Original Article

Effect of Rosmarinic Acid on Dog Semen Quality after Freeze-drying by Applying Freeze-drying Machines for Vaccine Production

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Abstract

Freeze-drying (FD) is a sperm preservation method that eliminates the need for liquid nitrogen. However, the FD process can expose sperm to free radicals and temperature variations. This study aimed to determine the impact of rosmarinic acid (RA) as an antioxidant, initial sperm motility, and storage temperature on the DNA integrity of freeze-dried dog sperm. Forty-four ejaculates from 15 dogs of various breeds were divided into control (FD extender) and RA (FD extender with RA) groups, further categorized by motility (above or below 70%). After FD, samples were stored for three months at room temperature and 4 °C. Sperm quality analysis, including rehydrated freeze-dried samples, was conducted at intervals (immediately post FD, 7, 14, 30, and 90 days). Results indicated reduced viability and motility post-FD, with predominant abnormal sperm tail morphology. Initial motility had no impact on sperm quality. Storage temperature and the antioxidant RA improved DNA integrity but did not affect sperm morphology. DNA fragmentation occurred after 7 days at room temperature, slower at 4 °C, and further slowed with RA. The percentage DNA integrity after 3 months ranked from best to worst: RA group at 4 °C (1.41 ± 0.51), RA group at room temperature (4.59 ± 0.51), control group at 4 °C (4.76 ± 0.56), and control group at room temperature (38.80 ± 1.01). While sperm motility is crucial for frozen semen, freeze-dried semen presents an alternative for dogs with low fertility, given its easy storage, reduced shipping costs, and applicability in locations without liquid nitrogen. However, the FD extender and storage temperature choice significantly impacts DNA integrity in freeze-dried dog sperm.

Keywords: dog semen, freeze-drying, Rosmarinic Acid

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Introduction

At present, dog breeding is taking place worldwide, and assisted reproductive technologies are constantly being used to improve breeding in the future. Semen cryopreservation plays an important role in long-term sperm storage. There are many methods of cryopreservation, including chilled, frozen, and freeze-drying (FD). Chilled semen is simple and inexpensive, stored at 4 °C, but should be used as soon as possible after collection, ideally within the first 48 hours (Pena *et al.,* 2006). Frozen semen required specific extenders to prevent ice crystal formation due to a rapid temperature drop and maintained in liquid nitrogen at -196 °C. Freeze drying is the removal of water by sublimation of frozen water to a vapor state in a vacuum. Freeze-dried semen does not require liquid nitrogen for storage, which reduces the cost and increases convenience. In addition, it also facilitates semen transportation, and the freeze-dried semen can temporarily be stored at room temperature (25 °C). Therefore, it might be an alternative method for genetic preservation.

FD procedure or lyophilization is the one method used for semen cryopreservation which was applied to many species, for example, in rabbits (Domingo *et al.,* 2018), horses (Olaciregui *et al.,* 2016) mice (Kaneko and Serikawa, 2012) boar (Garcia-Campos *et al.,* 2014) and bull (Hara *et al.,* 2014). The fertilization of freeze-dried sperm and oocyte is performed using the intracytoplasmic sperm injection (ICSI) technique. The production of viable offspring from ICSI has been reported in rabbits (Kusakabe *et al.,* 2001), mice (Liu *et al.,* 2004), rats (Hirabayashi *et al.,* 2005), and horses (Choi *et al.,* 2011). The good point of this method is that it can be stored and transported at 4 °C without using liquid nitrogen.

During the process of FD, sperm are exposed to mechanical stress or oxidative stress, which can damage the DNA. Reactive oxygen species (ROS) can also damage DNA, which increases by the storage time (Domingo *et al.,* 2018). Rosmarinic acid (RA) is a natural antioxidant. Since RA extracted from plants, therefore, does not affect the genetic material, when compared to the negative controls, the mutagenicity assay results for Swiss mice treated with varying doses of RA did not reveal an increase in the frequency of micronuclei (Furtado *et al.,* 2008). On the other hand, synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are associated with chronic toxicity related to BHT to the possibility of promoting tumors when consumed in excess (Kaczmarski *et al.,* 1999; Witschi, 1981) and causing other toxicity in the body of the animal (Scott, 1988). The previous study showed that RA had antioxidant and resist oxidative stress properties. Adding RA to the semen extender in the frozen porcine semen (Luño *et al.,* 2014; Zhang *et al.,* 2012) and freeze-dried ovine semen (Olaciregui, Luno, *et al.,* 2017) could help to increase the quality of sperm cryopreservation. Sperm motility was used as the fertility criteria for the evaluation of frozen semen techniques in dogs (Ivanova-Kicheva *et al.,* 1997). A dog with sperm motility <70 % is unsuitable for frozen semen cryopreservation (Thomassen *et al.,* 2006). The acceptable range and ideal motility of frozen semen for artificial insemination were 30-50 % and 60 %, respectively (Concannon and Battista, 1989). On the other hand, motility may not be essential for freeze-dried semen. However, a correlation between sperm motility and DNA fragmentation in the canine semen samples is expected. To identify the effect of motility on freeze-dried semen, dogs with sperm motility >70 % and <70 % were used for the study as the normal and low fertility dog semen. Therefore, this study aimed to determine the effect of RA as an antioxidant on DNA integrity, the effect of motility on freeze-dried semen, and the effect of stored temperature on the DNA integrity of freeze-dried dog sperm.

Materials and Methods

Reagent and media: Tris egg yolk glucose extender consisted of Tris 2.4 g, citric acid 1.3 g, glucose 1 g, egg yolk 20 ml, Penicillin 1,000,000 I.U. Streptomycin 100 mg and added distilled water to 100 ml (Zorinkimi *et al.,* 2017). The freeze-dried media in this study composed of 1) Control freeze-drying media consisting of 10 mM Tris-HCl buffer, 50 mM NaCl, and 50 mM EDTA 2) RA freeze-drying media consisting of 10 mM Tris-HCl buffer, 50 mM NaCl, 50 mM EDTA and RA (CAS:20283-92-5; Sigma Aldrich, USA) 105 µM (Luño *et al.,* 2014). The final pH of the solutions was adjusted between 8.2 to 8.5.

