mM Tris and 1 mM EDTA (TE buffer) adjusted to pH 8.0 protect sperm DNA from physical damage by the freeze-drying process and from endogenous nuclease activity during storage [20,21]. When damaged DNA sperm is used for ICSI, fertilization occurs but the rates of embryo development and implantation

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

We performed two experiments in this study. First, we evaluated the level of DNA damage that occurred to cat sperm during the freeze-drying procedure by analyzing DNA integrity (experiment 1). We then generated cat embryos using freeze-dried sperm that had been stored for several months (experiment 2).

2. Material and methods

2.1. Animals included in the study

The ovaries and testes with epididymides were collected from domestic short-haired cats at a local veterinary clinic following routine ovariohysterectomy and castration, respectively. The male cats were between 8 mo and 6 y of age and the female cats were between 6 mo and 7 y of age. We do not have any informations regarding estrous cycle stage of ovary donors. All the animals were clinically healthy. The collected ovaries and testes were maintained in 0.9% (w/v) saline solution and transported at room temperature (24ºC) to our laboratory within 6 h of collection. The animals did not receive surgery nor were they euthanized specifically for the study. All cats were privately owned and the owners provided consent for collection and use of the tissues.

2.2. Sperm freeze drying

Freeze drying of the sperm was carried out using a partial modification of the procedure suggested by Kaneko *et al.* [23]. Briefly, after removing the external tissues from the testes, the epididymides were separated and cut into small pieces in Tris-EDTA buffer (TE buffer), pH 8.0 (Ambion, Austin, TX, USA). The processed epididymis tissues were then cultured for 10 min at 38.5°C under 5% CO_2 in humidified air. After filtering through a 20-µm filter (Nipro, Osaka, Japan), the semen was centrifuged for 5 min at 500 × *g* and the supernatant was removed by aspiration. The pelleted sperm were resuspended in 1 mL TE buffer and 100 µL aliquots were transferred into long-necked glass ampoules for freeze drying (Wheaton, Millville, NJ, USA). The ampoules were plunged into liquid nitrogen for 20 s and were then connected to the manifold of an FZ-compact freeze-drying machine (Labconco, Kansas City, MO, USA). Sperm suspensions were dried for 4 h at a pressure of 0.04 hPa at -50℃. The ampoules containing the freeze-dried sperm were flame-sealed and stored at 4℃ until use.

2.3. Sperm collection and cryopreservation

Sperm collection and cryopreservation were carried out as previously described

[24]. Epididymides prepared from cat testes were cut into small pieces in Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS (−); Nacalai, Kyoto, Japan) and cultured at 38.5°C under 5% CO_2 in humidified air for 10 min. After filtering through a 20-µm filter (Nipro), the semen was centrifuged for 5 min at 500 × *g* and the supernatant was removed by aspiration. To prepare a sperm suspension, the pelleted sperm were resuspended in 1 ml modified Human Tubal Fluid (m-HTF) medium (Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan).

The sperm suspension was mixed with egg yolk Tris-fructose citrate (EYT-FC) solution, which was comprised of 20% (v/v) egg yolk supplemented with 13 µg/mL citric acid (Nacalai), 10 µg/mL d-fructose (Nacalai), 24 µg/mL Tris aminomethane (Nacalai), 1,000 IU/mL penicillin (Sigma–Aldrich, St. Louis, MO, USA), and 1 mg/mL streptomycin (Sigma–Aldrich). The mixture was incubated at 4°C for 1 h and then EYT-FC solution containing 14% (v/v) glycerol (Nacalai) was added to obtain a final concentration of 12.5 × 10^6 cells/mL with a final glycerol concentration of 7%. This solution was then loaded into 0.25-mL straws (Fujihira, Tokyo, Japan). After sealing, the straws were laid horizontally on a rack 4 cm above liquid nitrogen vapor for 5 min, plunged into liquid nitrogen, and then stored in a liquid nitrogen storage tank until use.

2.4. Thawing of cryopreserved sperm

Straws containing the cryopreserved sperm were thawed in a 37ºC water bath for 30 s. The sperm were then transferred into 10 ml m-HTF medium and the solution was centrifuged for 5 min at 500 × *g*. The pelleted sperm were resuspended in 5 ml Human Tubal Fluid medium (Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) and centrifuged again for 5 min at 500 × *g* with the final sperm pellet being resuspended in 1 ml m-HTF medium (1×10^6 cells/mL).

