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Original Article

Production of offspring from vacuum-dried mouse spermatozoa and assessing the effect of drying conditions on sperm DNA and embryo development

Natsuki USHIGOME[1)], Sayaka WAKAYAMA[2)], Kango YAMAJI[1)], Daiyu ITO[1)], Masatoshi OOGA[1)] and Teruhiko WAKAYAMA[1, 2)]

[1)]*Faculty of Life and Environmental Science, University of Yamanashi, Kofu 400-8510, Japan*
[2)]*Advanced Biotechnology Center, University of Yamanashi, Kofu 400-8510, Japan*

Abstract. Freeze-dried sperm (FD sperm) are of great value because they can be stored at room temperature for long periods of time, However, the birth rate of offspring derived from FD sperm is low and the step in the freeze-drying process particularly responsible for low offspring production remains unknown. In this study, we determined whether the drying process was responsible for the low success rate of offspring by producing vacuum-dried sperm (VD sperm), using mouse spermatozoa dried in a vacuum without being frozen. Transfer of embryos fertilized with VD sperm to recipients resulted in the production of several successful offspring. However, the success rate was slightly lower than that of FD sperm. The volume, temperature, and viscosity of the medium were optimized to improve the birth rate. The results obtained from a comet assay indicated that decreasing the drying rate reduced the extent of DNA damage in VD sperm. Furthermore, even though the rate of blastocyst formation increased upon fertilization with VD sperm, full-term development was not improved. Analysis of chromosomal damage at the two-cell stage through an abnormal chromosome segregation (ACS) assay revealed that reduction in the drying rate failed to prevent chromosomal damage. These results indicate that the lower birth rate of offspring from FD sperm may result from the drying process rather than the freezing process.
Key words: Abnormal chromosomal segregation (ACS), Freeze-dry, Intracytoplasmic sperm injection (ICSI), Spermatozoa, Vacuum-dry

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Long-term spermatozoa preservation plays a significant role in assisted reproductive technology, maintenance of genetically modified animals, and preservation of genetic resources for rare animal species. In general, liquid nitrogen (LN_2) is used to permanently preserve spermatozoa, as they regain motility after thawing; however, the use of LN_2 has several limitations. Most importantly, the maintenance costs are high because of the need for a constant supply, making it difficult for some countries to maintain their specimens. Furthermore, transporting a small number of spermatozoa samples to another laboratory requires expensive dry shippers or a large amount of dry ice, which results in high costs.

The use of freeze-dried spermatozoa (FD sperm) was developed as a preservation method to solve the problems associated with LN_2-based preservation. Although mouse spermatozoa are unable to survive after freeze-drying, their DNA is still viable, and normal offspring may be obtained when the head of the FD sperm is injected into oocytes [1]. Subsequently, this technique has been successfully employed to obtain offspring from the FD sperm of rats [2], hamsters [3], rabbits [4], and horses [5] and has been applied to domestic animals, such as sheep [6] and cattle [7, 8]. In addition, several studies have aimed at long-term FD sperm preservation [9–13]. In our previous study, we succeeded in producing healthy offspring from FD mouse sperm stored for more than one year at room temperature (RT) by improving the vacuum conditions of the ampoules [12]. Under refrigerated conditions, FD rat sperm may be stored for five years [14]. Interestingly, offspring have been obtained from FD mouse sperm exposed to high or low temperatures [15], high radiation [16] or even those stored for six years on the International Space Station [17, 18]. In addition, FD sperm can be stored in thin plastic sheets, allowing the sperm to be mailed on postcards [19]. These studies suggest that FD sperm are highly tolerant to extreme environments and enable long-term preservation of sperm DNA. However, more than 20 years after the first success with FD mouse sperm, the birth rate remains low at approximately one-third of that of fresh spermatozoa [12, 20, 21]. Although improving the quality of FD sperm and the success rate of offspring production to facilitate stable storage and reliable transport is crucial, the underlying cause of the low birth rate remains unclear [22].

The procedure for FD sperm production comprises five steps: preculture, freezing, drying, preservation, and rehydration. However, the step that causes the highest damage to spermatozoa has not yet been examined in detail. Previous studies have demonstrated that freezing spermatozoa without a cryoprotectant kills all spermatozoa; however, when the sperm head is injected into oocytes after thawing, healthy offspring may be obtained with only a slight decrease in birth rate [21, 23, 24]. Therefore, the freezing process of FD sperm is possibly not the main reason for the low birth rate of fertilized embryos.

