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CHARACTERISTICS AND KINEMATICS OF RAM FROZEN-THAWED SPERM MOVEMENT WITH DIFFERENT LECITHIN TYPES

Karakteristik dan Kinematika Pergerakan Sperma Domba yang Dibekukan dengan Beberapa Jenis Lesitin

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ABSTRACT

This study evaluated the effectiveness of animal, plant, and synthetic lecithin-based extenders in preserving local Indonesian ram semen during cryopreservation. Post-thaw sperm quality was assessed using *Computer-Assisted Sperm Analysis* (CASA), focusing on motility, viability, membrane integrity, and kinematics. Data were analyzed using ANOVA and *Duncan's Multiple Range Test* (DMRT) at a 95% significance level. Results showed that sperm motility, viability, and membrane integrity were significantly higher ($P<0.05$) in the animal lecithin-based extender (53.66±1.33%, 66.45±2.50%, and 67.68±2.33%, respectively) compared to plant (51.84±0.48%, 62.98±1.94%, and 64.27±1.51%) and synthetic (50.14±0.22%, 60.72±0.81%, 62.44±1.37%) extenders. While kinematic parameters were not significantly different among groups, synthetic lecithin showed slightly higher values in velocity-related measures, whereas animal lecithin resulted in the most consistent improvement in overall sperm quality. All lecithin-based extenders met the Indonesian National Standard (SNI) for semen quality, and sperm kinematics remained within optimal fertilization values after cryopreservation.

Keywords: *Computer Assisted Sperm Analysis, Kinematics, Lecithin, Sperm*

ABSTRAK

Lesitin dari sumber nabati, hewani, dan sintetis telah diidentifikasi sebagai pengencer yang potensial untuk mencegah kerusakan pada sperma akibat proses pembekuan. Penelitian ini mengevaluasi efektivitas penggunaan pengencer berbasis lesitin hewani, nabati, dan sintetis dalam menjaga kualitas semen domba lokal Indonesia selama proses kriopreservasi. Kualitas sperma pasca-thawing dianalisis menggunakan *Computer-Assisted Sperm Analysis* (CASA), dengan fokus pada motilitas, viabilitas, integritas membran, dan parameter kinematik. Data dianalisis menggunakan *Analysis of Variance* (ANOVA) dan *Duncan's Multiple Range Test* (DMRT) pada tingkat signifikansi 95%. Hasil menunjukkan bahwa motilitas, viabilitas, dan integritas membran sperma secara signifikan lebih tinggi ($P<0,05$) pada penggunaan ekstender lesitin hewani (53,66±1,33%; 66,45±2,50%; dan 67,68±2,33%) dibandingkan dengan lesitin nabati (51,84±0,48%; 62,98±1,94%;

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64,27±1,51%) dan sintetis (50,14±0,22%; 60,72±0,81%; 62,44±1,37%). Meskipun parameter kinematik tidak menunjukkan perbedaan signifikan antar kelompok, lesitin sintetis menunjukkan nilai yang sedikit lebih tinggi pada parameter kecepatan, sementara lesitin hewani memberikan peningkatan kualitas sperma yang paling konsisten secara keseluruhan. Semua jenis ekstender lesitin memenuhi Standar Nasional Indonesia (SNI) untuk kualitas semen, dan parameter kinematik sperma tetap berada dalam kisaran optimal untuk fertilisasi setelah kriopreservasi.

***Kata kunci**: Computer Assisted Sperm Analysis, Kinematika, Lesitin, Sperma*

INTRODUCTION

Artificial insemination (AI) is one of the reproductive technologies that have great potential to increase productivity (Madrigali et al. 2021), genetic quality, and reduce the risk of spreading reproductive diseases in livestock (Patel et al. 2017). The effectiveness of AI can be improved by freezing semen, which allows for prolonged storage (Fannessia et al. 2015) and distribution to distant locations (Kumar et al. 2019). AI's success in ram is influenced by the cervix's complex anatomical structure (Siregar et al. 2023) and the quality of frozen semen used (Triyaningrum et al. 2024).

