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Original Article

Evaluation of the optimal thawing conditions for dog spermatozoa frozen in cryovials

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ABSTRACT

Background: Using cryovial for freezing dog spermatozoa provides a practical method to increase extended sperm volume and shorten the time required for equilibration by using a simple freezing techniques. The purpose of this study was to determine the optimal thawing condition for dog sperm cryopreservation using cryovials.

Methods: For sperm freezing, cryovials with 200 × 10^6 sperm/mL were cooled after the addition of tris egg yolk extender (TEY) at 4℃ for 20 min, then TEY with 4% glycerol was added and equilibrated for another 20 min before being aligned over LN_2 vapor for another 20 min and plunged directly into LN_2. Spermatozoa were thawed in a water bath at 37℃ for varying times (25 sec, 60 sec, 90 sec, and 120 sec) in the first experiment. In the second experiment, spermatozoa were thawed in a water bath at various temperatures and times (37℃ for 1 min, 37℃ for 1 min with gentle stirring, 24℃ for 24 min, and 75℃ for 20 sec). In these experiments, the effect of thawing conditions on motility parameters, viability (SYBR-14/PI), and acrosome integrity (PSA/FITC) of spermatozoa were investigated.

Results: The post-thaw sperm motility parameters, viability, and acrosome integrity were not significantly different across the experimental groups.

Conclusions: In this study, the characteristics of spermatozoa frozen using cryovials were not significantly affected by various thawing conditions.

Keywords: cryopreservation, cryovial, dog spermatozoa, thawing condition

INTRODUCTION

Semen cryopreservation is considered as the most significant step in artificial insemination and the most commonly used assisted reproductive technology (ART) in canine practice (Abdillah et al., 2019). The process of cryopreservation tends to reduce the biological characteristics of canine spermatozoa (Strzezek et al., 2012). One of the influencing factors that interact with thawing procedures to affect the post-thawing motility of the sperm is sperm-freezing vessels such as straws and cryovials. Other variables include the type of extender, glycerol concentration, cooling rate, and handling of semen during cryopreservation procedures (Robbins et al., 1976). For the cryopreservation of dog spermatozoa, the most widely used protocol is the conventional freezing method with straws and LN_2 (Farstad, 2010). However, the straws used for sperm freezing are available in only two volumes

(0.25 mL or 0.5 mL), whereas cryovials are available in a wide range of volumes (Li et al., 2019; Beirão et al., 2021). At -196℃, straws are much more fragile than cryovials because cryovials become hard and inflexible (Talha et al., 2020). Using cryovial provides a practical way to increase the volume of the extended sperm and shorten the time required for equilibration by using a simple freezing technique (Ibrahim et al., 2024). Previous studies of cryovials for human, goose, and spotted wolffish sperm have provided valuable results (Watson, 2000; Li et al., 2019; Beirão et al., 2021).

The thawing procedure significantly impacts the survival of viable spermatozoa (Nur et al., 2003). Numerous studies have focused on cryopreservation of sperm in straws to determine the optimal temperature, duration, and rate to achieve the highest percentage of viable spermatozoa after the post-thawing process of cryopreserved sperm (Correa et al., 1996). Furthermore, considering the aforementioned information and the lack of detailed published research on the temperature and thawing period of dog sperm frozen in cryovials, we aimed to develop a more precise and efficient thawing procedure using cryovials. Therefore, we determined motility, viability, and acrosome integrity of dog sperm frozen using cryovials with comparing the various thawing conditions.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Four 2-5-year-old male dogs (two Pomeranian and two Beagles) were used as the semen sources. All experimental dogs were maintained in individual pens, fed daily with commercial dog food (BioMill®, Jindo, South Korea), and given fresh drinking water ad libitum. All dogs were trained for routine semen collection.

Semen collection and preparation

One ejaculate from each of the four dogs was obtained by digital manipulation. Sperm-rich second fraction of each ejaculate was collected using pre-warmed (37℃) calibrated plastic tubes (SPL Life Sciences Co., Pocheon, Korea) connected to a funnel. The samples were immediately transferred to the laboratory in a Thermus flask at 37℃. Normal ejaculates with sperm concentrations ≥ 2×10^8 sperm/mL, motility ≥ 70% and normal morphology ≥ 80% were included in this study. To reduce individual variations in the current study, each ejaculate from the four dogs was centrifuged (12 min at 300 × g) for each replicate, and the pellets were pooled.