To obtain clear results regarding the effects of the drying process on spermatozoa using FD sperm is challenging. To date, evaporative (or convective) drying of mouse sperm and heat-drying of rat sperm have been employed as methods of drying spermatozoa without

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Correspondence: T Wakayama (e-mail: twakayama@yamanashi.ac.jp)

freezing [25–27, 28]. Each method has been successfully used in offspring production, and microwave drying of feline spermatozoa also produces blastocysts [29]. However, it is possible that evaporation, heating, and microwave treatment used in these studies damaged sperm DNA; therefore, the results cannot be directly compared with those of FD sperm. To elucidate whether DNA damage occurs in FD sperm during the drying process, it is important to examine vacuum-dried (VD sperm) sperm, which are dried under conditions similar to those of FD sperm, without freezing them. To date, VD sperm has only been produced from rhesus monkey sperm, and it has been confirmed that monkey embryos fertilized with VD sperm can develop into blastocysts [30, 31].

In this study, we investigated whether offspring can be produced from embryos fertilized with mouse VD sperms. We optimized the drying conditions, including the volume of the medium, drying temperature, and viscosity of the medium to determine how the drying process affects the integrity of sperm DNA and embryonic development.

Materials and Methods

Animals

Female and male ICR mice (8–10 weeks old) were obtained from SLC, Inc. (Hamamatsu, Japan). Surrogate pseudo-pregnant ICR females were prepared for use as recipients of the embryos by mating with vasectomized ICR males whose sterility had been previously demonstrated. On the day of the experiment or after completion of all experiments, the mice were euthanized *via* CO_2 inhalation or cervical dislocation and used in the experiments described further. All animal-based experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Committee of Laboratory Animal Experimentation of the University of Yamanashi (reference number: A29–24), which followed the ARRIVE guidelines.

Media

HTF medium was used for capacitation, freeze-thawing, freeze-drying, and vacuum-drying of spermatozoa [32]. HEPES-CZB [33] and CZB [34] media were used for oocyte/embryo manipulation and were incubated in 5% CO_2 at 37°C.

Preparation of VD spermatozoa

Both epididymides were collected from male ICR mice and the ducts were severed using sharp scissors. A few drops of dense spermatozoa mass were then placed into a centrifuge tube containing 1-ml HTF medium, which was then incubated for 30 min at 37°C and 5% CO_2. The concentration and motility of spermatozoa were determined using a microscope, and 800 µl of the supernatant was collected. Aliquots (10–200 µl) of the spermatozoa suspension were dispensed into glass ampoules. The ampoules were connected to an FDU-2200 freeze dryer (EYELA, Tokyo, Japan) for vacuum-drying. The cork of the freeze-dryer was opened for at least 6 h until all samples were completely dry. Subsequently, the ampoules were sealed by melting the ampoule necks using a gas burner under vacuum, as described previously [35] (Figs. 2A–C). All ampoules were stored in a freezer at −30°C until further use to avoid the effects of damage suffered during storage and preserved for 3 days to 2 weeks before use [15]. No additional protective measures were taken to preserve spermatozoa.

Experimental design of vacuum-drying

The experimental procedure for vacuum-drying of spermatozoa has been described in Fig. 1. The effects of the following methods were analyzed.

Experiment A. Effect of freezing, freeze-drying, or vacuum drying on the spermatozoa used for fertilization and development after Intracytoplasmic sperm injection (ICSI). After dispensing 50 µl of sperm suspension into ampoules, they were treated as follows: no treatment (fresh sperm), freeze-thawed (FT sperm), freeze-dried (FD sperm), and vacuum-dried (VD sperm) (Fig. 1A).

Experiment B. Effect of the volume of the drying medium on VD sperm: 10, 25, 50, 100, or 200 µl of the sperm suspension was placed in glass ampoules and then vacuum-dried at RT (Fig. 1B).

Experiment C. Effect of temperature during vacuum-drying on VD sperm: 50 µl sperm suspension was transferred to glass ampoules, placed in a water bath at 37°C, in iced water (0.5°C), or at RT, and then vacuum-dried (Fig. 1C).

Experiment D. Effect of viscosity of the drying medium on VD sperm: The sperm suspension was centrifuged at 2300 × *g* for 10 min. Different quantities of the supernatant (most, 1/2, 3/4, or 1/8) were removed and replaced with the same amount of 12% polyvinylpyrrolidone (PVP; 360 kDa) and mixed by pipetting. The concentration of spermatozoa remained constant, whereas the concentrations of PVP were 12%, 6%, 3%, and 1.5%. Then, 50 µl of sperm suspension with PVP was placed in glass ampoules and vacuum-dried at RT (Fig. 1D).

E. Effect of combined method: Methods of Experiment B and C above were combined by transferring 50 µl sperm suspension with or without PVP into glass ampoules and vacuum-drying at 37°C, RT, or 0.5°C (Fig. 1E).

Measurement of drying speed

The drying speed of each sample mixture was determined and compared, using the following procedure. First, empty 1.5 ml PCR tubes or 1 ml glass ampoules were weighed and the instrument set to zero. Next, the medium or medium with PVP was added to the tubes or glass ampoules and then vacuum-dried using the procedure described above. The weight was measured every 2 min for the first 10 min of drying and every 5 min thereafter for up to 30 min after turning off the vacuum drier and removing the tubes or ampoules from the machine.