Animals: The sample was collected from 15 small (<10 kg), large (27-45 kg), and giant breed dogs (>45 kg) composed of Cavalier King Charles Spaniel (n = 1), Scottish Terrier (n = 1), Caucasian Shepherd (n = 2), Central Asian Shepherd (n = 3), English Mastiff (n = 2), Kangal (n = 1), Presa Canario (n = 1), Saint Bernard (n = 1), Spanish Mastiff (n = 1), and Standard Poodle (n = 2) dogs aged between 2.5 to 5 years of age. Fertile dogs are defined by their history of producing offspring. Giant breed dogs over 6-8 years old or dogs with health problems were not included in the experiment such as the presence of red-colored semen caused by inflammation of the prostate gland, absence of sperm in the sample, and dogs having hip problems preventing them from standing for semen collection. All the dogs were raised under the same management conditions in a dog kennel in Samut Sakhon province, Thailand. Each dog was maintained in an individual cage at 22 to 25 °C. All animals were fed a commercial pellet diet, and water was given ad libitum.

All procedures described here were submitted and reviewed by the Kasetsart Institutional Animal Care and Use Committee and found to be in accordance with the guidelines of animal care and used under the Ethical Review Board of the Office of National Research Council of Thailand (NRCT) for the conduction of the scientific research. (ID# ACKV64-VET-064)

Semen collection and experimental groups: Dog semen was collected by manual stimulation technique by using an artificial vagina with a female teaser at the dog kennel. The sperm-rich fraction of each dog was diluted with a pre-warmed 37 °C tris egg yolk glucose extender (1:2) and stored in a foam box that had put the

ice pack while traveling back to the semen laboratory faculty of Veterinary Medicine, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom Province (1 hour and a half to 2 hours). After that, semen samples were made for sperm motility evaluation by using Computer Assisted Sperm Analysis (CASA) (Hamilton Thorne Biosciences IVOS, Version 12 TOX IVOS, Beverly, MA, USA) that adjusted the samples to the same concentration before analysis. The semen samples were justified for sperm with motility >70 % or <70 %. Then, the samples in each motility were pooled together to eliminate individual differences and had enough volume for the FD process and evaluation. A pooled semen sample was processed for complete semen evaluation, and sperm DNA fragmentation was analyzed manually. The semen samples were divided into 2 groups according to freeze-dried media in the control group and RA group, and each group was divided into 2 subgroups according to their motility (>70 % and <70 %). The entire procedure was replicated 4 times, equivalent to 44 ejaculations from 15 dogs. In each replication, the dogs utilized for sample collection comprised both the same dogs and new ones, contingent upon their availability on the day of sampling.

Group 1 Control group
Subgroup 1.1 Control group with sperm motility >70 %
Subgroup 1.2 Control group with sperm motility <70 %

Group 2 RA group
Subgroup 2.1 RA group with sperm motility >70 %
Subgroup 2.2 RA group with sperm motility < 70 %

Macroscopic examination of semen: A macroscopic examination was performed to assess the color and the volume. Semen must be white and not contaminated with urine or blood.

Microscopic examination of semen: A progressive motility exam was performed by dropping the semen on a glass slide and covering it with a coverslip, then observing the sample under a light microscope (x100, x400) and recording in percentage.

Concentration is the number of sperm per ejaculation. A concentration examination was performed by gently combining the fresh semen 10 µl with 10 % formaldehyde 990 µl and then dropping 20 µl of semen mixture into a hemocytometer and observing the sample under a light microscope (x400). Counted the number of sperm heads in the middle table of hematocytometer 1 head of sperm equal to 1 million sperm per milliliter. Total sperm count per ejaculation was calculated by multiplying sperm count by semen volume (Freshman, 2002).

Sperm vitality is the percentage of live sperm in the semen sample. The two common methods of sperm vitality assessment are the Hypo-osmotic swelling test (HOS test) and eosin-nigrosin staining. HOS-test determines the sperm osmoregulatory function under hypo-osmotic conditions. HOS test was performed by combining the semen with citrate buffer in a ratio of 25 to 250 and incubating at 37 °C in a water bath for 30 min. After incubation, drop the semen mixture on a glass slide and cover it with a coverslip. Observed the sample under a light microscope (x400) (Kumi-Diaka, 1993). Sperm with a curling (swelling) tail is alive; sperm with a straight tail is dead. The other method is eosin-nigrosin staining, performed by gently mixing 2 drops of eosin-nigrosin solution with 1 drop of the semen sample and smearing on a glass slide, air dried, and observed under a light microscope (x1000) (Freshman, 2002). Eosin stains only the dead sperm and turns into a dark pink head sperm, whereas live sperm is white. Eosin-nigrosin staining provides a rapid assessment of sperm viability based on membrane integrity, whereas the HOS test evaluates both membrane integrity and functional competence related to osmoregulation. Using multiple assays allows for cross-validation of results, enhancing the reliability of sperm quality assessment.

Sperm morphology analysis was examined using Diff-Quik staining. Dropped the semen sample on a glass slide to create a smear, let the slide air-dried, and then dipped the slide in fixative solution (methanol), eosin solution, and methylene blue, respectively, for 5 minutes each and then gently washed the excess stain with water. Allow the slide to air dry and observe the sample under a light microscope (x1000) (Freshman, 2002). The morphology of the head, midpiece, and tail were examined. Sperm morphology was characterized as a normal, primary abnormality, or secondary abnormality.

