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Research Article

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Determination of Equilibration Time on Frozen Dog Semen in Caniplus® Extender

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ABSTRACT

Background: Dog semen is ejaculated in several fractions, and each fraction may differ in quality characteristics. In semen cryopreservation, equilibration time prior to freezing is an important factor influencing post-thaw sperm quality. However, information regarding optimal equilibration time using commercial extenders remains limited.

Aims: This study aimed to analyse the characteristics of fresh dog semen from two fractions and to determine the optimal equilibration time for freezing dog semen using a commercial extender.

Methods: Semen was collected from three large dog breeds aged between two and five years from a private kennel. The ejaculates were separated into fractions and analysed for quality. The highest quality semen fraction was diluted with Caniplus® extender, packaged into 0.25 ml mini straws, and arranged in three freezer racks. Samples were frozen after equilibration periods of 1, 2, or 3 hours. Post-thaw evaluations included total sperm motility, progressive motility, plasma membrane integrity, sperm viability, and sperm abnormalities.

Results: The results showed variations in semen quality between the two fractions. Different equilibration times did not significantly affect total sperm motility, progressive motility, or plasma membrane integrity ($p>0.05$). The highest sperm viability was observed in semen equilibrated for 1 hour ($p<0.05$). The lowest sperm abnormalities were found in semen equilibrated for one or two hours. Sperm motility was descriptively noted in frozen semen with a 1-hour equilibration time.

Conclusion: This study concluded that semen fractions differ in quality characteristics, and an equilibration time of one to two hours in Caniplus extender is recommended for optimal semen freezing results.

INTRODUCTION

Conventional dog breeding programs in Indonesia are still conducted in a conventional manner and are considered impractical. Reproductive technologies, including artificial insemination (AI), have not been extensively adopted. Semen cryopreservation is a technology with the potential to support AI programs. Semen cryopreservation is defined as the storage of frozen male gametes to facilitate the transport of genetic material without animal movement (Sicherle et al., 2020). This method is of particular significance for breeds of high economic value because it facilitates the storage of genetic material over extended periods, enhances mating efficiency, and serves as a substitute

in instances of natural mating failure (Hermansson et al., 2021; Ponglowhapan et al., 2004).

Semen is defined as a secretion from male reproductive organs during ejaculation, consisting of sperm and seminal plasma. A distinguishing feature of canine semen is its composition, which differs from that of the semen of other animals. The ejaculate consists of multiple fractions of varying qualities. Therefore, it is imperative that proper dilution, mixing, and cooling procedures are followed to prevent cold shock and reduce the risk of damage to the sperm cell structure (Ezzati et al. 2020). The process of cooling semen prior to freezing, also known as equilibration, was performed at 5°C. The objective of equilibration is to balance the osmotic conditions between the intracellular and extracellular environments, facilitate the penetration of cryoprotectants, and remove water from cells, thereby reducing the risk of damage (Passarelli et al., 2020).

The optimal equilibration time varies among species: 2–4 h for bovine semen (Amal et al., 2019), 2 h for pig semen (Passarelli et al., 2020), 4 h for buffalo semen (Rastegarnia et al., 2014), 6 h for sheep semen (Vozaf et al., 2021), and 1–3 h for horse semen (Arifiantini et al., 2010). Furthermore, the equilibration time depends on the freezing technique employed. For instance, in the event of automatic freezing, equilibration is required for a duration of only 0.5 h (Kyselová et al., 2022). The equilibration time for canine semen varied. Belala et al. (2016) conducted a six-hour equilibration process in beagle dogs using an egg-yolk-based extender.

Abe et al. (2020) utilised a duration of 120 minutes or 2 hours with skim milk extender. In a study conducted by Berghe et al. (2018), a period of 2.5 hours was dedicated to observing African wild dogs. Ibrahim et al. (2024) equilibrated Pomeranian and Beagle dogs for 40 min using a Tris egg yolk extender. As demonstrated in previous research, there are variations in equilibration time contingent on the dog breed, extender type, and individual cryotolerance (Lechner et al., 2022). The absence of a standardized protocol for canine semen cryopreservation is attributable to the considerable diversity of the breeds involved and the characteristics of the semen (Buriak et al., 2020). The objective of this study is to analyse the characteristics of fresh semen from two different semen fractions and to determine the equilibration time in several dog breeds using the commercial extender Caniplus®.