Detection of trapped air in ampoules using a Tesla coil leak detector

Ampoules containing air were identified using a Tesla coil leak detector (Sanko Electronic Laboratory, Kanagawa, Japan), according to the manufacturer's protocols. Ideally, when the tip of the Tesla coil is brought near the ampoule, sparks are observed around the glass. If a substantial amount of air is trapped inside the ampoule, it cannot be ionized. However, if the ampoule contains only a small amount of residual air, ionization generates a spark inside the ampoule. Only Tesla-positive ampoules, considered highly vacuumed ampoules, were used in the experiments [12].

Measurement of sperm heads severance rate

After rehydration, 5–10 µl of sperm suspension was placed into a drop of PVP medium. About 15–20 photographs were taken for each drop, and 150–300 sperm were observed using microscope (Olympus IX-73, Tokyo, Japan). The severance rate was measured by dividing the number of sperm without heads by the total number of sperm.

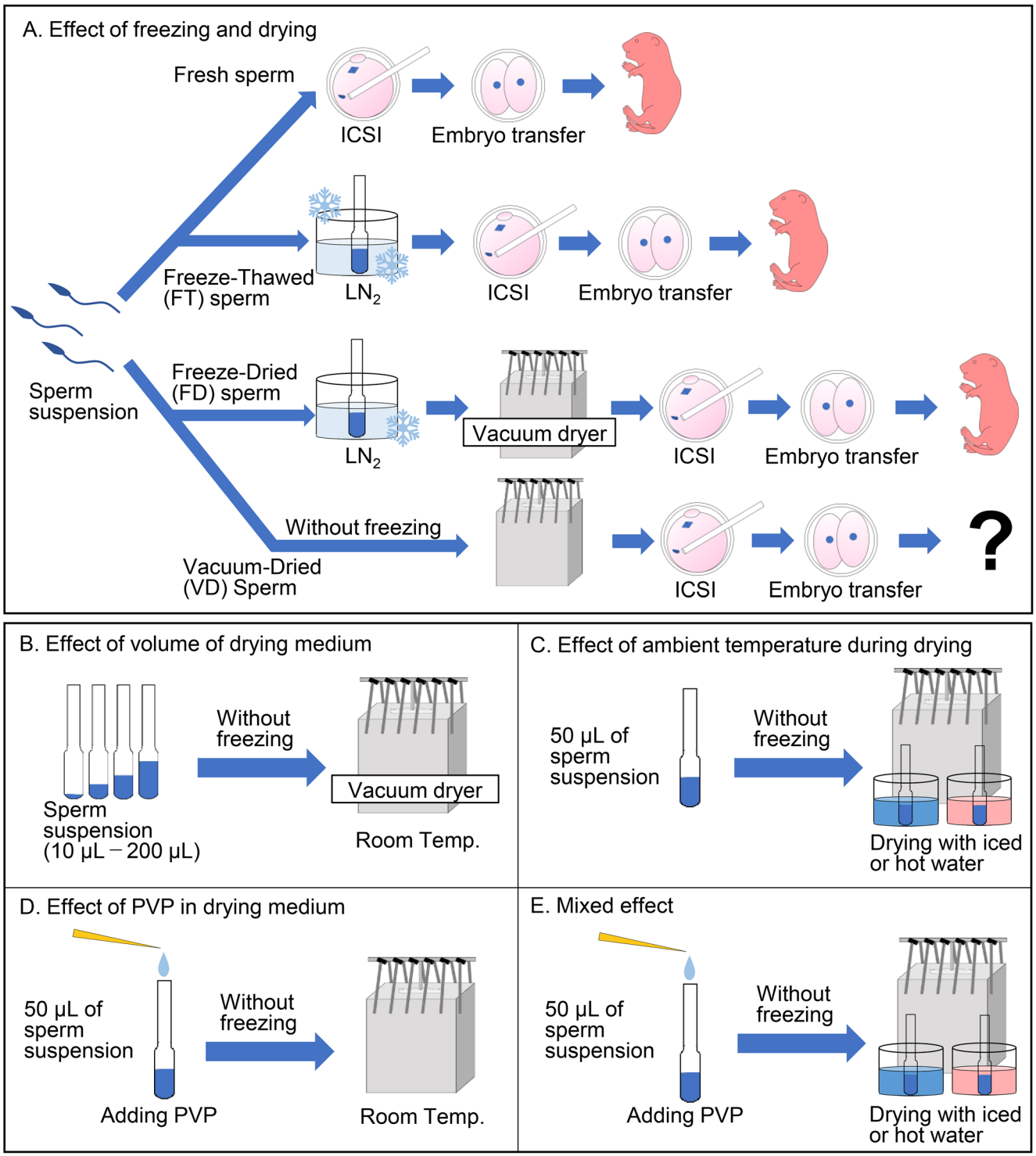

Fig. 1. Schematic diagram of the preparation of vacuum-dried (VD) sperm and the type of experiments conducted.