Sperm cryopreservation at -196 ℃ and ending with thawing at 37 ℃ affects sperm quality (Masir et al. 2017). The freezing process has adverse effects due to temperature changes, including decreased motility, viability, mitochondrial function, premature acrosome reaction, damage to membrane structure, and decreased DNA integrity (Ozimic et al. 2023). Damage due to cold shock is irreversible (Citraesti et al. 2021) and can be prevented by adding extenders containing cryoprotectants before the freezing process. Extenders generally contain permeable glycerol and non-permeable cryoprotectants such as lecithin (Ezzati et al. 2019).

Lecithin, or phosphatidylcholine, is a phospholipid that maintains the integrity of the sperm plasma membrane during freezing. Lecithin plays a role by coating the sperm plasma membrane to prevent damage during freezing (Tar et al. 2021). Lecithin can generally be categorized based on its source: plant, animal, and synthetic lecithin. Plant lecithins, such as those derived from soybeans, can replace animal lecithins in semen extenders with equivalent or better results in various species, including sheep (Chelucci et al. 2015). Liposome-based synthetic lecithin has also shown promising results in maintaining sperm motility after thawing in multiple animals (Kumar et al. 2015). Conventionally used egg yolk and milk-based extenders contain lipoproteins and lecithin that protect sperm from cold shock and provide the necessary nutrients to maintain sperm motility and viability (Tarig et al. 2017). However, the risk of microorganism contamination from animal-based lecithin still threatens AI's success (Bielanski 2012). Research on extenders based on lecithin sources needs to be conducted to evaluate and determine the most appropriate and effective type of lecithin-based extender for use in local ram to ensure optimal quality and fertility of ram sperm after cryopreservation.

MATERIALS AND METHODS

Study Design, Time, and Location

This study was experimental laboratory-based research conducted between July 2024 and January 2025 at the In Vitro Fertilization Laboratory, Reproduction and Obstetrics Division, School of Veterinary Medicine and Biomedical Sciences, IPB University and Reproduction Laboratory, Genomics, National Research and Innovation Agency, Cibinong.

Fresh Semen Collection

Semen samples were derived from 3 local rams ranging in age from 18-24 months with a weight of 30-40 kg obtained from the Reproductive Rehabilitation Unit, School of Veterinary Medicine and Biomedical Sciences, IPB University. Semen was collected using the artificial vagina method with an inner liner filled with water at 40-

42°C for four replicates. After collection, semen was immediately brought to the laboratory for evaluation. Furthermore, samples with sperm motility of more than 70% were immediately processed and used in this study.

Type and Characteristic of Lecithin-based Extenders

In this study, we use three types of extenders based on the lecithin source that were prepared following their procedure on the package. Each type of extender has the following characteristic:

1. Animal lecithin-based extenders, such as those formulated from egg yolk, are rich in phospholipids like phosphatidylcholine and cholesterol. These components play a crucial role in stabilizing sperm membranes during the cryopreservation process. The protective effect is attributed to the ability of these lipids to integrate into the sperm plasma membrane, thereby enhancing its resilience against cold shock and osmotic stress encountered during freezing and thawing (Moussa et al. 2002).
2. Plant lecithin-based extenders, such as those containing soybean-derived phospholipids, offer a plant-based alternative to egg yolk. They possess antioxidant properties and present a reduced risk of microbial contamination, while maintaining cryoprotective efficacy. These characteristics make soybean lecithin a suitable substitute in semen cryopreservation protocols (Layek et al. 2016).
3. Synthetic lecithin-based extenders, formulated with purified phospholipids and cholesterol analogs produced through chemical synthesis, offer consistency, shelf stability, and minimal biological contaminants. These chemically defined media have been shown to effectively replace egg yolk in semen cryopreservation without compromising post-thaw sperm motility and survival (Sicchieri et al. 2021).

Frozen Semen Processing

The dilution method used was one-step dilution. Fresh semen was divided into three parts, each diluted with three different types of extenders. Semen and extender were gently homogenized and packed into 0.25 mL mini-straws (Minitüb, Germany), with a mean of ±50 million sperm cells in each straw. The straws were then placed on a freezing rack and equilibrated for 3 hours in a refrigerator at 4-5 °C. It was freezing using styrofoam for 10 minutes over liquid nitrogen vapor and dipping in liquid nitrogen. Frozen semen was stored in containers for post-thawing evaluation 24 hours after freezing.