MATERIALS AND METHODS

The research was conducted from February to March 2024 at two locations: the Dogs Ministry private kennel In Jakarta, Indonesia and the Reproductive Rehabilitation Unit Laboratory, Division of Reproduction and Obstetrics, School of Veterinary Medicine and Biomedicine, Bogor Agricultural University, Bogor, Indonesia. The research procedures were approved by the Animal Ethics Committee, the School of Veterinary Medicine and Biomedical Sciences, Bogor Agricultural University (approval number: 180/KEH/SKE/2024).

Research Procedures

Fresh Semen Collection

Semen was collected from three large breeds of dogs: Samoyed, Alaskan Malamute (A. Malamute), and Siberian Husky (S. Husky). The dogs were aged between two and five years and were sourced from the Dogs Ministry's private kennel. The three canines were subjected to health examinations and an acclimatization process for a period of one month prior to semen collection. Notably, no mating was performed for a period of one week before the collection of semen. The reproductive status of the three canines was confirmed to be fertile, as evidenced by the presence of offspring on multiple occasions.

Canine semen was collected using the penis massage technique outlined by Kalkan and Ömer (2022) with minor adjustments. Semen collection was conducted once a week over a period of one month, at a frequency of once a week. Semen samples were then divided into three fractions: fraction I (pre-sperm), II (rich sperm), and III (post-sperm). The present study exclusively compared the semen characteristics of fractions I and II. The third fraction was not used because preliminary research has indicated that it contains an almost negligible amount of sperm. Total ejaculation from the three individuals was repeated four times for each sample.

Semen Quality Testing

Semen samples were immediately evaluated. Sperm motility, kinematics, and concentration were assessed using a portable AndroScope (Minitübe, Tiefenbach, Germany) computer-assisted semen analysis (CASA) device with Leja slides. Semen (3 µL) was placed on a Leja slide and observed in four fields of view, with approximately 300 sperm cells per field. Sperm viability and morphology were assessed using eosin-nigrosin staining, a technique that involves the deposition of 5 µL of semen on an object glass. Semen was added to 20 µL of eosin-nigrosin stain (1:4), homogenized, and smeared. The preparation was then subjected to desiccation via heating, after which it was examined under a microscope (Olympus CH 23) at 10×40 magnification. Live sperm did not absorb the stain,

whereas dead sperm exhibited an absorption response. Sperm samples were evaluated by counting both normal and abnormal cells, with a minimum of 200 cells or 10 fields of view recorded.

Plasma membrane integrity was assessed using the hypoosmotic swelling test (HOST), as described by Agarwal et al. (2016). This involved mixing 30 µL of semen with 300 µL of HOST solution (1:10), followed by homogenization and incubation at 37°C for 30 min. Following the incubation period, a 5 µL sample was applied to an object glass, which was then covered with a cover glass and observed under a 10×40 magnification microscope. Sperms with intact plasma membranes exhibited curled tails, whereas those with compromised membranes had straight tails. The number of sperm was enumerated in ten fields of view.

Extender Preparation

The Caniplus® (Minitüb, Tiefenbach, Germany) is a commercial extender in the form of a clear solution containing a buffer and antibiotics. Before use, 20% egg yolk was added to the diluting agent and homogenized.

Semen Dilution and Freezing

It is imperative to note that only the optimal fractions from fractions I and II were used for freezing. The semen is then subjected to evaluation and dilution with Caniplus® at a 1:1 ratio. Semen and extender were then homogenized and packaged into 0.25 ml Minitubes (Minitube, Germany). The straws were then arranged in three freezing racks and equilibrated for 1, 2, and 3 h. Following equilibration for the specified time, the semen was subjected to liquid nitrogen vapor for 10 min and stored in a liquid nitrogen container for subsequent testing.