Analysis and scoring of comet slides

Spermatozoa DNA damage, potentially caused by single- and double-strand breaks [36], was measured using the CometAssay™ Kit (Trevigen, Gaithersburg, MD, USA) according to the manufacturer's protocol. Briefly, spermatozoa specimens were collected from the ampoules immediately after opening and rehydrated in water. The specimen was mounted on the slide, and 100–300 spermatozoa heads on each slide were analyzed *via* electrophoresis. To standardize the results obtained from different conditions under which the spermatozoa were produced, the length of each DNA comet tail was divided by the mean length of the one-sided results for each experiment. Fresh and FT spermatozoa could not be used as controls, since the requirement of a different preparation technique would prevent adequate comparison between specimens on the same slide.

Oocyte preparation

Female mice were superovulated *via* injection with 5 IU of equine chorionic gonadotropin, followed by 5 IU of human chorionic gonadotropin injected after 48 h. After 14–16 h, cumulus-oocyte complexes (COCs) were collected from the oviducts of female mice and transferred to a Falcon dish containing HEPES-CZB medium. To disperse the cumulus, COCs were transferred to a 50 µl droplet of HEPES-CZB medium containing 0.1% bovine testicular hyaluronidase for 3 min. Cumulus-free oocytes were washed twice and added to a 20 µl droplet of CZB for culture.

ICSI and embryo transfer

ICSI was performed as described previously [33]. Immediately prior to ICSI, the neck of the ampoule was broken off, and 50 µl of sterile distilled water was immediately added and mixed using a pipette. Then, 1–2 µl of the sperm suspension was transferred directly to the injection chamber. The sperm suspension was replaced every 30 min during ICSI. Several piezo pulses were applied to separate the sperm head from the tail and the head was then injected into the oocyte. The oocytes that survived ICSI were incubated in CZB medium at 37°C in 5% CO_2. Pronuclear formation was verified 6 h after ICSI. The embryos at the two-cell stage were transferred to a pseudo-pregnant mouse at day 0.5 that had been mated with a vasectomized male the night prior. Five to 12 embryos were transferred into each oviduct. On day 18.5, the offspring were delivered *via* cesarean section and allowed to mature. The remaining unused embryos were cultured for up to four days to evaluate their potential to develop into blastocysts.

Detection of abnormal chromosome segregation (ACS)

The day after ICSI, embryos in the two-cell stage were fixed and permeabilized with 4% PFA and 0.5% Triton X-100 for 15 min. The embryos were observed *via* fluorescence microscopy (Olympus IX-73, Tokyo, Japan) in PBS containing DAPI and 1% BSA. ACS was categorized into four groups: light, moderate, heavy, and lethal, as previously described [18]. Light ACS was considered when only one micronucleus was detected. Moderate ACS was noted when two small, one to two medium micronuclei, or a single large micronucleus were detected. Heavy ACS was designated when three small or medium or two or three large micronuclei were detected. Lethal ACS was considered when the embryos contained multiple micronuclei. In several instances, two conditions occurred simultaneously, making the more severe evaluation. For instance, if one medium and two small micronuclei were observed in an embryo, it was considered as “heavy”.

Statistical analysis

The results of the comet assay were analyzed using the Wilcoxon–Mann–Whitney nonparametric test. Blastocyst and birth rates were evaluated using the chi-square test or Tukey’s WSD test. The statistical significance of the differences between variables was set at $P<0.05$.

Results

DNA damage of VD sperm and its developmental potential after fertilization

To determine the quality of VD sperm, we compared the extent of DNA damage, fertilization rate, *in vitro* development to the blastocyst stage, and full-term development between fresh, FT, FD, and VD sperm (Fig. 1A). The morphology of VD sperm after rehydration did not differ from that of FD sperm (Figs. 2 D–F); however, more spermatozoa displayed separated heads and tails than fresh spermatozoa. The extent of DNA damage between FD and VD sperms was similar, as determined by the comet assay (Figs. 2G–I). When spermatozoa were injected into oocytes (Fig. 2J), most of the oocytes were fertilized normally (Table 1). The rate of blastocyst generation did not differ between FD (39%) and VD (32%) sperms; however, both were significantly lower than that of the fresh (58%) and FT (63%) sperms (Table 1, Fig. 2K, L, $P < 0.05$). After transferring the embryos at two-cell stage to the recipient females, healthy offspring were obtained from embryos derived from VD sperm (Fig. 2M); however, the success rate was lower than that of FD sperm (11% *vs*. 23%, $P < 0.05$).