Sperm Motility and Kinematics

Frozen semen that has gone through the thawing process is taken as much as 4 µL, dripped on a warm object glass, and covered with a cover glass. Then, the sperm were observed on a microscope equipped with a warm stage at 37 °C. Sperm motility was evaluated using CASA Sperm Vision 3.7 (Minitüb, Tiefenbach, Germany) in four fields with ±500 sperm cells per field of view. Parameters measured included total motility (percentage of motile spermatozoa), motility, progressive (percentage of progressively moving spermatozoa), velocity curvilinear (VCL: µm/second), velocity straight line (VSL: µm/second) and velocity average path (VAP: µm/second), linearity (LIN: %), straightness (STR: %), wobble (WOB: %) dan beat cross frequency (BCF: Hz) and amplitude lateral head displacement (ALH: µm).

Sperm Viability

Sperm viability assessment using eosin-nigrosin staining. A sample of 4 µL was dripped on an object glass, and then 20 µL of eosin-nigrosin dye was added, homogenized, and a smear slide was made. The slides were dried using a heating table and then observed using a 10x40 magnification microscope. Live sperm are not colored (transparent), and dead sperm are colored (red head). Live and dead sperm cells are counted from at least 200 cells.

HOST (Hypo-Osmotic Swelling Test)

HOST method sperm membrane integrity test based on Pardede et al. (2022). The HOST solution was incubated in a water bath at 37 °C. Semen samples of as much

as 10 µL were put into 500 µL of HOST solution, homogenized, and incubated at 37 °C. Evaluation of frozen semen membrane integrity was carried out after 30 minutes of incubation by dripping 4 µL of the solution mixture on an object glass, covered with a cover glass, and observed under a 10x40 magnification microscope. Sperm with intact plasma membrane integrity shows a curled tail reaction, while a straight tail will characterize sperm with a damaged membrane. Reacted and unreacted sperm were counted from at least 200 cells.

Data Analysis

Sperm assessment is presented as mean and standard deviation. Quantitative data on all stages of the study are presented as percentages and standard deviations. All data were tested for normal distribution using the Shapiro-Wilk Test. Sperm quality data was tested using ANOVA at a 95% significance rate, then post hoc with DMRT.

RESULTS AND DISCUSSION

Frozen Semen Quality

The quality of frozen semen of local rams was observed based on three interrelated parameters: progressive motility, viability, and sperm plasma membrane integrity. The progressive motility of sperm from each extender showed significant differences ($P<0.05$), with the highest to lowest results being animal-, plant-, and synthetic-based lecithin extenders, respectively. Animal lecithin-based extenders also showed significantly better results than plant and synthetic lecithin extenders in maintaining the viability and integrity of the plasma membrane post-thawing semen (Figure 1). The plant (soybean) and synthetic (liposome) lecithin-based extenders used in the study were considered to have an equally good ability to maintain the quality of frozen semen of local rams during the cryopreservation process.