2.5. DNA integrity

DNA integrity was evaluated using acridine orange (AO) following a slight modification of the method described by Tejada *et al*. [25]. The staining solution was prepared by adding 10 mL of 10 mg/mL AO (Sigma-Aldrich, St. Louis, MO, USA) in distilled water to 40 mL of 0.1 M citric acid (Sigma–Aldrich) and 2.5 mL of 0.3 M $Na_2HPO_4 \cdot 7H_2O$ (Nacalai) at pH 2.5. The freeze-dried sperm were rehydrated by adding 100 µL of distilled water. Air-dried smears on microscope slides that were prepared using 10 µL of rehydrated sperm were fixed overnight in ethanol-acetic acid (3:1, v:v) at room temperature. Thereafter, the smears were air dried, stained with AO solution for 5 min, and rinsed with distill water. Two hundred spermatozoa were evaluated under confocal

laser microscopy (TE2000E; Nikon, Tokyo, Japan) and characterized as having normal DNA integrity (green fluorescence), or denatured or single stranded DNA (yellow, orange, or red fluorescence). Fresh sperm and cryopreserved sperm were stained with AO in the same manner and also evaluated under confocal laser microscopy.

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

Oocyte collection and IVM were conducted as previously described [26]. Briefly, cumulus–oocyte complexes (COCs) were collected after mincing the ovaries in Medium 199 (Thermo Fisher Scientific, Waltham, MA, USA). Oocytes completely surrounded with more than four layers of compacted cumulus cells were used. The COCs were collected and rinsed three times in IVM medium, which was comprised of Medium 199 supplemented with 0.4% (w/v) bovine serum albumin (BSA; Sigma–Aldrich), 10 IU/mL 17β-estradiol (Sigma–Aldrich), 100 μg/mL gentamycin (Sigma–Aldrich), 137 μg/mL sodium pyruvate (Sigma–Aldrich), 0.02 IU/mL human recombinant follicle-stimulating hormone (Follistim®; MSD, Tokyo, Japan), and 25 ng/mL epidermal growth factor (Sigma–Aldrich). The COCs were then incubated for 28 h in 100 μL IVM medium droplets covered with mineral oil (Sigma–Aldrich) in an incubator at 38.5°C with 5% CO_2.

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

oocyte was pierced by applying several piezo pulses to the sperm-injection pipette. After the sperm was pushed forward to the point where its head was near the tip of injection pipette, the pipette was advanced until its tip almost reached the opposite side of the oocyte cortex. The oolemma was broken by applying a single piezo pulse and the entire sperm was expelled into the ooplasm. The pipette was then gently withdrawn. All Piezo-ICSI procedures were performed at room temperature. The injected oocytes were maintained at room temperature for approximately 10 min.

Fresh epididymal sperm were injected into oocytes using the same procedure described above for the freeze-dried sperm. Only morphologically normal and progressively motile fresh epididymal sperm were used.

2.8. Embryo cultures

Embryo cultures were maintained as previously described [27]. After Piezo-ICSI, oocytes were cultured in Only-One medium (Nippon Medical & Chemical Instruments Co., Ltd.) supplemented with 0.3% (w/v) BSA for 2 d at 38.5ºC with 5% O_2, 5% CO_2, and 90% N_2. Subsequently, cleaved embryos were washed and cultured for 5 d in Only-One medium containing 5% (w/v) fetal bovine serum (Biosolutions International, Melbourne, Australia) at 38.5ºC with 5% O_2, 5% CO_2, and 90% N_2. The percentages of

cleaved embryos, morulae, and blastocysts relative to the number of injected oocytes were determined on days 2, 5, and 7 after microinjection, respectively. All blastocysts were rinsed twice with PBS (−). The blastocysts were then fixed and stained with 25 μg/mL bisbenzimide (Hoechst 33342, Sigma–Aldrich) in 100% ethanol for 2 to 3 h at 4°C. The stained blastocysts were examined under a fluorescence microscope to determine the numbers of cells.

2.9. Statistical analyses

Experiment 1 was performed nine times. Experiment 2 was independently performed seven times. Results are expressed as the mean ± standard error of the mean (SEM). Statistical differences were evaluated by ANOVA and the Tukey–Kramer test using StatCell 3 (OMC Publishing Inc., Saitama, Japan). For all cases, differences were considered statistically significant when P values were < 0.05.