Sperm freezing extenders and freezing procedure

In a cryovial (CryoTube™ Vials, Nunc A/S, Roskilde, Denmark), spermatozoa were slowly diluted (200×10^6 sperm/mL) with extender 1, which contained Tris (3.025 g), citric acid (1.7 g), fructose (1.25 g), Na benzylpenicillin (0.06 g), streptomycin sulfate (0.1 g), and egg yolk (20 mL) per 100 mL ultra-pure water (ProGen Life Sciences, NY, USA). The extended sperm was then incubated for 20 min at 4℃, diluted with extender 2 (extender 1 supplemented with 8% glycerol) in a 1:1 ratio (4% glycerol at the final concentration), and allowed to equilibrate for an additional 20 min. Each cryovial with sperm was placed 7 cm above LN_2 vapor for 20 min (cooling rate of 3.75℃/min) and then plunged directly into the LN_2 (cooling rate of 276.2℃/min).

Thawing procedure

In the first experiment, sperm in cryovials were thawed in a water bath set at a constant temperature of 37℃ for different periods of time [Group 1: 37℃ for 25 sec and then kept at 24℃ for 20 min (thawing rate: 27℃/min); Group 2: 37℃ for 60 sec and then kept at 24℃ for 15 min (21.9℃/min); Group 3: 37℃ for 90 sec and then incubated at 24℃ for 5 min (22.3℃/min); Group 4: 37℃ for 120 sec (42℃/min)].

The second experiment was conducted by thawing sperm in cryovials at various temperatures and durations [Group 1: 37℃ for 1 min and then kept at 24℃ for 15 min (21.9℃/min); Group 2: 37℃ for 1 min (with light stirring) and then incubated at 24℃ for 15 min (21.7℃/min); Group 3: 24℃ for 24 min (2.9℃/min); Group 4: 75℃ for 20 sec and then incubated at 37℃ for 150 sec (45.6℃/min)]. Thawing rates were measured using a thermocouple (Thermocouple thermometer type T, Cole-Parmer®, Vernon Hills, IL, USA). Frozen sperm in cryovials were thawed according to the procedure described above, and the sperm quality variables were analyzed as described below.

Post-thaw sperm evaluation

1) Sperm motility

To evaluate the sperm motility parameters, a computer-assisted sperm analysis (CASA) using a Vet edition SCA analyzer (version 6.3.0.32, CASA; SCA-CASA; Microptic SL., Barcelona, Spain) was used. This CASA system was connected to a phase-contrast microscope (Nikon Eclipse E-200; Nikon Corporation, Kanagawa, Japan) with a minitherm heating stage (Thermo plate, Tokai Hit, Shizuoka-ken, Japan) and camera (acA1300-200uc, Basler AG, Ahrensburg, Germany). A 2 μL of thawed spermatozoa was placed into a preheated (37℃) Leja standard-count 8 chambered slide with a 20 μm (Leja Products B.V., NieuwVennep, The Netherlands). For each sperm sample, five videos lasting three seconds each were recorded in different fields. Sperm motility parameters were analyzed, including total motility (MOT, %), progressive motility (PMOT, %), rapid progressive (%), medium progressive (%), non-progressive (%), average path velocity (VAP, μm/sec), curvilinear velocity (VCL, μm/sec), straight line velocity (VSL, μm/s), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), amplitude of the lateral head displacement (ALH, μm), and beat cross frequency (BCF, Hz).