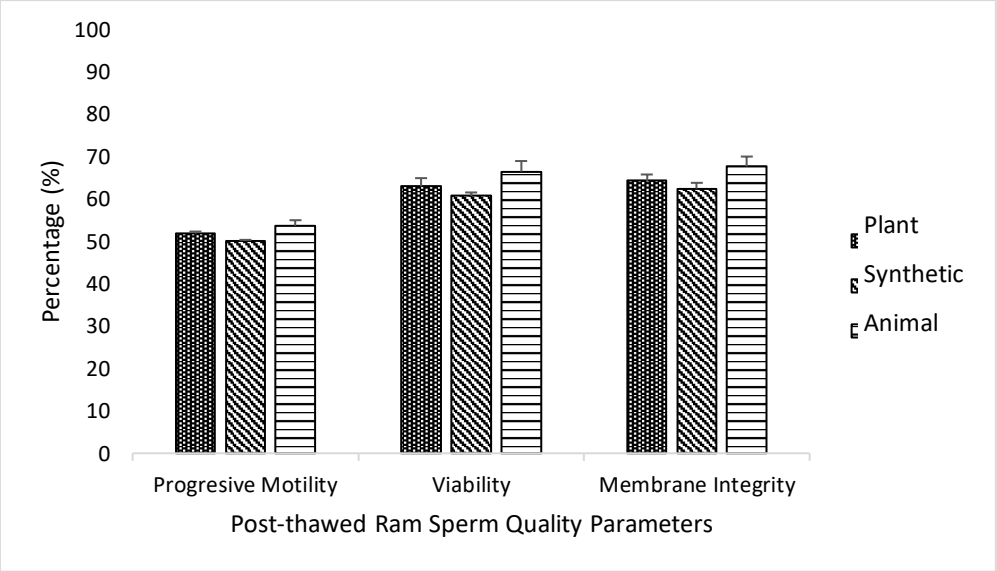


Figure 1 Characteristics of local ram frozen semen with various lecithins. Different letters in each parameter indicate significant differences ($P<0.05$).

The results of this study show that the type of lecithin-based extender significantly affects the post-thaw sperm quality of local rams. The animal lecithin-based extender resulted in the highest overall quality in terms of progresive motility, viability, and membrane integrity, supporting its suitability for semen cryopreservation. This superior performance is likely due to the presence of egg yolk-derived phospholipids, cholesterol, and low-density lipoproteins (LDL), which help stabilize the sperm plasma membrane during freezing and thawing (Swelum et al. 2019; Nikitkina et al. 2020; Aybazov et al. 2021). These components are known to interact with sperm membranes, reduce the effects of cold shock, and maintain viability (Citraesti et al. 2021).

In contrast, plant lecithin and synthetic lecithin extenders showed slightly lower

post-thaw quality. The reduced motility and viability observed with plant lecithin may be related to its higher viscosity, which can hinder sperm movement and cryoprotectant diffusion (Forouzanfar et al. 2010). Although both animal and plant lecithin contain phospholipids, their mechanisms of action differ. LDL in egg yolk includes membrane-binding proteins that enhance the protective interaction with sperm membranes, while soybean-derived LDL lacks these proteins (Nguyen et al. 2019). The lecithin content in egg yolk is also higher than that in soybeans, further contributing to its cryoprotective effectiveness (Zhao et al. 2023). While consistent and free of biological contaminants, synthetic lecithin lacks the natural cofactors and structural diversity of egg yolk components, which may explain its lower performance. These findings highlight the importance of lecithin origin and composition in determining sperm cryotolerance.

Frozen Semen Kinematics

Sperm kinematics are essential parameters for fertility analysed using CASA, such as velocity curvilinear (VCL), velocity straight line (VSL), velocity average path (VAP), linearity (LIN), straightness (STR), wobble (WOB), beat cross frequency (BCF), and amplitude lateral head displacement (ALH).

The analysis of sperm kinematic parameters revealed minimal variation across the three lecithin-based extenders (Table 1). VCL (curvilinear velocity), VAP (average path velocity), and VSL (straight-line velocity) were slightly higher in sperm frozen with synthetic lecithin (93.61 ± 12.38 µm/s, 63.73 ± 6.53 µm/s, and 46.55 ± 4.55 µm/s, respectively) compared to those frozen with plant and animal lecithin. However, the differences were not statistically significant, and overall values remained within the optimal fertilization range. Parameters related to movement precision (LIN (linearity), STR (straightness), and WOB (wobble)) were consistent across all groups, suggesting similar directionality and beat patterns of sperm movement. BCF (beat cross frequency) was highest in the plant group (29.03 ± 1.31 Hz), while ALH (amplitude of lateral head displacement) was highest in the animal group (4.26 ± 0.27 µm), indicating slightly more lateral head motion in sperm extended with animal-based lecithin. These findings suggest that while kinematic values across all extenders were generally comparable, the animal-based lecithin showed more favorable results in other sperm quality parameters (e.g., motility and viability), making it the most suitable extender overall.

Table 1. Sperm kinematics of local ram frozen semen post thawing in different types of lecithin.