2.10. Experiment design

2.10.1. Experiment 1: DNA integrity

In experiment 1, samples obtained from nine male cats were used. Semen from the each individual animal was divided into the following three experimental groups:

fresh sperm (n = 9), cryopreserved sperm stored in liquid nitrogen for 1 mo (n = 9), and freeze-dried sperm stored at 4°C for at 1 mo (n = 9). To evaluate the effect of the freeze-drying procedure on the DNA integrity of cat sperm, the above three groups were stained with AO solution. The fresh sperm group was used as the control.

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

In experiment 2, morphologically normal freeze-dried sperm and morphologically normal and progressively motile fresh sperm were used. A total of 455 M II oocytes from 37 female cats were divided among the following three treatment groups: Piezo-ICSI using freeze-dried sperm (n = 260), Piezo-ICSI using fresh sperm (n = 93), and sham-treated oocytes that were injection without sperm (n = 102). The sham-treated oocytes served as the control group. The freeze-dried sperm used in this experiment was stored at 4°C for at least 1 mo and for a maximum of 5 mo. The developmental competence of the microinjected oocytes was assessed on days 2, 5, and 7 of *in vitro* culturing (day 0 = day of ICSI) in terms of the percentages of all the oocytes that underwent cleavage, morula formation, and blastocyst formation, respectively. Embryos with ≥ 16 cells without a blastocoel were classified as morulae. Only embryos

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

3. Results

3.1. DNA integrity

DNA integrity of the sperm is shown in Fig. 1. As shown in Fig. 2, based on percentage of DNA fragmentation there was no difference between the freeze-dried sperm and cryopreserved sperm samples ($P > 0.05$). However, the DNA damage rate of the fresh sperm was lower than that of the freeze-dried and cryopreserved sperm samples ($P < 0.05$).

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

As shown in Table 1, there were no differences in the proportions of cleaved embryos, morulae, or blastocysts comparing the fresh sperm and freeze-dried sperm groups ($P > 0.05$). Blastocysts generated using fresh sperm and blastocysts generated using freeze-dried sperm were not morphologically different. In addition, the blastocyst quality as measured by the number of cells in each blastocyst were not different in the fresh sperm group relative to that in the freeze-dried sperm group (Fig.3A and 3B). No blastocyst development was observed in the sham group. The proportions of cleaved embryos, morulae, and blastocysts to the number of cells in each blastocyst using freeze-

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

4. Discussion

In this study, we succeeded in preserving freeze-dried cat sperm for 1-5 mo at 4ºC. When preserving sperm, motility, cell membrane integrity, and the rate of DNA damage are primary indicators used in utility evaluation of the preservation method [28]. When DNA-damaged sperm is used for ICSI, fertilization may occur but embryo development and the implantation rate both decrease [22]. Therefore, the DNA damage rate of sperm may serve as an important index when long-term stored sperm is used for ICSI.

The DNA damage rate for cryopreserved cat spermatozoa has been previously reported to be 12-15% [28,29] with Prochowska et al. reporting it to be 3% [30]. The DNA damage rate is affected by the cryopreservation methods and by the individual sperm condition [28-32]. In Experiment 1 of the current study, to avoid the effect of individual differences in DNA damage rates, we used nine male cats and divided the semen from individual animals into three groups (fresh, cryopreserved, and freeze-dried). As a result, the DNA damage rate of freeze-dried sperm was 9.6% while the DNA damage rate of sperm cryopreserved in liquid nitrogen was 6.6%. Our results suggest that

preservation of cat sperm by freeze-drying is equivalent to conventional cryopreservation methods with respect to damaging DNA. The cryopreservation of sperm using conventional methods results in damage to the sperm cell membranes during the cryopreservation treatment and the thawing process [28]. Freeze drying physically damages sperm DNA at the time of preparation, in addition to calcium-dependent endonucleases being present in sperm during storage that also damage sperm DNA upon freeze drying [23,33]. To prevent damage to sperm DNA, TE buffer containing EDTA, a calcium chelating agent, was used based on the reports of Kaneko *et al.* [23,34]. In boars, rabbits, and dogs, the rate of DNA damage for freeze-dried sperm changes depending on the type of calcium chelating agent and the addition of substances that protect cells from physical damage during freezing [35-37]. We used TE buffer, which has been used for preparing freeze-dried sperm of mice and rats. We expect that damage to DNA may be further reduced in the future by identifying the most appropriate solvent for use with freeze-dried cat sperm.