The quality of the frozen semen was tested 24 h after freezing. Frozen semen samples were thawed individually at 37°C for 30 s. Semen found in the straw was extracted by severing both straw caps and transferred into microtubes for storage at 37°C for subsequent observation. Sperm motility and kinematic tests were performed using an AndroScope. Sperm viability, membrane integrity, and abnormalities were assessed using a methodology analogous to that used for fresh semen, with minor adjustments to accommodate the unique characteristics of frozen semen.

Data Analysis

Research data on the characteristics of fresh dog semen in the two fractions were analyzed using the Independent-sample T-test, while research data on the quality of frozen semen at different equilibrium times were analyzed using a Randomized Block Design (ANOVA), followed by Duncan's Multiple Range Test (DMRT) if there were differences with a 95% confidence level.

RESULTS AND DISCUSSION

Characteristics of Fresh Canine Semen in Two Different Fractions

The characteristics of fresh canine semen in fractions I and II differed in this study. The macroscopic characteristics of the semen samples in all fractions were found to be thin, with almost identical pH values ranging from 6.20±0.00 to 6.22±0.05. The color of the semen varied, with the majority exhibiting a cloudy white appearance, except for fraction II of A. The Malamute and Siberian Husky breeds displayed a milky white hue. Furthermore, no significant variations in semen volume were observed between different semen fractions or between dog breeds within the same fraction (Table 1).

Quality of Frozen Dog Semen with Different Equilibration Times

The quality of the semen examined in this study was found to be substandard in terms of sperm motility. The present study demonstrated that equilibration time during canine semen freezing did not affect the total or progressive motility ($p > 0.05$). Total sperm motility ranged from 13.46% to 19.01%. Furthermore, progressive motility exhibited no significant variance, with a range of 8.47–13.50% (Table 2).

Kinematic analysis of sperm from three dogs demonstrated that Curvilinear Velocity (VCL) exhibited no variation between equilibration times of 67.86 to 73.47 µm/s. The velocity average path (VAP) and velocity straight line (VSL) were found to be at their highest in sperm that had been equilibrated for a duration of three hours, with no discernible difference observed between the two-hour and three-hour equilibration periods. The Amplitude of Lateral Head Displacement (ALH) values were consistent across various equilibration times, ranging from 1.77 to 1.84 µm (Table 3).

The volume of canine semen examined in this study ranged from 1.40 ± 0.45 mL to 2.45 ± 0.96 mL (Table 1). Zorinkimi et al. (2017) stated that in Mongrel dogs, the ejaculate volume of fractions I and II was 2.96 ± 0.41 mL. The investigation revealed that

differences in plasma membrane integrity were only evident in A Malamute dogs, whereas the other two dog breeds exhibited no such differences. The plasma membrane integrity of the subjects in this study did not vary between breeds in Fraction I. However, in Fraction II, the plasma membrane integrity was found to be optimal in Siberian Husky, with Malamutes and Samoyeds demonstrating comparable results. Sperm abnormalities were observed in fractions I and II; however, these abnormalities differed exclusively in the Samoyed breed. The prevalence of sperm abnormalities was 25.52% ± 11.26% in fraction I, whereas fraction II had a significantly lower prevalence of 7.82% ± 8.25%. The prevalence of sperm abnormalities in other dog breeds ranged from 6.51 ± 3.34% to 12.76 ± 4.87%.

The semen characteristics presented in Table 1 demonstrate variations between the fractions from different dog breeds. This finding suggests that there are differences in the characteristics of fresh semen fractions I and II among various dog breeds. Lechner et al. (2022) explain that variations in semen characteristics are influenced by individual variations such as breed, body size, body weight, age, prostate gland size, and semen collection frequency. The results of the study by Wicaksono and Arifiantini (2009) demonstrated that the semen volume of fraction II in Retriever dogs was 1.95 ± 0.02 mL, with a cloudy white appearance and moderate consistency. The pH of retriever dog semen was found to be 6.43 ± 0.02, with sperm motility recorded at 70 ± 0.08%, sperm viability at 84.51 ± 0.03%, and a concentration of 407.50 ± 1.02 × 10^6 mL^{-1}.