2) Sperm viability

To evaluate sperm viability, we used the fluorescent double stain technique with SYBR-14/propidium iodide (PI) as the double stain (LIVE/DEAD™ sperm viability kit, L#7011, Invitrogen™, Thermo Fisher Scientific, Carlsbad, CA, USA), following Yu (2014). To 50 μL of spermatozoa, 5 μL of SYBR-14 (100 nM) was added and kept for 5 min at room temperature in the dark. Then, 5 μL of PI (12 μM) was added, and the sample was incubated for an additional 5 min in the same conditions. Two smears were prepared, and at least 200 spermatozoa were counted per slide. The number of spermatozoa with green (live) and red (dead) fluorescence was counted under a fluorescence microscope (Axio, Carl Zeiss, Goettingen, Germany) equipped with a 488 nm excitation filter, and the percentage of membrane-intact spermatozoa (green fluorescence) was calculated.

3) Acrosome integrity

To assess the integrity of the sperm acrosome membrane, Pisum sativum agglutinin (PSA) conjugated with fluorescein isothiocyanate (FITC) stain was used according to the method of Yu (2014). Briefly, a smear was prepared using 20 μL of spermatozoa. It was air dried, fixed by adding methanol and alcohol, and air dried again for 10 min. Thereafter, 30 μL of PSA-FITC (100 mg/mL) was added to the slide, which was then covered with parafilm for 20 min in the dark. The slide was then immersed in a jar with distilled water for 15 min and air-dried. For each sample, two slides were examined under a fluorescence microscope (Axio, Carl Zeiss, Goettingen, Germany). At least 200 spermatozoa were counted per slide. No fluorescence or pale green on the sperm anterior acrosomal region was regard as damaged acrosome. Spermatozoa with intense green fluorescence (intact acrosomal membrane) on the sperm anterior acrosomal region were examined, and the percentage of sperm with intact acrosomes was calculated.

Statistical analysis

The experimental procedures for each group were repeated in three separate experiments. The different thawing conditions on motility, viability, and acrosome integrity of dog sperm were analyzed using the Statistical Package for the Social Sciences (IBM SPSS Statistics 26.0, Armonk, NY, USA). All results are presented as mean ± SEM and assessed with one-way ANOVA and Duncan statistical tests. $p < 0.05$ was considered significant.

RESULTS

The effect of the thawing condition at 37℃ for different durations on the sperm motility parameters, and viability

The motility parameters of the sperm thawed at a constant temperature of 37℃ for various durations of time (experiment 1) are shown in Fig. 1A and Table 1, the percentage of the sperm total, progressive, rapid progressive, and medium progressive motility was not significantly different between groups (Fig. 1A). In data for motility kinematic parameters (VCL, VAP, VSL, STR, LIN, WOB, ALH and BCF), no differences were detected in the kinematic parameters between groups (Table 1). There was no statistically significant difference in the viability percentage between the groups, if 60 sec group was a little higher than other groups (Fig. 1B, Fig. 2A and 2B).