Sperm DNA fragmentation was analyzed by acridine orange staining. Dropped the semen sample on a glass slide and make a smear, let the slide air dry and then dipped the slide in cranoy's solution for overnight, air dry and then dipped the slide in acridine orange for 5 minutes and gently wash the excess stain with a little water (Tejada *et al.,* 1984). Allow the slide to air dry and observe the sample under *a* fluorescence microscope (x400) in a dark room and report as a percentage of DNA fragments. Acridine orange staining is a relatively simple and inexpensive procedure compared to other DNA fragmentation assays. The fluorescence emitted by acridine orange varies depending on the structure of the DNA it binds to. Specifically, acridine orange emits green fluorescence when bound to double-stranded DNA (dsDNA) and red fluorescence when bound to single-stranded DNA (ssDNA) or denatured DNA. Research indicates that the acridine orange test is effective in evaluating DNA fragmentation in sperm cells, particularly in canine semen (Qamar *et al.,* 2020)

Freeze-drying process: In this study, the FD machine used in the production of animal vaccines was applied, and the FD procedure was applied by Wakayama and Yanagimachi (1998). The samples were divided into 4 subgroups following the experimental protocol. Each sample was placed into a falcon tube for centrifuge at 700 g for 10 mins. And then discarded the supernatant. After that, each falcon tube was resuspended with 1 ml freeze-dried extender for each group, control freeze-dried media, and RA freeze-dried media. Gently combined and incubated at 37 °C in a water bath for 10 mins, a 1 ml semen mixture from each falcon tube was placed into a glass vial. Afterwards, glass vials were placed into a -180 °C freezer for 30 mins. When the

samples are frozen, place the 2 legs of the vial rubber stopper at the vial opening. The samples were transferred to the FD machine (FreeZone Plus Freeze-Dryer System #7960034 and Stoppering Tray Dryer #7948030; LABCONCO, MO, USA). The temperature was set to -40 °C, and the machine worked automatically. When finished, the rubber stopper closed the vial. Removed the sample from the machine and then sealed the vial with an aluminum cap. Store the freeze-dried semen at 4 °C at room temperature by group for 3 months, waiting for rehydration and evaluation.

Rehydration: The time for assessing the sperm quality was immediately after freeze-drying, 7, 14, 30, and 90 days, respectively. The rehydration of freeze-dried spermatozoa was performed by adding 0.5 ml of distilled water to the sample, facilitating the evaluation, especially for samples with low concentrations. The samples were analyzed for sperm motility, vitality, morphology, and DNA fragmentation.

Statistical analysis: The data results of DNA fragmentation, abnormal sperm morphology, sperm vitality, and sperm motility were expressed in percentages of mean ± SD and analyzed by *t-test*. The data were used to analyze the following data. Compare the various parameters between the groups (control group and RA group) and subgroups (>70 % of sperm motility and <70 % of sperm motility) storage at room temperature and 4 °C assessed before and immediately after the FD process. After storing the samples for 7, 14, 30, and 90 days, data were analyzed using IBM SPSS Statistics 23 for Windows. The level of significance was set at $P < 0.05$.

Result

Dog semen quality before the freeze-drying process: Pooled fresh semen evaluation before the FD process is shown in Table 1. The parameters of pooled dog semen control and RA group with sperm motility >70 % were in the normal range for canine species (Oettlé, 1993; Johnston *et al.*, 2001). The averaged motility, concentration per milliliter, HOS-test, eosin-nigrosin, and sperm abnormality were 86.75 %, 72 x 10^6/ml, 94.50 %, 95.50 %, and 8 %, respectively. The sperm with motility <70 % had normal vitality and sperm morphology but low sperm motility (37.50 %) and concentration (36.75 x 10^6/ml). No DNA fragmentation was observed in dog semen before the FD process.

Freeze-dried sperm motility and viability: After the FD process, dog sperms lost their viability and motility both in the control and RA group, including immediately after the FD process and after being stored for 3 months both at 4 °C and room temperature. Therefore, there was no statistically significant difference between groups.

Freeze-dried sperm morphology: Abnormal sperm morphology significantly increased after the lyophilization process. More than 90 % of freeze-dried sperm morphology abnormalities were tail defects that were identified immediately after the FD process. In subgroups with motility >70 %, head, midpiece, and tail defects before freeze-dry ranged from 0, 0-1, and 2-10 %, respectively, in 4 pooled semen samples. Immediately after freeze-drying, the percentage of midpiece defect was zero, the head defect was still low, and the average head defect was 1.75 % in the control group and 1.00 % in the RA group. Contrary to tail defect, the percentage increased from 7.50 % to 43.50 % in the control group and 39.25 % in the RA group (Table 2). The same result was also observed in subgroups with sperm motility <70 %. The tail defect increased from 10 % before freeze-drying to 43.25 % in the control group and 41.75 % in the RA group (Table 3). Between groups, immediately after freeze-dry, sperm morphology abnormalities increased from 9.63 to 45.25 % in the control group and 9.63 to 41.87 % in the RA group. The average tail defect was 8.75 % before freeze-dry and increased to 43.38 % in the control group and 40.50 % in the RA group immediately after freeze-dry (Table 4). After keeping the sample for 7, 14, 30, and 90 days, there were no differences in abnormal sperm morphology (%) between the control and RA groups and within the subgroup both at room temperature and 4 °C (Table 5). In control and RA groups, the comparison of abnormal sperm morphology between 2 motility subgroups at 4 °C and room temperature did not show any change in abnormal sperm morphology every time of observation at 7, 14, 30, and 90 days of sample storage (Table 6).