The quality of canine semen is influenced by several factors, including genetics, age, and physiological conditions (Tesi et al., 2018). Genetic adaptation affects lipid composition, which in turn directly affects semen quality. Physiological conditions, including hormonal status and testicular health, have been shown to have detrimental effects on semen quality. A decline in testosterone levels has been linked to decreased sperm motility (Ezzati et al., 2020). As reported by Kumar et al. (2023), the mean volume of semen ejaculated by mongrel dogs was 3.55 ± 0.31 mL. This range is reported to extend from 2.0 to 7.0 mL in a single ejaculation.

This investigation revealed that the sperm motility of fraction I in A. Malamute and Samoyed dogs did not differ from that of S. Siberian Husky. The highest sperm motility in fraction II was demonstrated by S. Husky, followed by A. Malamute, and the lowest in samoyed semen. The sperm concentration varied between the fractions. Fraction 1 was lower than that of fraction II. The sperm concentration of fraction II in S. Husky was the highest ($p<0.05$) compared to the other two breeds (Table 1). The sperm viability data from Fraction I revealed no significant differences between the various dog breeds. However, in Fraction II, the highest sperm viability was observed in S. Husky. Additionally, there

Table 1. Characteristics of fresh dog semen in two different fractions

Variable	A. Malamute Fraction		S. Husky Fraction		Samoyed Fraction	
	1	2	1	2	1	2
SV (ml)	1.40±0.45	1.72±0.55	2.45±0.96	2.02±1.39	1.45±0.42	1.27±0.33
SC	Thin white	Milky white	Thin white	Cloudy white	Cloudy white	Cloudy white
Consistency	thin	thin	thin	Thin	thin	thin
Semen pH	6.22±0.05	6.20±0.00	6.20±0.00	6.20±0.00	6.20±0.00	6.20±0.00
MT (%)	33.81±5.82^{b}	57.77±13.01^{ab}	25.27±9.49^{a}	79.96±4.21^{b}	31.57±14.56^{b}	53.81±30.36^{ab}
MP (%)	26.85±3.93^{a}	52.97±11.01^{ab}	21.40±8.41^{a}	74.21±4.47^{b}	28.58±14.86^{a}	48.30±28.28^{ab}
SC (10^6/ml)	165.00±21.60^{*b}	215.00±38.72*^{b}	208.00±35.29*^{a}	385.50±285.05*^{a}	116.25±8.53*^{c}	162.25±41.52*^{c}
SV (%)	91.03±5.48^{a}	93.76±3.16^{ab}	87.13±11.43^{a}	97.12±2.15^{b}	86.32±5.02^{a}	91.03±3.62^{ab}
SIMP (%)	81.29±4.83*^{a}	88.16±1.71*^{b}	85.69±5.63^{a}	92.19±0.76^{a}	81.43±5.97^{a}	87.31±2.16^{b}
SA (%)	12.76±4.87^{b}	8.35±4.02^{a}	6.51±3.34^{a}	8.54±5.81^{a}	25.52±11.26*^{c}	7.82±8.25*^{a}

SV, Semen Volume; SC, semen color; TM, total motility; PM, progressive motility; SC =Sperm concentration; SV, sperm viability; SIMP= Sperm membrane plasma integrity; SA=Sperm abnormality.
Note: The asterisk (*) indicates a significant difference ($P<0.05$) between fractions 1 and 2 in the same male patient. a, b, and c indicate significant differences ($p<0.05$) between males in the same fraction group.

was no significant difference in sperm viability between the Malamute and Samoyed breeds (Table 1).