In experiment 2, we succeeded in obtaining blastocysts from freeze-dried cat sperm that had been preserved for several months. There are species-specific differences in the fertilizing ability of freeze-dried sperm [21,38]. While freeze-dried rat sperm have equivalent fertilizing abilities relative to that of fresh sperm [21], the rate of blastocyst

stage embryogenesis is lower for freeze-dried boar sperm relative to fresh boar sperm [38]. The proportions of cleaved embryos, morulae, and blastocysts to the cell number of blastocysts were not significantly different between groups using fresh sperm and freeze-dried sperm in experiment 2 of our study. The proportion of blastocysts was 14.1% with fresh sperm and 8.2% with freeze-dried sperm. Since approximately 9% of M II oocytes developed into blastocysts after Piezo-ICSI with cryopreserved sperm in our previous report [24], we suggest that freeze-dried cat sperm has the same level of fertilizing potential as do cryopreserved and fresh sperm. Therefore, we suggest the feasibility of preserving cat sperm through freeze-drying. Freeze-dried mouse sperm may be stored for at least 3 y at 4ºC and up to 3 mo at room temperature and still be successfully used [34]. In phase 2 of the current study, cat blastocysts were successfully produced using freeze-dried cat sperm that had been stored at 4ºC for 1-5 mo. In the future, if freeze-dried spermatozoa can be stored at room temperature or at 4ºC for extended time, it may become unnecessary to purchase/supplement liquid nitrogen at the time of preservation, which would make the preservation of spermatozoa considerably easier. Furthermore, by applying this technique to the sperm of wild cats, it would be possible to transport sperm internationally at room temperature and to use it for breeding rare wild cats around the world.

In summary, we found that cat sperm freeze dried using TE buffer had no significantly different rate of DNA damage compared to that of cryopreserved sperm. In addition, we successfully generated cat blastocysts using freeze-dried sperm that had been stored for 1-5 mo after being freeze dried. The techniques described in our report represent an expansion of the repertoire of available ARTs that have potential application for enhancing the propagation of both domestic and endangered wild cats.

Supplementary material

Table S1. Proportions of cleaved embryos, morulae and blastocysts, and the number of cells in each blastocyst obtained using freeze-dried sperm stored for 5 months.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Numbers JP15K07747, JP18K19273 and JP18H02349. The sponsors had no role in designing the study; in the collection, analysis, or interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

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.

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Figure legends

Fig. 1. Acridine orange (AO) staining of cat sperm evaluated under confocal laser microscopy (magnification, 200x). (A) Fresh sperm. (B) Cryopreserved sperm. (C) Freeze-dried sperm. The scale bar represents 40 μm. Arrow head indicates normal DNA integrity. Arrow indicates fragmented DNA.