Sperm DNA fragmentation: The sperm DNA fragmentation was analyzed in freeze-dried semen samples by using acridine orange staining. The result revealed no DNA fragmentation before and immediately after the FD process both in the control group and RA group (Fig. 1). During sample storage, after the FD process to 90 days, DNA fragmentation in the control group at room temperature increased percentage of DNA damage than the control group stored at 4 °C and RA group in both temperatures ($P < 0.05$). In the control group at room temperature, DNA fragmentation continued to increase from 0.43, 0.34, 11.38, and 38.80 at 7, 14, 30, and 90 days after storage, respectively. After 3 months of storage, DNA fragmentation in the RA group stored at 4 °C (1.41 ± 0.51) was significantly less than that in the control group at 4 °C (4.76 ± 0.56), control group at room temperature (38.80 ± 1.01) and RA group at room temperature (4.59 ± 0.51) ($P < 0.05$). DNA fragmentations in the control group at 4 °C were significantly greater than in the RA group at 4 °C but equal to the RA group at room temperature. The difference in DNA fragmentations among the control and RA groups at 4 °C was observed after keeping the samples for 3 months. Additionally, in the RA group, the samples stored at room temperature (4.59 ± 0.51) had a DNA fragmentation significantly increased more than the sample at 4 °C (1.41 ± 0.51). According to the result, the minimum percentage of DNA fragmentation was observed in the RA group at 4 °C.

Table 1 The pooled dog semen quality data before the freeze-drying process in the control and RA groups within 2 subgroups with sperm motility >70 % and subgroups with sperm motility <70 %

Pooled Samples	Number of dogs	Motility (%)	Conc. (M/ml)	Vitality (%)		Abnormal morphology (%)	DNA fragmentation (%)
				HOS-test	E/N		
Control and RA group: Subgroup with sperm motility >70 %							
1	6	92	67	90	95	2	0
2	6	90	78	97	96	8	0
3	5	79	73	95	96	15	0
4	6	86	70	96	95	7	0
mean	5.75	86.75	72.00	94.50	95.50	8.00	0.00
Control and RA group: Subgroup with sperm motility <70 %							
1	5	41	40	88	85	10	0
2	5	46	35	94	94	11	0
3	6	34	37	90	92	19	0
4	5	29	35	90	92	5	0
mean	5.25	37.50	36.75	90.50	90.75	11.25	0.00

Abbreviation: Conc. = Concentration E/N = Eosin-nigrosin staining

Table 2 The percentage of head, midpiece, and tail defects in the control and RA groups within 2 subgroups with sperm motility >70 %

Pooled Samples	Abnormal sperm morphology (%)															
	Before freeze-dry								Immediately after freeze-dry							
	Control group				RA group				Control group				RA group			
	H	M	T	%	H	M	T	%	H	M	T	%	H	M	T	%
1	0	0	2	2	0	0	2	2	2	0	42	44	1	0	40	41
2	0	0	10	10	0	0	10	10	1	0	47	48	0	0	38	38
3	0	0	8	8	0	0	8	8	3	0	45	48	2	0	40	42
4	0	1	10	11	0	1	10	11	1	0	40	41	1	0	39	40
mean	0.00	0.25	7.50	7.75	0.00	0.25	7.50	7.75	1.75	0.00	43.50	45.25	1.00	0.00	39.25	40.25

Abbreviation: H = Head M = Midpiece T = Tail; % = percentage of abnormal sperm morphology

Table 3 The percentage of head, midpiece, and tail defects in the control and RA groups within 2 subgroups with sperm motility <70 %

Pooled Samples	Abnormal sperm morphology (%)															
	Before freeze-dry								Immediately after freeze-dry							
	Control group				RA group				Control group				RA group			
	H	M	T	%	H	M	T	%	H	M	T	%	H	M	T	%
1	3	0	12	15	3	0	12	15	5	0	39	44	2	0	40	42
2	0	1	18	19	0	1	18	19	1	0	45	46	2	0	42	44
3	1	0	6	7	1	0	6	7	2	0	41	43	2	0	41	43
4	1	0	4	5	1	0	4	5	0	0	48	48	1	0	44	45
mean	1.25	0.25	10.00	11.50	1.25	0.25	10.00	11.50	2.00	0.00	43.25	45.25	1.75	0.00	41.75	43.50

Abbreviation: H = Head M = Midpiece T = Tail; % = percentage of abnormal sperm morphology

Table 4 The percentage of head, midpiece, and tail defects in the control and RA group before freeze-dry and immediately after freeze-dry

Sperm morphology	Abnormal sperm morphology (%)			
	Before freeze-dry		Immediately after freeze-dry	
	Control group	RA group	Control group	RA group
Head	0.63 ± 1.06	0.63 ± 1.06	1.86 ± 1.55	1.38 ± 0.74
Midpiece	0.25 ± 0.46	0.25 ± 0.46	0.00 ± 0.00	0.00 ± 0.00
Tail	8.75 ± 5.00	8.75 ± 5.00	43.38 ± 3.34	40.50 ± 1.85
Total	9.63 ± 5.45	9.63 ± 5.45	45.25 ± 2.66	41.87 ± 2.23

Table 5 The percentage of abnormal sperm morphology in the control and RA groups before freeze-dry, immediately after freeze-dry, and 7, 14, 30, and 90 days after storage

		Abnormal freeze-dry sperm morphology (%)			
		Control group (mean ± SD)		RA group (mean ± SD)	
		4 °C	Room temp	4 °C	Room temp
Before freeze-dry		9.63 ± 5.45	9.63 ± 5.45	9.63 ± 5.45	9.63 ± 5.45
Immediately after freeze-dry		45.25 ± 2.66	45.25 ± 2.66	41.87 ± 2.23	41.87 ± 2.23
Storage time (day)	7	44.12 ± 7.01	45.13 ± 6.78	40.65 ± 6.10	43.29 ± 6.57
	14	45.71 ± 8.11	44.06 ± 5.69	42.76 ± 4.60	41.76 ± 5.97
	30	45.88 ± 7.71	44.31 ± 6.13	42.88 ± 5.09	43.88 ± 6.43
	90	44.00 ± 6.46	45.27 ± 6.33	40.82 ± 6.31	43.06 ± 6.56