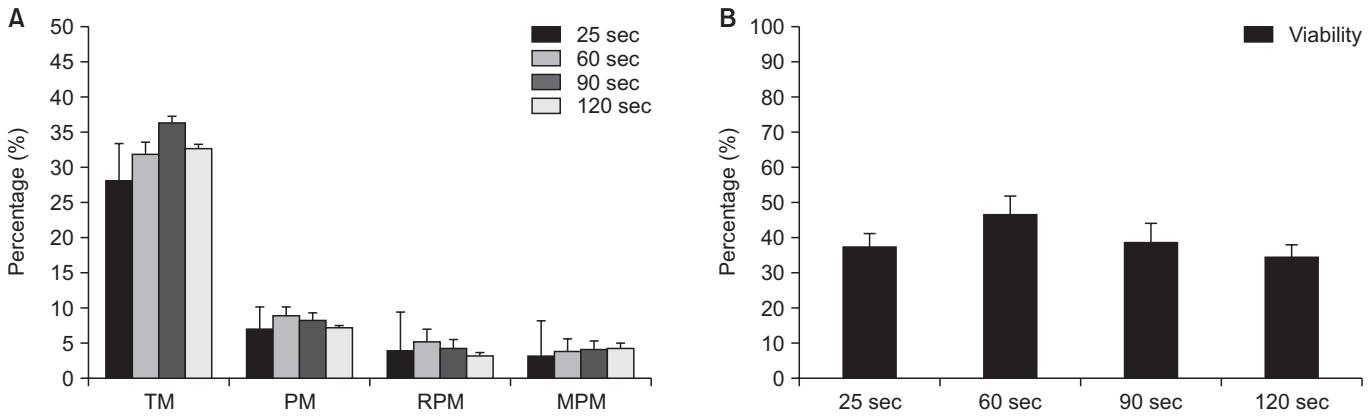

Fig. 1. The effect of thawing temperature at 37℃ and different durations of dog sperm frozen in cryovials on (A) total motility (TM), progressive motility (PM), rapid progressive motility (RPM), medium progressive motility (MPM), and (B) viability. Values are expressed as the mean ± SE.

Table 1. The effect of thawing temperature at 37℃ and different durations on motility kinematic parameters of dog sperm frozen in cryovials

Time	VCL (μm/S)	VAP (μm/s)	VSL (μm/s)	STR (%)	LIN (%)	WOB (%)	ALH (μm/s)	BCF (Hz)
25 sec	29.1 ± 1.8	20.1 ± 1.7	15.7 ± 1.5	62.3 ± 1.3	42.9 ± 1.0	63.5 ± 0.9	1.3 ± 0.1	5.5 ± 0.3
60 sec	30.0 ± 2.0	21.4 ± 2.0	16.8 ± 1.8	63.7 ± 1.1	45.3 ± 1.1	66.0 ± 0.8	1.3 ± 0.0	5.6 ± 0.2
90 sec	27.9 ± 1.8	18.8 ± 2.0	13.7 ± 1.9	58.6 ± 1.2	37.7 ± 3.9	62.1 ± 2.2	1.3 ± 0.0	5.2 ± 0.2
120 sec	27.8 ± 1.7	18.2 ± 1.6	13.1 ± 1.4	59.9 ± 0.8	40.4 ± 0.6	62.5 ± 0.7	1.3 ± 0.0	5.1 ± 0.1

VCL, curvilinear velocity; VAP, average path velocity; VSL, straight-line velocity; STR, straightness; LIN, linearity; WOB, wobble; ALH, amplitude of the lateral head displacement; BCF, beat cross frequency. Values are expressed as the mean ± SE.

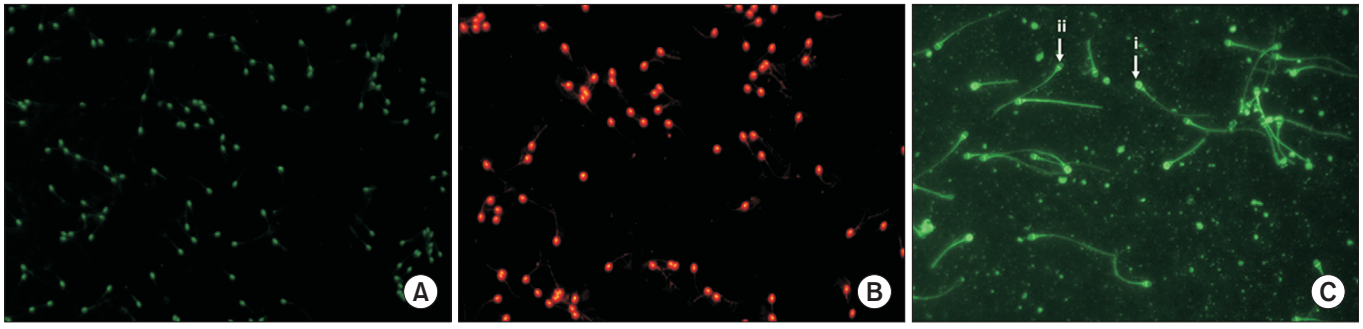

Fig. 2. The effect of different thawing temperatures and durations on post-thaw viability and acrosome integrity of dog sperm after freezing and thawing in cryovials. For viability, spermatozoa were stained with SYBR-14 for live spermatozoa (A) and with propidium iodide for dead spermatozoa (B). Frozen-thawed spermatozoa were stained for acrosome integrity (C) with Pisum sativum agglutinin labeled with FITC and examined under a fluorescent microscope; (i) sperm with an intact acrosomal membrane, and (ii) sperm with a damaged acrosomal membrane. Values are expressed as the mean ± SE.