The semen criteria employed for the freezing process should have an initial sperm motility value of ≥70%, sperm viability of ≥80%, and a sperm concentration of ≥100 x10^6 sperm ml^{-1} (Qamar et al. 2020). The total and progressive motility results in this study were low. Ibrahim et al. (2024) also reported findings analogous to those of the present study, with progressive motility levels of less than 10%. The researchers employed two Pomeranian dogs and two Beagles in a Tris egg yolk extender. The VCL and VSL values indicate the ability of sperm to move faster and more efficiently towards the egg, whereas the ALH value reflects a wider head movement pattern and can be associated with readiness for hyperactive motility, an important phase in the fertilization process (Suarez and Ho 2003).

The uniformity of progressive motility and VAP values indicates that despite differences in trajectory speed, the direction of sperm movement between dog breeds is relatively similar. Therefore, this combination of parameters is closely related to fertility potential, as progressive motility is often reported as an important indicator of successful penetration of the zona pellucida. It is evident that sperm kinematics vary between species; for instance, an ALH value of >2.5 for bovine sperm is indicative of the capacity to penetrate cervical mucus. The ALH value for canine sperm reported by Ibrahim et al. (2024) was 1.3 µm.

However, the optimal equilibration time for canine semen remains unclear. The range of equilibration times varies from a minimum of 30 min (Kovalyová et al., 2024) to 40 min (Ibrahim et al., 2024) and a maximum of 6 h (Belala et al., 2016). Equilibration time is influenced by the extender used (Abe et al., 2020) and the dog breed (Berghe et al., 2018). As demonstrated in previous research (Lechner et al., 2022), there are variations in equilibration time that are contingent on the dog breed, extender type, and

Table 2. Sperm motility and kinematics of frozen dog semen with different equilibration times in Caniplus® extender

Variable	Equilibration times (hours)			p-value
	1	2	3	
Total Motility (%)	13.46±2.12^a	19.01±1.80^a	15.44±1.99^a	0.07
Progressive Motility (%)	8.47±1.83^a	13.50±1.59^a	9.99±1.55^a	0.05
Velocity Curvilinear (µm/s)	67.86±3.49^a	72.17±3.64^a	73.47±3.81^a	0.31
Velocity Average Path (µm/s)	27.19±0.95^b	29.87±1.48^{ab}	31.15±1.31^a	0.04
Velocity Straight Line (µm/s)	19.74±0.67^b	21.61±1.21^{ab}	23.56±0.97^a	0.01
Amplitude of Lateral Head Displacement (µm)	1.77±0.09^a	1.84±0.0^a	1.83±0.09^a	0.06

Note: Different superscript letters following numbers in the same row indicate significant differences ($p<0.05$).

Table 3. Effect of equilibration time on viability, plasma membrane integrity, and sperm abnormalities in dogs in Caniplus® extender

Variable	Equilibration times (hours)			p-value
	1	2	3	
Sperm viability (%)	92.50±0.70^a	91.39±2.15^b	91.54±2.54^b	0.03
Sperm plasma membrane integrity (%)	46.64±3.33^a	42.44±5.55^a	57.52±6.86^a	0.06
Sperm abnormality (%)	8.72±3.06^b	7.66±1.42^b	14.18±0.18^a	0.02

Note: Different superscript letters following numbers on the same row indicate significant differences ($p<0.05$).

individual cryotolerance. The quality of frozen dog semen is also influenced by supplementation with substances that reduce cell damage during freezing. The addition of 50 µM metformin enhances the quality of frozen canine semen following an equilibration period of 1.5 h (Grandhaye et al., 2020).

As demonstrated in Table 3, following one hour of equilibration, the viability of frozen dog semen was higher than that after two and three hours. The integrity of the sperm plasma membrane was unaffected by equilibration time after freezing. The presence of abnormalities in the sperm of canines treated with Caniplus® extender was found to be influenced by the equilibration time. Equilibration for a duration of three hours resulted in an increase in the number of sperm abnormalities (Table 3).