Table 6 The percentage of abnormal sperm morphology in the control and RA groups within 2 subgroups with sperm motility >70 % and subgroups with sperm motility <70 % at 7, 14, 30, and 90 days after storage

Storage time (day)	Abnormal freeze-dry sperm morphology (%)							
	Control group (mean ± SD)				RA group (mean ± SD)			
	4 °C		Room temp		4 °C		Room temp	
	Motility >70 %	Motility <70 %	Motility >70 %	Motility <70 %	Motility >70 %	Motility <70 %	Motility >70 %	Motility <70 %
7	46.88±7.08	41.67±6.33	48.00±7.96	42.63±4.72	43.25±5.12	38.33±6.23	46.75±5.50	40.22±6.12
14	48.75±8.70	43.00±6.91	47.14±5.52	41.67±4.80	44.88±4.09	40.89±4.40	43.63±6.12	40.11±5.67
30	49.00±8.26	43.75±6.52	48.14±6.79	41.33±3.61	46.25±3.24	39.89±4.60	46.50±6.33	41.56±5.90
90	46.00±6.63	42.22±6.12	47.86±7.49	43.00±4.41	43.88±4.12	38.11±6.86	46.38±5.85	40.11±5.95

Table 7 The percentage of DNA fragmentation of sperm motility in more than 70 % of subgroups and motility in <70 % of subgroups

Storage time (day)	DNA fragmentation (%)							
	Control group (mean ± SD)				RA group (mean ± SD)			
	Control 4 °C		Control room temp		RA 4 °C		RA room temp	
	Motility >70 %	Motility <70 %	Motility >70 %	Motility <70 %	Motility >70 %	Motility <70 %	Motility >70 %	Motility <70 %
7	0.00±0.00[2]	0.00±0.00[2]	0.43±0.19[1,3,4]	0.44±1.77[1,3,4]	0.00±0.00[2]	0.00±0.00[2]	0.00±0.00[2]	0.00±0.00[2]
14	0.00±0.00[2]	0.00±0.00[2]	0.29±0.27[1,3,4]	0.39±0.22[1.3.4]	0.00±0.00[2]	0.00±0.00[2]	0.00±0.00[2]	0.00±0.00[2]
30	1.63±0.52[2]	1.50±0.54[2]	11.14±1.22[1,3,4]	11.56±1.01[1,3,4]	1.38±0.52[2]	1.22±0.44[2]	1.75±0.46[2]	1.56±0.18[2]
90	4.63±0.52[2,3]	4.89±0.60[2,3]	24.00±1.15[1,3,4]	24.63±0.52[1.3.4]	1.25±0.46[2,4]	1.56±0.53[2,4]	4.50±0.54[2,3]	4.67±0.50[2,3]

Superscripts a, b, c, d, e, f, g, and h in the third row referred to subgroup.
[1]Statistically significant difference ($P < 0.05$) compared with control 4 ◦C motility >70 % and <70 % subgroup; [2] Statistically significant difference ($P < 0.05$) compared with control room temp motility >70 % and <70 % subgroup; [3] Statistically significant difference ($P < 0.05$) compared with RA 4 ◦C motility >70 % and <70 % subgroup; [4] Statistically significant difference ($P < 0.05$) compared with RA room temp motility >70 % and <70 % subgroup.

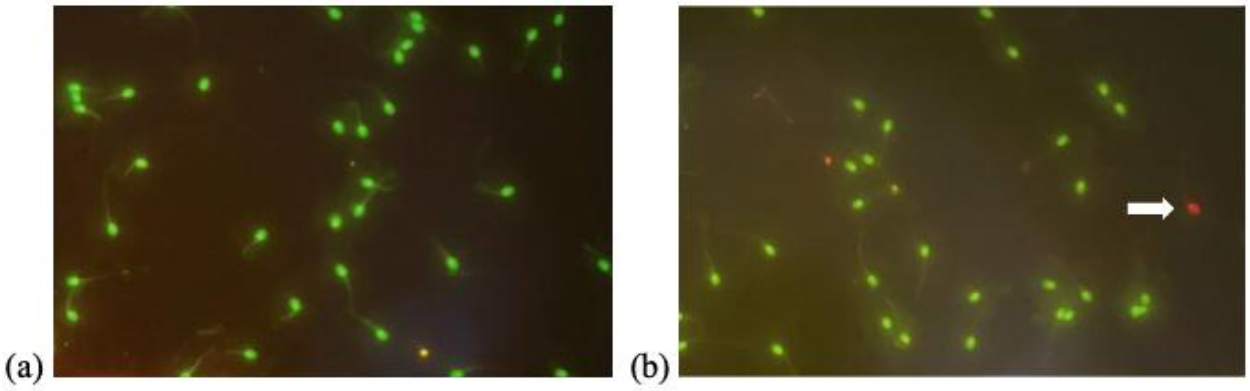


Figure 1 (a) Fluorescence microscopic analysis of DNA fragmentation in freeze-dried dog sperm stained with acridine orange. Spermatozoa with intact chromatin fluoresce as green head were analyzed immediately after the freeze-drying process (x400). (b) Acridine orange binds to the fragmented DNA sperm and fluoresces as a red sperm head (arrow) 90 days after storage (x400)

The comparison of DNA fragmentation between 2 motility subgroups at 4 °C and room temperature did not show any difference in DNA damage during observations. At 7, 14, 30, and 90 days after storage, the percentages of DNA fragmentation in the control group at room temperature with motility >70 % and <70 % were significantly greater than in the control group at 4 °C, RA group at room temperature, and RA group at 4 °C (Table 8). A comparison of the quality of freeze-dried semen between 4 subgroups from the best to the worst were the RA group at 4 °C, the RA group at room temperature, the control group at 4 °C, and the control group at room temperature, respectively. DNA fragmentation changed very quickly when freeze-dried semen was stored at room temperature without antioxidants after the first 2 weeks (Table 8). From the result, sperm motility did not influence DNA integrity. However, DNA fragmentation was slow when adding an antioxidant (rosmarinic acid) for freeze-drying, even kept at room temperature.