Relationship between drying rate and DNA damage to VD sperm

The factors that regulate the drying rate during vacuum drying include the amount of medium, drying temperature, and viscosity of the medium. We investigated the drying rate and degree of DNA damage in VD sperm obtained using each method. When VD sperm was prepared with different volumes of the medium, varying from 10 to 200 µl (Fig. 2N), the drying rate decreased with increasing volume of the medium. In contrast, DNA damage tended to decrease with increasing volume of the medium, and the lowest DNA damage was observed when 200 µl of medium was added (Fig. 2O).

Next, we examined the drying rate of the medium under different temperatures, varying from 37°C to 0.5°C. The drying rate decreased concomitantly with temperature, with the lowest rate observed at 0.5°C (Fig. 2P). DNA damage evaluated using the comet assay showed the same pattern. As the medium temperature decreased, DNA damage also decreased, and the lowest DNA damage was observed when spermatozoa were vacuum-dried at 0.5°C (Fig. 2Q).

When PVP was added to the medium, the viscosity increased and the drying rate decreased with increasing PVP concentration (Fig. 2R). At 6% PVP or higher, complete drying of the medium was not achieved even after 30 min of vacuum drying. The DNA damage of VD sperm also exhibited a similar pattern; with increased concentrations of PVP or viscosity, DNA damage decreased, and the lowest DNA damage was observed when VD sperm was generated in 6% PVP (Fig. 2S).

Finally, we combined the aforementioned optimized parameters to produce VD sperm, in which 50 µL of medium with 6% PVP was vacuum-dried at 37°C to 0.5°C. The drying rate and DNA damage decreased with decreasing temperature (Figs. 2T, U).

Relationship between drying rate and in vitro developmental potential of embryos derived from VD sperm

Next, we investigated the *in vitro* development of embryos obtained after fertilization with VD sperms prepared using these methods. When the VD sperm produced in different volumes of medium were used, the blastocyst formation rate increased with an increase in the volume of the medium and reached a maximum at 100 µl (30%) (Table 2). However, since further samples could not be generated with such high volume of sperm suspension within a single ampoule, and considering that comet assay revealed similar extents of DNA damage with 100 and 50 µl, we selected 50 µl of sperm suspension for subsequent experiments.

VD sperm produced at different temperatures did not display any significant difference; however, the rate of blastocyst formation increased as temperature decreased (37°C: 21%, RT: 29%, and 0.5°C: 29%). Furthermore, when 6% PVP was added to the medium and the temperature was varied as above, the highest rate of blastocyst formation (44%) was obtained from the embryos derived from the