The effect of the various thawing condition with different thawing temperature on the sperm motility parameters, viability, and acrosome integrity

In experiment 2, the post-thaw percentage of the sperm total, progressive, rapid progressive, and medium progressive motility was not significantly different across the groups (Fig. 3A). In addition, motility kinematic parameters of motility were not significantly different, as shown in Table 2. The percentage of viability (Fig. 3B) and acrosome integrity (Fig. 2C and 3B) was not significantly different throughout the groups.

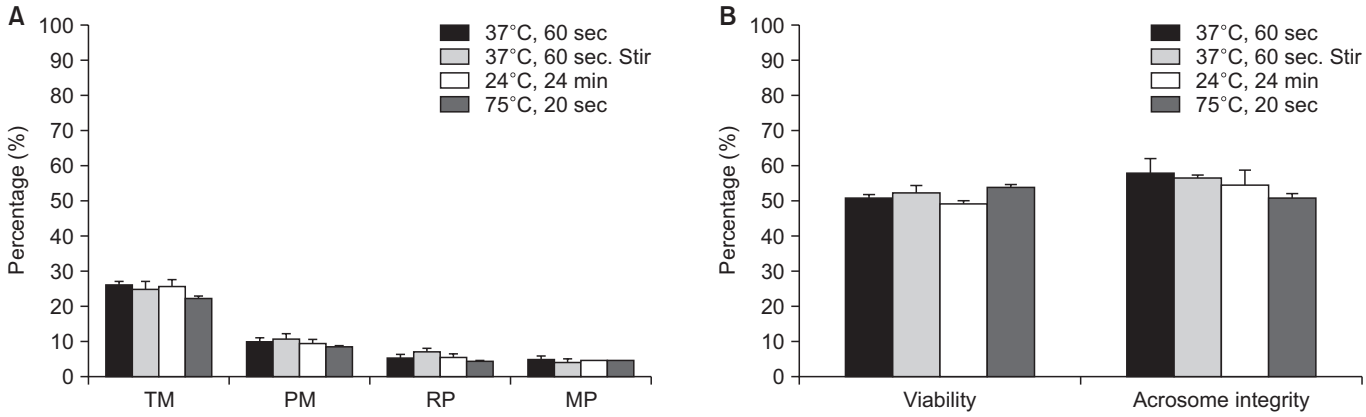

Fig. 3. The effect of different thawing temperatures and durations of dog sperm frozen in cryovials on (A) total motility (TM), progressive motility (PM), rapid progressive motility (RPM), medium progressive motility (MPM), and (B) viability and acrosome integrity. Values are expressed as the mean ± SE.

Table 2. The effect of different thawing temperatures and durations on motility kinematic parameters of dog sperm frozen in cryovials

Temperature	Time	VCL (μm/s)	VAP (μm/s)	VSL (μm/s)	STR (%)	LIN (%)	WOB (%)	ALH (μm/s)	BCF (Hz)
37℃	60 sec	34.2 ± 1.5	26.2 ± 1.9	21.9 ± 2.0	75.5 ± 2.0	56.6 ± 1.9	74.8 ± 1.3	1.3 ± 0.0	6.4 ± 0.2
37℃	60 sec. Stir	40.5 ± 2.5	34.2 ± 2.3	30.7 ± 2.5	76.6 ± 1.6	64.0 ± 1.4	69.9 ± 10.2	1.2 ± 0.0	7.0 ± 0.3
24℃	24 min	34.3 ± 2.0	28.2 ± 1.7	24.5 ± 1.9	76.7 ± 0.6	62.4 ± 1.5	78.2 ± 1.3	1.2 ± 0.0	6.2 ± 0.2
75℃	20 sec	35.2 ± 0.4	29.0 ± 0.1	24.9 ± 0.4	73.8 ± 3.3	60.0 ± 3.4	77.3 ± 2.0	1.2 ± 0.0	6.1 ± 0.0

Stir, stirring; VCL, curvilinear velocity; VAP, average path velocity; VSL, straight-line velocity; STR, straightness; LIN, linearity; WOB, wobble; ALH, amplitude of the lateral head displacement; BCF, beat cross frequency. Values are expressed as the mean ± SE.