Discussion

Many factors influence the FD efficacy. Sperm quality decreasing due to the FD process was indicated by the strongly damaged cell membrane, which was evaluated by viability and motility tests. From the result of this study, an eosin stain was able to penetrate the sperm, and no viability was observed under light microscopy, and there was no sperm motility after the FD process. The percentage of abnormal sperm morphology showed no significant difference between the control and RA groups, but it was affected by the FD process. Abnormal sperm morphology was mostly found immediately after FD at the sperm tail, typically the bent tail, which occurred due to dehydrated cells during the FD process. Consequently, these freeze-dried sperms cannot support natural fertilization. Thus, artificial insemination was not the proper technique for freeze-dried semen. To overcome this point, ICSI is used as a selective fertilization technique.

The presence of chelating agents in FD media, such as EDTA, was effective in inhibiting endonucleases and DNA fragmentation (Kaneko and Nakagata, 2006). The addition of RA in freeze-dried media can reduce DNA damage when stored at room temperature or 4 °C. In this study, DNA damage was slow when RA was added. The difference in DNA fragmentations in the control and RA groups was observed at room temperature and 4 °C during the whole period of study, supported by the property results of many studies (Domingo *et al.,* 2018; Olaciregui, Luño, *et al.,* 2017; Wang and Zhu, 2021). In assessing the ability of antioxidants and preventing damage to DNA of 10 types of phenolic acid, such as RA, caffeine, gallic, protocatechuic, chlorogenic, cinnamic acid, ferulic, syringic, vanillic, and P-Hydroxybenzoic acid (PHBA), found that RA is the most effective in antioxidants. When testing with Beta-Carotene Bleaching Assay, it was found that RA had more antioxidant ability than butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Sevgi *et al.,* 2015). In studying the effects of RA on various cells, it was found that RA had the property of resisting free radicals. Vostálová *et al.* (2010) investigated the impact of UVB radiation on human keratinocytes. It was found that RA can help reduce ROS and decrease the interleukin-6 release, which is an inflammatory cytokine that stimulates the production of ROS and increases oxidative stress. In the study of a Chinese hamster, lung fibroblast cells (V79) treated with RA, it was found that RA can protect against chromosomal damage due to doxorubicin and reduce DNA damage. Furthermore, RA does not cause damage to the DNA of V79 cells (Furtado *et al.,* 2010). Olaciregui and Gil (2017) suggested that the percentage of DNA damage in EDTA media without an RA supplement (3 %) was higher than spermatozoa in RA media (1.4 %). In addition, the DNA damage was also decreasing in the boar semen sample, to which RA was added (Olaciregui, Luño, *et al.,* 2017).

In the analysis of DNA fragmentation in the control group, DNA damage was significantly found at room temperature more than in the 4 °C control group ($P < 0.05$) and the RA group ($P < 0.05$). At 3 months of storage, the sample in the control group, which was stored at 4 °C, had more DNA damage than the RA group at the same storage temperature. Furthermore, the percent of DNA fragmentation in the RA group and the group stored at room temperature significantly had more damage than in the 4 °C storage group. In the current study, storage temperature affects DNA damage, which can be reduced by storing the sample at 4 °C instead of at room temperature. Olaciregui *et al.* (2015) reported that storage temperature influenced the DNA integrity of freeze-dried dog spermatozoa, while the sample stored at a low temperature had less damage likewise; Domingo *et al.* (2018) observed that rabbit semen samples stored at 4 °C had lesser damage to the DNA. However, a mouse study found that no DNA fragmentation occurred when freeze-dried semen was stored at 4 °C for a year (Ward *et al.,* 2003). The freeze-dried dog semen samples in the Olaciregui and Gil (2017) study reported that DNA fragmentation evaluated by sperm chromatin dispersion test increased with storage times and had a positive effect on storage temperature. DNA fragmentation was 3.20, 9.30, and 23.60 % at 1, 6, and 12 months after storage, respectively. In addition, room temperature (22 °C) significantly decreased the percentage of DNA damage compared with samples stored at 4 °C. Recent studies results demonstrated that the comparison of the quality of freeze-dried semen between 4 subgroups from the best to the worst RA group at 4 °C, RA group at room temperature, control group at 4 °C and control group at room temperature. DNA fragmentation was observed when freeze-dried semen was stored at room temperature without antioxidants after the first 2 weeks. Therefore, the semen sample that has been stored for an extended period should be transferred from room temperature to 4 °C within 2 weeks.

An FD machine that was used to produce animal vaccines was applied to the FD process. Owing to laboratory protocol, the process of FD was different from other studies. In this study, the drying temperature was set at -40°C for the process, and the vial volume was fixed at 1 mL due to the limitations of the FD machine. While other studies used the drying temperature of -50 °C and the quantity was 150 µl. According to the results of this study, DNA was damaged more. This may be caused by the application of the FD procedure. More volume and higher temperatures may result in incomplete drying and more moisture remaining than usual, as well as may affect the structure of the sample, known as cake collapse. Cake collapse is considered to influence the stability of the protein product (Passot *et al.,* 2007) and could negatively affect sperm function (Hara *et al.,* 2014). Furthermore, compared to other studies, more damage to DNA was found when samples were stored at room temperature. In this study, the room temperature varied from 26 to 34 °C instead of 25 °C, which might be the factor that affects DNA integrity.