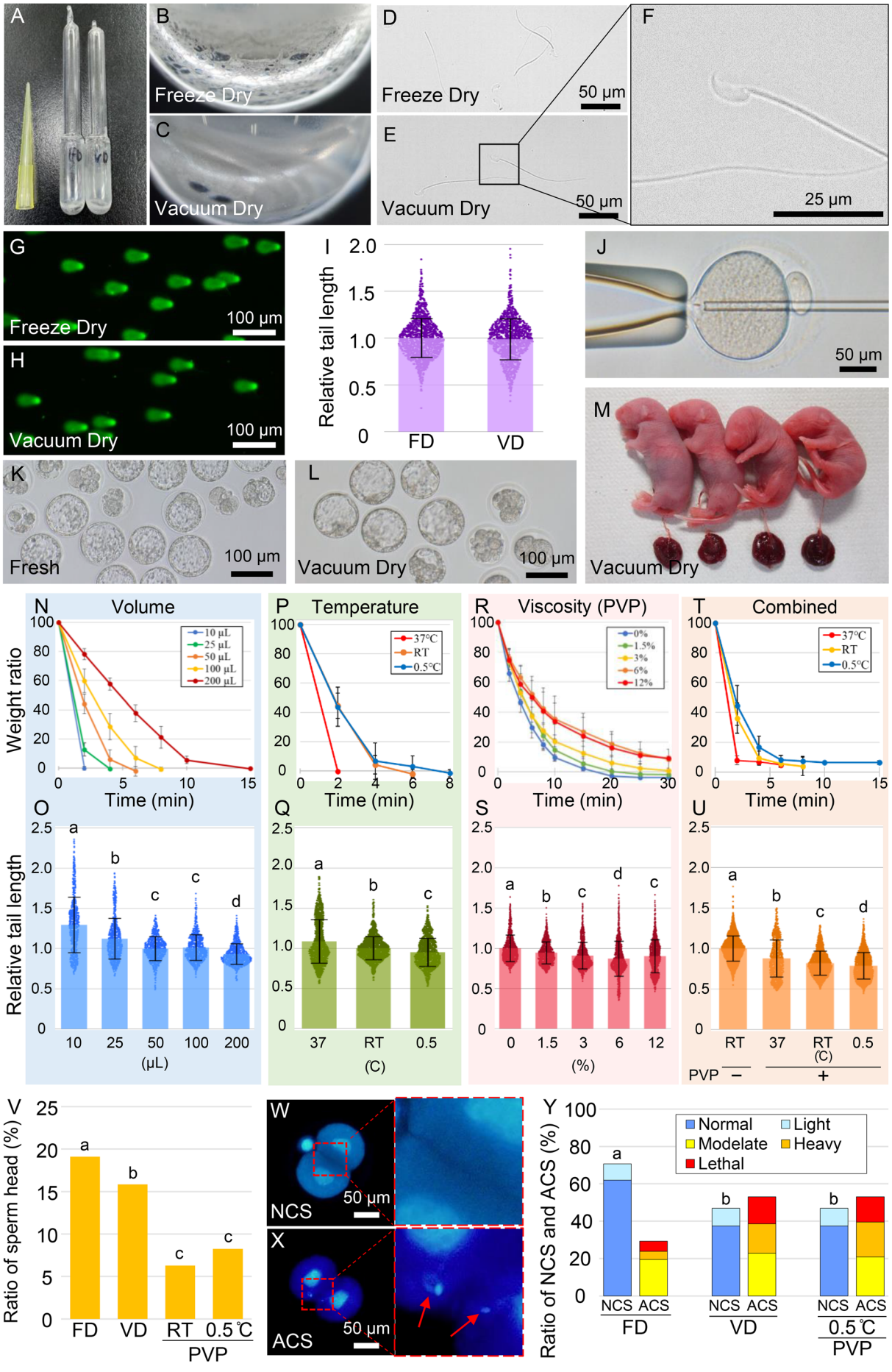
A
B
Freeze Dry
C
Vacuum Dry
D
Freeze Dry
50 μm
E
Vacuum Dry
50 μm
F
25 μm
G
Freeze Dry
100 μm
H
Vacuum Dry
100 μm
I
Relative tail length
2.0
1.5
1.0
0.5
0
FD
VD
J
50 μm
M
Vacuum Dry
K
Fresh
100 μm
L
Vacuum Dry
100 μm
N
Volume
Weight ratio
10 μL
25 μL
50 μL
100 μL
200 μL
Time (min)
P
Temperature
37℃
RT
0.5℃
Time (min)
R
Viscosity (PVP)
0%
1.5%
3%
6%
12%
Time (min)
T
Combined
37℃
RT
0.5℃
Time (min)
O
Relative tail length
a
b
c
c
d
10
25
50
100
200
(μL)
Q
a
b
c
37
RT
0.5
(℃)
S
a
b
c
d
c
0
1.5
3
6
12
(%)
U
a
b
c
d
RT
37
RT
0.5
(℃)
PVP
–
+
V
Ratio of sperm head (%)
a
b
c
c
FD
VD
RT
0.5℃
PVP
W
NCS
50 μm
X
ACS
50 μm
Y
Ratio of NCS and ACS (%)
Normal
Light
Modelate
Heavy
Lethal
a
b
b
NCS
ACS
NCS
ACS
NCS
ACS
FD
VD
0.5℃
PVP

Fig. 2. Examination of the sperm and DNA damage after vacuum-drying. (A) Ampoule carrying vacuum-dried (VD) sperm. (B, C) Freeze-dried (FD) and VD sperm inside the ampoule. (D, E) Fresh and VD sperm after rehydration. (F) Higher magnification of the image presented in E. (G, H) Comet DNA assays of FD and VD sperm. (I) Comparison of comet tail lengths of FD and VD sperm. The lengths of the comet tails were standardized against the average lengths of the FD sperm. Each plot shows the relative value of each sperm, and the bars indicate the average of the relative values. Error bar indicates standard deviation. (J) Image representing VD sperm injected into fresh ICR oocytes. (K, L) Blastocysts derived from embryos fertilized with fresh and VD sperm. (M) Offspring obtained from VD sperm. (N, P, R, T) Measurement of the sample drying rate. Horizontal axis indicates drying time and vertical axis shows weight ratio compared to before drying. (O, Q, S, U) Comet DNA assays of VD sperm with different amounts of medium, temperatures, and viscosities, and with a combined method. (V) The rate of sperm head severance from the tail. (W) Embryo at the two-cell stage derived from FD sperm showing normal chromosomal segregation (NCS) detected by DAPI staining. (X) Embryo at the two-cell stage derived from VD sperm showing abnormal chromosome segregation (ACS). (Y) The ratio of NCS and ACS in embryos derived from different types of sperm. Embryos showing light ACS were included in NCS. Different characters show significant difference ($P < 0.05$).