DISCUSSION

The aim of this study was to investigate the effect of various thawing temperature and duration on the sperm motility parameters, viability, and acrosome integrity in order to standardize the thawing procedure for spermatozoa frozen in cryovials, as straws are commonly used in cryopreservation of dog spermatozoa. Due to the differences in thickness and volumes between straw and cryovial, different cooling, freezing, and thawing rates are required for cryovials. To address this, a fixed technique was needed to be developed.

Firstly, we tried to compare various thawing methods with constant temperature at 37℃ and different durations; however, the thawing rate was narrow (21.9-42.0℃/min). Secondly, sperm was thawed in cryovials at various temperatures (24-75℃) and durations, and a wide range of thawing rates was used (2.9-45.6℃/min). The thawing temperatures and exposure periods in the current experiment had no significant effect on sperm motility parameters, viability, and acrosome integrity of dog spermatozoa frozen using cryovials. In order to determine several sperm motility characteristics, such as total motility, progressive motility linearity, and multiple velocity parameters, we used CASA, which provides an accurate and rapid assessment (Verstegen et al., 2002). As sperm motility influences the success of fertilization, it is an important factor to consider when evaluating the quality of spermatozoa (Volpe et al., 2009; da Cunha et al., 2017). Additionally, according to Preece et al. (2017), it serves as an indirect indicator for sperm viability of domestic dog. The analysis of velocity parameters (VAP, VSL, and VCL), as well as BCF, appears to be the most effective way to discriminate between the semen of fertile and infertile dogs (Domosławska et al., 2013), as well as an indicator of the ability of sperm to penetrate the zona pellucida of oocytes (Donnelly et al., 1998; Verstegen et al., 2002; Sallam et al., 2003).

The thawing method enables the spermatozoa maintain their fertility after thawing, along with additional factors including spermatozoon concentration, glycerol concentration, cooling rate, and cryopreservation techniques (Robbins et al., 1976). According to Foote (1989), sperm must thaw quickly in order to prevent damage from

the ice crystals during the procedure. This will maintain motility, acrosomal integrity, as well as fertility. The researchers recommended thawing condition of mini straws with a capacity of 0.25 mL at 38 ± 2℃ for 25 sec (Yilmaz et al., 2019). Gilbert and Almquist (1978) reported that frozen bull spermatozoa in 0.3 mL straws had better acrosomal integrity and motility after thawing at 65℃ for 7.5 sec and 95℃ for 6 sec than at 35℃ for 10 sec. The variations between straw and cryovials that were previously highlighted might be the cause of the differences between this research and the other investigations. Cryovials have a higher surface-to-volume ratio than straws, which influences thawing rates. It is widely considered that rapid thawing rates are required for optimal sperm survival (Shah et al., 2016). The thickness of the cryovial wall influences complete thawing in fast thawing methods because the interior part of the sperm sample remains frozen. However, if the temperatures at 75℃ were attempted in this study, there is a limitation to develop the higher thawing rates for cryovials with spermatozoa, due to the thickness of the cryovial wall. According to Farrant et al. (1977), a slow rate of thawing, less than 50℃/min, will permit the cell to rehydrate and maintain an osmotic balance. In conclusion, the thawing temperatures and exposure periods had no effect on dog spermatozoa frozen using cryovials at a generally lower thawing rate (2.95-45.6℃/min). Therefore, a thawing rate of 2.95-45.6℃/min resulted in generous results of sperm parameters. This range will produce consistent results for sperm frozen in cryovials.

CONCLUSION

In this study, the characteristics of spermatozoa frozen using cryovials were not significantly affected by various thawing conditions.

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Ethical Approval: The dogs used in this study were treated and received care under the Guiding Principles for the Care and Use of Research Animals, as established by Jeonbuk National University (NON2023-019).

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Consent to Publish: Not applicable.

Availability of Data and Materials: Not applicable.

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