According to the study, the quality of the semen is unaffected by the uneven sperm concentration of two subgroups, which is correlated with Silva and colleagues' study in 2005. The comparison between the semen sample with motility >70 % and <70 % before freeze-drying did not find any difference at the time of observation. Since sperm membranes were severely harmed by FD, the process of lyophilization resulted in sperm that were neither motile nor viable. However, compared to frozen semen, this approach caused less harm to DNA (Gianaroli *et al.,* 2012). In addition, in studies on equine spermatozoa, less DNA damage was observed in freeze-drying. Compared with frozen semen, 0.02% and 0.03%, respectively (Restrepo *et al.,* 2019). The adverse effects of the cryopreservation process, such as cold shock, osmotic stress, and cryoprotectant intoxication, negatively impact semen quality (Prinosilova *et al.,* 2012). DNA fragmentation was observed within 3 hours of storage in frozen dog semen (Koderle *et al.,* 2009). Freeze-dried semen could be an alternative method for dogs with low fertility to preserve their genetic resources and a model for keeping the semen of another canine family. In sudden or unexpected death animal, viable and motile sperm from epididymis could be processed for chilled or frozen semen and were achievable to produce offspring via artificial insemination (Hori *et al.,* 2009; Hori *et al.,* 2015; Varesi *et al.,* 2013). However, motility and viability were the important criteria before sperm

cryopreservation. FD can also preserve genetic material from dead animals despite the loss of motility and viability of sperm. Sperm collected from chimpanzees and giraffes that naturally ejaculated on the ground or even testicles and epididymis from dead jaguars, weasels, and long-haired rats could succeed in freeze-dried semen (Kaneko *et al.,* 2014). The sperm that had been collected was already immobile but had a normal morphology with a head and tail attached. Therefore, in dead animals, it is advised that the sperm morphology before FD should have a connected head and tail and have the integrity of DNA. In conclusion, there was a relationship between storage temperature, periods of storage, and DNA fragmentation of freeze-dried dog spermatozoa. DNA fragmentation was slower when adding antioxidants. Another advantage is that it was easy to store, reduced shipping costs, and applied in locations without liquid nitrogen. However, this technique requires an FD machine, which is not readily available in some laboratories.

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References

Choi YH, Varner DD, Love CC, Hartman DL and Hinrichs K 2011. Production of live foals *via* intracytoplasmic injection of lyophilized sperm and sperm extract in the horse. Reproduction. 142(4); 529–538.

Concannon PW and Batista M 1989. Canine semen freezing and artificial insemination. Current Veterinary Therapy. v.10; 1247-1259.

Domingo P, Olaciregui M, González N, De Blas I and Gil L 2018. Long-term preservation of freeze-dried rabbit sperm by adding rosmarinic acid and different chelating agents. Cryobiology. 81; 174-177.

Freshman JL 2002. Semen collection and evaluation. Clin Tech Small Anim Pract. 17(3); 104-107.

Furtado MA, de Almeida LC, Furtado RA, Cunha WR and Tavares DC 2008. Antimutagenicity of rosmarinic acid in Swiss mice evaluated by the micronucleus assay. Mutat Res. 657(2); 150-154.

Furtado RA, de Araújo FR, Resende FA, Cunha WR and Tavares DC 2010. Protective effect of rosmarinic acid on V79 cells evaluated by the micronucleus and comet assays. J Appl Toxicol. 30(3); 254-259.

Garcia-Canpos A, Gil L, Malo C, Martinez F, Kershaw-Young C and de Blas I 2014. Effect of different disaccharides on the integrity and fertilising ability of freeze-dried boar spermatozoa: a preliminary study. Cryo Letters. 35(4); 277-85.

Gianaroli L, Magli MC, Stanghellini I, Crippa A, Crivello AM, Pescatori ES and Ferraretti AP 2012. DNA integrity is maintained after freeze-drying of human spermatozoa. Fertil Steril. 97(5); 1067-1073.e1.

Hara H, Tagiri M, Hwang IS, Takahashi M, Hirabayashi M and Hochi S 2014. Adverse effect of cake collapse on the functional integrity of freeze-dried bull spermatozoa. Cryobiology. 68(3); 354-360.

Hirabayashi M, Kato M, Ito J and Hochi S 2005. Viable rat offspring derived from oocytes intracytoplasmically injected with freeze-dried sperm heads. Zygote. 13(1); 79–85.

Hori S, Sengupta A, Shukla CJ, Ingall E and McLoughlin J 2009. Long-term outcome of epididymectomy for the management of chronic epididymal pain. J Urol. 182(4); 1407-1412.

Hori T, Atago T, Kobayashi M and Kawakami E 2015. Influence of different methods of collection from the canine epididymides on post-thaw caudal epididymal sperm quality. J Vet Med Sci. 77(5); 625-630.

Ivanova-Kicheva MG, Bobadov N and Somlev B 1997. Cryopreservation of canine semen in pellets and in 5-ml aluminum tubes using three extenders. Theriogenology. 48; 1343-1349.

Johnston SD, Root Kustritz MV and Olson PNS 2001. Canine and Feline Theriogenology. W.B. Saunders Company, Philadelphia. 287–306.

Kaczmarski M, Wójcicki J, Samochowiec L, Dutkiewicz T and Sych Z 1999. The influence of exogenous antioxidants and physical exercise on some parameters associated with production and removal of free radicals. Pharmazie. 54(4); 303-306.

Kaneko T, Ito H, Sakamoto H, Onuma M and Inoue-Murayama M 2014. Sperm preservation by freeze-drying for the conservation of wild animals. PLoS One. 9(11); e113381.

Kaneko T and Nakagata N 2006. Improvement in the long-term stability of freeze-dried mouse spermatozoa by adding of a chelating agent. Cryobiology. 53(2); 279-282.

Kaneko T and Serikawa T 2012. Successful long-term preservation of rat sperm by freeze-drying. PLoS One. 7(4); e35043.

Koderle M, Aurich C and Schäfer-Somi S 2009. The influence of cryopreservation and seminal plasma on the chromatin structure of dog spermatozoa. Theriogenology. 72(9); 1215-1220.

Kumi-Diaka J 1993. Subjecting canine semen to the hypo-osmotic test. Theriogenology. 39(6); 1279-1289.