Table 1. Rate of blastocyst formation and full-term development of embryos after fertilization with fresh, freeze-thawed (FT), freeze-dried (FD), and vacuum-dried (VD) spermatozoa

Treatment	Observation	No. of oocytes surviving after ICSI	No. (%) of fertilized Zygotes	No. (%) of embryos developed to				No. of transferred embryos [no. of recipients]	No. (%) [min–max] of offspring
				2 cell	4–8 cell	Morula	Blastocyst		
Fresh	*In vitro* culture	116	111 (96) [a]	103 (93)	84 (76)	66 (59)	64 (58) [a]	—	—
	Full-term	142	130 (92)	117 (90)	—	—	—	82 [8]	39 (48) [a] [32–67]
Freeze Thaw	*In vitro* culture	118	112 (95) [a]	108 (96)	95 (85)	86 (77)	70 (63) [a]	—	—
	Full-term	168	149 (89)	125 (84)	—	—	—	82 [8]	38 (46) [a] [14–77]
Freeze Dry	*In vitro* culture	160	135 (84) [b]	98 (73)	89 (66)	81 (60)	52 (39) [b]	—	—
	Full-term	187	171 (91)	132 (77)	—	—	—	125 [10]	29 (23) [b] [0–43]
Vacuum Dry	*In vitro* culture	156	130 (83) [b]	90 (69)	77 (59)	66 (51)	41 (32) [b]	—	—
	Full-term	252	224 (89)	87 (39)	—	—	—	84 [8]	9 (11) [c] [0–20]

a *vs*. b, c; b *vs*. c: $P < 0.05$.

Table 2. Effect of different drying rates on fertilization rate and the *in vitro* development of vacuum-dried (VD) spermatozoa

Volume	Temperature	PVP (6%)	No. of oocytes surviving after ICSI	No. (%) of fertilized zygotes	No. (%) of embryos developed to			
					2 cell	4–8 cell	Morula	Blastocyst
10	RT	No	65	61 (94)	28 (46)	15 (25)	7 (11)	4 (7) [a]
25			79	76 (96)	42 (55)	25 (33)	11 (14)	10 (13)
50			136	133 (98)	99 (74)	62 (47)	48 (36)	24 (18)
100			83	79 (95)	68 (86)	51 (65)	40 (51)	24 (30) [b]
200			84	84 (100)	68 (81)	58 (69)	38 (45)	24 (29)
50	37ºC	No	114	103 (90)	50 (49)	28 (27)	24 (23)	22 (21)
	RT		246	207 (84)	135 (65)	109 (53)	82 (40)	59 (29)
	0.5ºC		271	227 (84)	141 (62)	114 (50)	92 (41)	65 (29)
50	37ºC	Yes	65	62 (95) [a]	49 (79)	34 (55)	24 (39)	17 (27)
	RT		209	169 (81) [b]	129 (76)	110 (65)	100 (59)	56 (33)
	0.5ºC		143	121 (85) [b]	85 (70)	76 (63)	74 (61)	53 (44)

a *vs*. b: $P < 0.05$. RT, Room temperature.

VD sperm produced with PVP at 0.5°C.

Relationship between drying rate and full-term development of embryos derived from VD sperm

To determine whether the increase in the rate of development of blastocysts was associated with an enhanced rate of offspring production, embryos derived from VD sperm produced using various methods were transferred to recipient females at the two-cell stage. As presented in Table 3, offspring were obtained in all experimental groups. Although the rate of offspring production was significantly higher at 0.5°C than at RT (20% *vs*. 7%, $P < 0.05$), no obvious difference was noted between the offspring production rates of embryos derived from VD sperm obtained at different temperatures with PVP in the medium.

Rate of sperm head severance from the tail

To determine the reason for the decrease in DNA damage without a reduction in the offspring production rate, we measured other types of sperm damage, including the rate of sperm head severance from the tail after rehydration (Fig. 2V). The results indicated that when VD sperm were prepared by adding PVP to the medium, the percentage of severed sperm heads was significantly reduced compared with VD sperm obtained without PVP. This clearly suggests that PVP in the medium can prevent physical damage to the spermatozoa during the drying process.

Table 3. Effect of ambient temperature and addition of 6% PVP during the production of vacuum-dried (VD) sperm on full-term development

Temperature	PVP (6%)	No. of oocytes surviving after ICSI	No. (%) of fertilized zygotes	2 cell (%)	No. of transferred embryos [no. of recipients]	No. (%) [min–max] of offspring
37ºC	No	134	130 (97)	104 (80)	89 [5]	11 (12) [0–20]
RT		281	257 (91)	169 (66)	161 [15]	12 (7) [a] [0–50]
0.5ºC		182	170 (93)	108 (64)	92 [6]	18 (20) [b] [5–44]
37ºC	Yes	275	249 (91) [b]	88 (35)	66 [4]	4 (6) [0–38]
RT		161	155 (96) [a]	115 (74)	95 [7]	7 (7) [0–15]
0.5ºC		140	119 (85) [b]	83 (70)	72 [6]	7 (10) [0–25]

a *vs.* b: $P < 0.05$. RT, Room temperature.