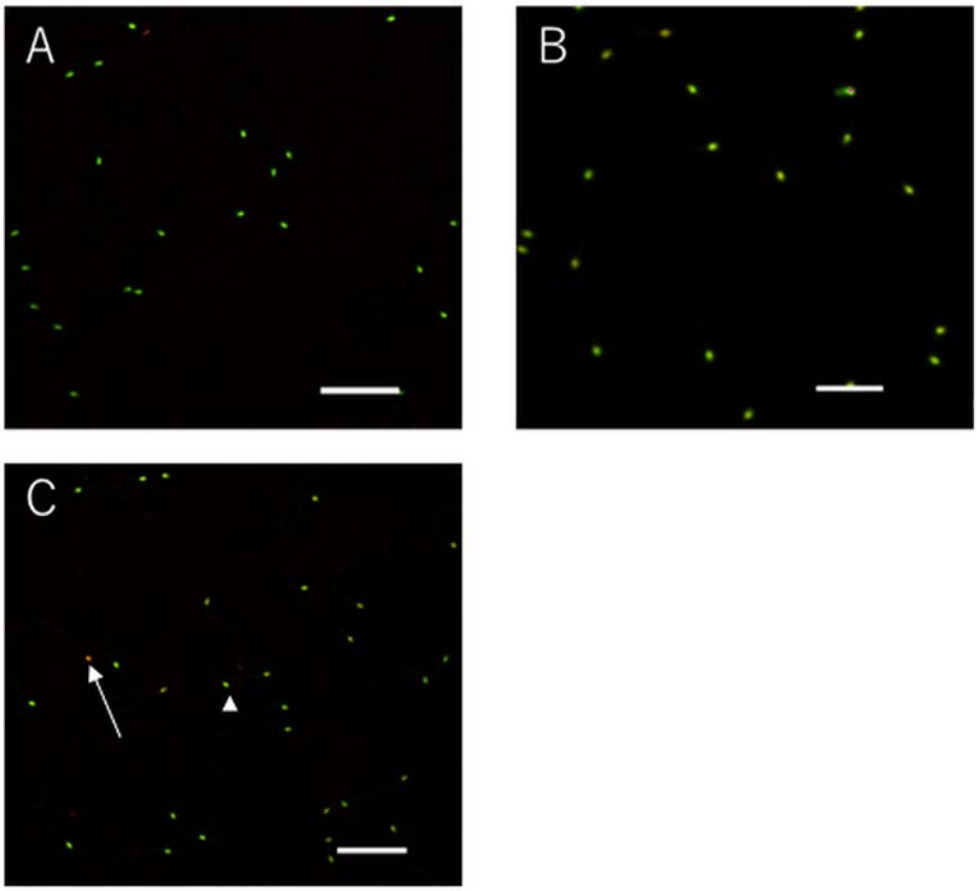


Fig. 2. Percentage of DNA fragmentation in three types of cat sperm specimens. Fresh cat sperm, cryopreserved cat sperm, and freeze-dried cat sperm were analyzed for DNA integrity and the percentage of fragmentation is plotted. Values are presented as mean ± SEM. Bars with different letters (a, b) are significantly different ($P < 0.05$).

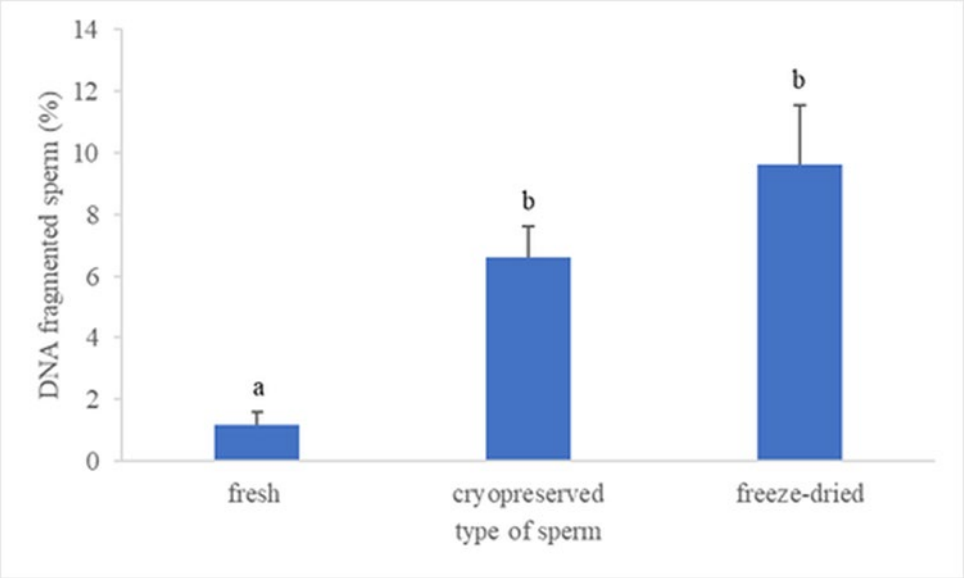
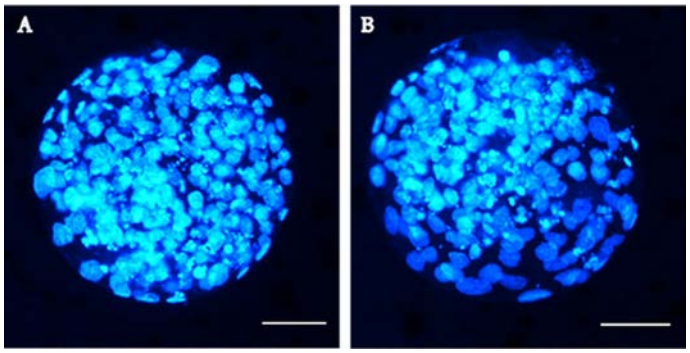


Fig. 3. Cat blastocysts stained with bis-benzimide (Hoechst 33342) were visualized under a fluorescence microscope (magnification, 200x). (A) Blastocyst derived from Piezo-ICSI using freeze-dried sperm. (B) Blastocyst derived from Piezo-ICSI using fresh sperm. The scale bar represents 20 μm.



Table

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

Data are expressed as the mean ± standard error of the mean (SEM). [a,b]Statistical differences within a column ($P < 0.05$). N.D, not determined.

Table S1 Proportions of cleaved embryos, morulae and blastocyst, and the number of cells in each blastocyst obtained using freeze-dried sperm stored for 5 month

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

Data are expressed as the mean.