Kusakabe H, Szczygiel MA, Whittingham DG and Yanagimachi R 2001. Maintenance of genetic integrity in frozen and freeze-dried mouse spermatozoa. Proc Natl Acad Sci USA. 98(24); 13501-13506.

Liu JL, Kusakabe H, Chang CC, Suzuki H, Schmidt DW, Julian M, Pfeffer R, Bormann CL, Tian XC, Yanagimachi R and Yang X 2004. Freeze-dried sperm fertilization leads to full-term development in rabbits. Biol Reprod. 70(6); 1776-1781.

Luño V, Gil L, Olaciregui M, González N, Jerez RA and de Blas I 2014. Rosmarinic acid improves function and in vitro fertilising ability of boar sperm after cryopreservation. Cryobiology. 69(1); 157-62.

Oettlé EE 1993. Sperm morphology and fertility in the dog. J Reprod Fertil Suppl. 47; 257-60.

Olaciregui M and Gil L 2017. Freeze-dried spermatozoa: A future tool?. Reprod Domest Anim. 52 Suppl 2; 248-254.

Olaciregui M, Luño V, Domingo P, González N and Gil L 2017a. In vitro developmental ability of ovine oocytes following intracytoplasmic injection with freeze-dried spermatozoa. Sci Rep. 7(1); 1096.

Olaciregui M, Luño V, Gonzalez N, De Blas I and Gil L 2015. Freeze-dried dog sperm: Dynamics of DNA integrity. Cryobiology. 71(2); 286-290.

Olaciregui M, Luño V, González N, Domingo P, de Blas I and Gil L 2017b. Chelating agents in combination with rosmarinic acid for boar sperm freeze-drying. Reprod Biol. 17(3); 193-198.

Olaciregui M, Luño V, Martí JI, Aramayona J and Gil L 2016. Freeze-dried stallion spermatozoa: evaluation of two chelating agents and comparative analysis of three sperm DNA damage assays. Andrologia. 48(9); 900-906.

Passot S, Fonseca F, Barbouche N, Marin M, Alarcon-Lorca M, Rolland D and Rapaud M 2007. Effect of product temperature during primary drying on the long-term stability of lyophilized proteins. Pharm Dev Technol. 12(6); 543-53.

Peña FJ, Núñez-Martínez I and Morán JM 2006. Semen technologies in dog breeding: an update. Reprod Domest Anim. 41 Suppl 2; 21-29.

Prinosilova P, Rybar R, Zajicova A and Hlavicova J 2012. DNA integrity in fresh, chilled and frozen-thawed canine spermatozoa. Veterinární medicina. 57; 133-142.

Qamar AY, Fang X, Kim MJ and Cho J 2020. Improved viability and fertility of frozen-thawed dog sperm using adipose-derived mesenchymal stem cells. Sci Rep. 10(1); 7034.

Restrepo G, Varela E, Duque JE, Gómez JE and Rojas M 2019. Freezing, Vitrification, and Freeze-Drying of Equine Spermatozoa: Impact on Mitochondrial Membrane Potential, Lipid Peroxidation, and DNA Integrity. Journal of Equine Veterinary Science. 72; 8-15.

Scott G 1988. Antioxidants. Bulletin of the Chemical Society of Japan. 61; 165-170.

Sevgi K, Tepe B and Sarikurkcu C. 2015. Antioxidant and DNA damage protection potentials of selected phenolic acids. Food Chem Toxicol. 77; 12-21.

Silva A, Cardoso RdC and Silva L. 2005. Comparison between different dilution rates on canine semen freezing using Tris-buffer with the addition of egg-yolk and glycerol. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 57; 764-771.

Tejada RI, Mitchell JC, Norman A, Marik JJ and Friedman S 1984. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. Fertil Steril. 42(1); 87-91.

Thomassen R, Sanson G, Krogenaes A, Fougner JA, Berg KA and Farstad W 2006. Artificial insemination with frozen semen in dogs: a retrospective study of 10 years using a non-surgical approach. Theriogenology. 66(6-7); 1645-1650.

Varesi S, Vernocchi V, Faustini M and Luvoni GC 2013. Morphological and acrosomal changes of canine spermatozoa during epididymal transit. Acta Vet Scand. 55(1); 17.

Vostálová J, Zdarilová A and Svobodová A 2010. Prunella vulgaris extract and rosmarinic acid prevent UVB-induced DNA damage and oxidative stress in HaCaT keratinocytes. Arch Dermatol Res. 302(3); 171-181.

Wakayama T and Yanagimachi R 1998. Development of normal mice from oocytes injected with freeze-dried spermatozoa. Nat Biotechnol. 16(7); 639-41.

Wang YY and Zhu WJ 2021. Effects of rosmarinic acid on DNA integrity and H19 differentially methylated region methylation levels in human sperm preserved by freeze-drying. Reproductive and Developmental Medicine. 5(1).

Ward MA, Kaneko T, Kusakabe H, Biggers JD, Whittingham DG and Yanagimachi R 2013. Long-term preservation of mouse spermatozoa after freeze-drying and freezing without cryoprotection. Biol Reprod. 69(6); 2100-2108.

Witschi HP 1981. Enhancement of tumor formation in mouse lung by dietary butylated hydroxytoluene. Toxicology. 21(2); 95-104.

Zhang W, Yi K, Chen C, Hou X and Zhou X 2012. Application of antioxidants and centrifugation for cryopreservation of boar spermatozoa. Anim Reprod Sci. 132(3-4); 123-8.

Zorinkimi A, Ahmed F and Lalrintluanga K 2017. Effect of Different Extenders on the Quality of Mongrel Dog Semen Preserved at 5°C on the Basis of Hypo-Osmotic Sperm Swelling Test (HOSST). International Journal of Current Microbiology and Applied Sciences. 6; 961-964.