Parameters	Lecithin types		
	Plant	Synthetic	Animal
VCL (µm/s)	92.09±13.16	93.61±12.38	87.98±7.38
VAP (µm/s)	62.80±6.38	63.73±6.53	60.30±4.07
VSL (µm/s)	45.65±4.48	46.55±4.55	42.56±4.38
LIN (%)	49±0.03	49±0.01	48±0.02
STR (%)	72±0.01	72±0.00	70±0.03
WOB (%)	68±0.03	68±0.02	68±0.02
BCF (Hz)	29.03±1.31	28.66±0.57	27.48±1.73
ALH (µm)	3.93±0.37	4.02±0.52	4.26±0.27

VCL = *velocity curve line*; VAP = *velocity average path*; VSL = *velocity straight line*; LIN = *linearity*; STR = *straightness*; WOB = *wobble*; ALH = *amplitude lateral head displacement* dan BCF = *beat cross frequency.*

High progressive sperm motility can improve sperm fertilization ability, which can be assessed using sperm kinematics parameters (Arif et al. 2022). The sperm kinematics values obtained showed that the three types of lecithin in each extender met the criteria of good sperm for fertilization. This is in line with the statement Gungor et al. (2018), which states that sperm must have a VCL value > 80 µm/s and must have

VAP and VSL values > 40 µm/s for sperm to penetrate the ovum successfully. The higher the post-thawing VCL and VSL values, the better spermatozoa motility for the fertilization process. (Singh et al. 2018). Parameters linearity (LIN), straightness (STR), and wobble (WOB) were analyzed to assess the efficiency and precision of sperm movement on sperm quality (Sarastina et al. 2007). LIN measures how straight the sperm movement trajectory is, STR measures the accuracy of straight sperm movement, and sperm movement path stability is measured by wobble (WOB) value. Linearity values >35% and straightness >60% indicate progressive motility (Selvaraju et al. 2016; González-Abreu et al. 2017).

Other essential parameters in CASA analysis are beat cross frequency (BCF) and amplitude lateral head displacement (ALH). ALH measures the vigor and energy of sperm movement, and BCF measures the rhythm and speed of sperm movement to ensure the sperm can reach the egg. The ALH and BCF values of frozen semen sperm with different lecithin source extenders were found to meet the criteria of optimal movement. BCF values >20 Hz and ALH between 2.5 - 6.5 µm indicate that spermatozoa movement is optimal and has high fertility potential (Belala et al. 2019). An ALH value of >4.5 µm is required for sperm to pass through cervical mucus (Shojaei et al. 2012). The sperm kinematics value obtained in this study is similar to the value obtained by Yotov et al. (2021) with VCL >70 µm/s, VAP >45 µm/s, VSL > 25 µm/s, ALH < 5 µm and BCF > 15 Hz. VAP, VCL, and VSL values showed a high correlation with sperm fertility and pregnancy rates obtained after artificial insemination (Nagy et al. 2015). ALH values >7 µm, LIN >50%, and VCL >80 µm indicate a sperm state transition from progressive motility to hyperactivity, which is unfavorable to be found in post-thawed sperm (Tanga et al. 2021).

Plant (soy) or synthetic (liposome) lecithin-based extenders have the same ability as cryoprotective as animal (egg yolk) lecithin-based extenders (Swelum et al. 2019). The protective mechanism of animal, plant, and synthetic lecithin-based extenders is phosphatidylcholine (Nadri et al. 2019). Phosphatidylcholine is the main component of lecithin, ~73.0%, which plays a role in the integrity of the plasma membrane (Zhao et al. 2023). Phospholipids, as a significant membrane component, also play a physiological role in lowering the freezing point so that ice crystal formation can be avoided and reduce the potential for mechanical damage to the sperm membrane. Egg yolk-based (animal), soy-based (plant-based), and liposome-based (synthetic) lecithin work as a protective outer layer of sperm and replace phospholipids that are lost or damaged in the sperm membrane during the freezing process (Mehdipour et al. 2018).

CONCLUSION

The sperm quality of local ram frozen with animal lecithin-based extenders was significantly better than that with plant and synthetic lecithin-based extenders. The animal lecithin-based extender is considered the most suitable option for freezing the semen of local rams due to its superior cryoprotective effects. Kinematics of frozen semen of local ram with animal, plant, and synthetic lecithin-based extenders showed optimal fertilization values after cryopreservation. Based on these results, the animal-based lecithin extender is the most effective for preserving sperm quality post-thaw. We suggest investigating the molecular mechanisms behind the protective effects of different lecithin types and assessing their influence on fertility outcomes under in vivo conditions.

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