Adverse effects of vacuum-drying treatment on chromosome segregation

To identify the cause of failure in offspring formation and to improve the offspring production rate, which were persistent issues even when the blastocyst formation rate was improved and physical damage was reduced; we examined the incidence of abnormal chromosome segregation (ACS) at the two-cell stage. The ACS incidence was considered to be a more profound DNA damage that could not be detected *via* the comet assay. In this study, we focused on moderate level of ACS, because embryos with a low level of ACS still have the potential to reach full-term development [37]. As presented in Figs. 2W and 2Y, when FD sperm were used, 71% of the embryos exhibited normal chromosome segregation (NCS); however, the rate was reduced to 47% when VD sperm were used (Figs. 2X, Y). Furthermore, the NCS rate was similar when the VD sperm was prepared by adding PVP to the medium and drying was performed at 0.5°C. This suggests that vacuum-drying treatment causes more severe DNA damage to the sperm nuclei compared with that of FD sperm even when slow drying is performed.

Discussion

Until now, whether pre-incubation, freezing, drying, preservation, or rehydration is the specific step responsible for the decline in sperm quality after freeze-drying has not been determined. In this study, we succeeded in producing offspring from mouse VD sperm. However, the success rate was lower than that for FD sperm. Indicating that the freezing process may produce dried sperm with less damage.

When oocytes and embryos are freeze-thawed without cryoprotectants, they do not survive due to ice crystal formation in the cytoplasm, in addition to severe osmotic stress [38]. We hypothesized that if the freezing process is omitted during the production of dry sperm, the offspring rate would increase. Contrary to our prediction, however, a lower birth rate resulting from VD sperm compared with FD sperm was noted, which was probably caused by the drying process rather than the freezing process. Damage to spermatozoa, such as the immobilization of spermatozoa and separation between the head and tail, was observed when the sperm was dried with or without the freezing process. However, it has been reported that ice crystals were not observed in horse spermatozoa frozen at various freezing rates [39]. Although we did not determine whether ice crystal formation occurred in mouse sperm after freezing, we suspect that because the DNA of spermatozoa was tightly packaged with protamine [40], the freezing process may not have damaged sperm DNA.

In the case of vacuum-drying, boiling occurs at RT because the vacuum conditions reduce the boiling point to approximately −30°C [41]. This may also cause significant physical damage to the intracellular organelles and DNA in spermatozoa. In contrast to freeze-drying, in vacuum-drying, if the drying rate of the medium under vacuum is high, evaporation occurs rapidly and causes a swift change in the osmotic pressure around the spermatozoa. A radical change in osmotic pressure can disrupt the lipid membrane structure and membrane proteins of spermatozoa, thereby causing leakage of membrane ions, which leads to morphological abnormalities [42, 43]. We previously demonstrated that when epididymides were placed directly into salt powder and rapidly dehydrated, spermatozoa could be stored for only 1 day [44], whereas mouse spermatozoa could be stored for 2 months when slowly dehydrated in a hyperosmotic solution [45]. These results indicate that when spermatozoa are rapidly vacuum-dried, the effect of boiling on intercellular organelles and rapid osmotic pressure changes may severely damage the sperm DNA, resulting in a lower success rate of offspring formation after fertilization. Therefore, in the present study, when VD sperm were produced *via* a slower drying process, DNA damage in the spermatozoa was reduced (Figs. 2O, Q, S, U). Interestingly, in the case of spermatozoa produced *via* evaporative drying, rapid drying resulted in a higher blastocyst formation rate than slow drying [46]. Although the sperm quality is clearly affected by the drying speed, it may be optimized for each method.

In the present study, we succeeded in significantly reducing DNA damage in VD sperm and improving the *in vitro* formation of blastocysts after fertilization by adjusting the drying speed. However, we were unable to improve the success rate of offspring production. Fertilized embryos derived from FD sperm with chromosomal abnormalities can develop into blastocysts relatively efficiently [20, 21, 47], and embryos fertilized with VD monkey sperm can develop into blastocysts with the same results as those using fresh sperm [30]. This indicates that the degree of DNA damage in VD sperm is not associated with the rate of development of embryos into blastocysts. We employed a comet assay for a comprehensive analysis of sperm DNA integrity as an indicator of global DNA damage, although it cannot measure damage such as chromosome breaks. Therefore, we also conducted an ACS assay that detects severe DNA damage in spermatozoa, which has been correlated with the offspring production rate [48]. In our study, ACS was more prominently observed in embryos with VD sperm than in those with FD sperm. Thus, even though reducing the drying rate decreased the overall DNA damage in VD sperm, no reduction was observed in severe DNA damage. Thus, to improve the success rate of offspring derived from VD or FD sperm, identification of the factor causing the severe DNA damage that occurs during the drying process is necessary.

In conclusion, we demonstrated for the first time that mouse embryos fertilized with VD sperm developed into offspring, although the success rate was inferior to that of FD sperm. We suspect that even though the freezing process is not essential for dry preservation of sperm, and can cause damage to the sperm, also reduce the damage

caused due to drying. Reducing the severe DNA damage caused by the drying process is crucial for improving the success rate of the offspring produced from FD spermatozoa.

Conflict of interests: The authors declare no conflicts of interest.

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