These results are consistent with those reported by Lechner et al. (2021), who found that equilibration for two hours at 5°C did not adversely affect frozen semen quality. Post-thawing sperm quality and optimal thawing time are associated with changes in the sperm plasma membrane that adapt to low temperatures and increased cryotolerance (Belala et al., 2016). The sperm plasma membrane undergoes lipid reorganization during equilibration to maintain membrane integrity (Rajoriya et al., 2016); however, dog sperm are known to be more sensitive to cooling, with plasma membrane damage occurring earlier than acrosomal damage (Ponglowhapan et al., 2004). Cooling and cryopreservation processes have been observed to induce alterations in the composition of the plasma membrane, which can lead to deterioration in sperm quality (Shahiduzzaman and Linde-Forsberg 2007).

The sperm plasma membrane is the primary site of structural and functional damage caused by cryopreservation (Belala et al., 2019). As Nguyen et al. (2019) explained, good sperm are characterized by intact sperm plasma membranes and acrosomes, as well as a high mitochondrial membrane potential compared to sperm motility. The equilibration process supports membrane reorganization, stabilization, and adaptation to reduce the effects of freezing and thermal shock, which have been shown to trigger cell death (Bencharif and Dordas-Perpinya, 2020).

Ponglowhapan et al. (2004) explained that dog sperm have subpopulations with different sensitivities to environmental changes, especially at low temperatures. A significant decrease in temperature from ambient temperature to 5°C during the equilibration stage has been shown to induce cold shock, a process that has been demonstrated to cause damage to the plasma membrane. As posited by Wicaksono and Arifiantini (2009), alterations in membrane configuration within the midpiece, occasioned by cold shock, result in the secretion of aspartate aminotransferase. This impedes the synthesis of energy essential for motility, thus diminishing sperm motility. Furthermore, canine sperm are sensitive to alterations in osmotic pressure (Ponglowhapan et al., 2004). Such fluctuations in temperature and osmotic pressure have been demonstrated to induce cellular stress, precipitating DNA damage, alterations in the plasma membrane structure, and an increase in reactive oxygen species (ROS) (Huang et al., 2022; Ugur et al., 2019). This, in turn, has been shown to result in a decline in semen quality.

As demonstrated in Table 3, following one hour of equilibration, the viability of frozen dog semen was higher than that after two and three hours. The integrity of the sperm plasma membrane was unaffected by equilibration time after freezing. The presence of abnormalities in the sperm of canines treated with Caniplus® extender was found to be influenced by the equilibration time. Equilibration for a duration of three hours resulted in an increase in the number of sperm abnormalities (Table 3).

The present study has certain limitations, primarily due to the relatively small number of canines used as semen sources, with only three dogs from three distinct breeds. This study investigated the correlation between dog breeds and the resistance of dogs to low temperatures, as well as differences in the lipid composition of sperm plasma membranes. This phenomenon can be attributed to the presence of cholesterol components within the membrane, which have been shown to play a regulatory role in membrane fluidity (Rajoriya et al., 2016).

It has been hypothesized that breeds with a higher proportion of polyunsaturated fatty acids may have more fluid membranes but are also more susceptible to lipid peroxidation, making them more prone to membrane damage during cryopreservation (Zhang et al., 2023). Sperm motility is the primary indicator of frozen semen that can be used in artificial insemination. As stated by Antonov and Ivanova (2023), the use of frozen semen with a sperm motility percentage of 30–50% is a viable option for artificial insemination of canines. Further research is required to enhance its quality, thereby enabling its utilization in the field of artificial insemination, for instance, through the incorporation of alternative extenders.

CONCLUSION

The study concluded that there were differences in several variables among semen fractions, and that the optimal equilibration time for canine semen in the Caniplus® extender was one to two hours.

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AUTHORS CONTRIBUTION

R.O. contributed as principal investigator, data collection, examinations, data analysis, and manuscript drafting. D.R.S. served as the second supervisor, contributing to the study design, research supervision, data collection, and data interpretation. T.P.N. contributed to the study design, research implementation, data collection, and the facilitation of the analysis facility. R.I.A. served as the first supervisor, participated in study design, research supervision, data collection, and data interpretation, and drafted the research and manuscript. All authors read, reviewed, and approved the final manuscript.

"The author declares that there are no conflicts of interest with any parties involved in this research."

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