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EFFECTIVENESS OF VARIOUS COMMERCIAL EXTENDERS IN CRYOPRESERVING SEMEN OF LOCAL INDONESIAN RAMS

Efektivitas Berbagai Pengencer Komersial dalam Kriopreservasi Semen Domba Jantan Lokal Indonesia

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ABSTRACT
Indonesian local sheep represent a particular species of sheep reared by farmers on small-scale farms. Semen freezing can be performed using either a homemade or commercial extender. The present study evaluated the effectiveness of several commercial extenders in freezing Indonesian local sheep. Semen was collected from three mature and healthy rams using an artificial vagina and evaluated. It was determined that only samples exhibiting sperm motility levels of greater than 70% would be included in the study. Subsequently, the semen from each ram was divided into three portions and diluted with Andromed, Sterydil, and OptiXcell, respectively. The frozen semen was then evaluated 24 hours post-freezing. The thawing process was conducted individually at 37°C for 30 seconds. The evaluation encompassed a range of metrics, including sperm motility, viability, and the presence of any abnormalities, in addition to the recovery rate. The results indicated that sperm frozen in Optixcell and Sterydil extenders exhibited superior motility, viability, and recovery rate than Andromed. The study also demonstrated no interaction between individual Ram and extender, and no differences were found between individuals. The study concluded that Optixcell and Sterydil extenders effectively froze Indonesian local sheep semen.

Keywords: *Commercial extenders, Frozen semen quality, Indonesian local sheep*

ABSTRAK
Domba lokal Indonesia adalah spesies domba yang dipelihara oleh peternak di peternakan skala kecil. Pembekuan semen dapat dilakukan menggunakan pengencer buatan sendiri atau pengencer komersial. Penelitian ini bertujuan untuk menguji efektivitas beberapa pengencer komersial untuk pembekuan semen domba lokal Indonesia. Semen dikoleksi dari tiga ekor domba dewasa yang sehat. Koleksi semen dilakukan menggunakan vagina buatan. Semen dievaluasi dan hanya sampel dengan motilitas lebih dari 70% digunakan dalam penelitian ini. Semen dari masing-masing domba dibagi menjadi tiga bagian dan diencerkan dengan Andromed, Sterydil, dan OptiXcell. Semen yang telah diencerkan dikemas dalam ministraw 0,25 ml, diekuilibrasi dan dibekukan. Pengujian kualitas semen beku dilakukan 24 jam setelah pembekuan. Semen di-*thawing* satu persatu pada suhu 37°C selama 30 detik. Semen dikeluarkan dari straw dan dipindahkan ke tabung mikro. Evaluasi semen meliputi motilitas dan viabilitas dan abnormalitas sperma, dan nilai *recovery rate*. Hasil menunjukkan sperma yang

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dibekukan dalam pengencer Optixcell dan Sterydil menunjukkan motilitas, viabilitas, dan *recovery rate* yang lebih baik dibandingkan dengan Andromed. Penelitian ini juga menunjukkan tidak ada interaksi antara individu pejantan dan pengencer, serta tidak ditemukan perbedaan antar individu. Penelitian ini menyimpulkan pengencer Optixcell dan Sterydil efektif dalam pembekuan semen domba lokal Indonesia.

Kata Kunci: *Domba lokal Indonesia, Kualitas semen beku, Pengencer komersial*

INTRODUCTION

Local sheep are a unique source of genes that can be used to improve sheep in Indonesia through crossbreeding between local and imported sheep. These sheep have a very strategic position in society because they have economic, social, and cultural functions (Sumantri et al. 2007). These sheep are raised by farmers on small-scale farms. The characteristics of these sheep include small stature, slow maturity, coarse hair, and relatively small meat yield. Breeding of the local sheep industry has been primarily based on natural mating. Artificial insemination (AI) of local sheep is still rare. Therefore, sheep inbreeding is very high. Many local male sheep have the potential to be used as studs, and their semen can be used for AI.

The effectiveness of AI in sheep is influenced by several endogenous and exogenous factors, which can vary considerably between individuals and breeds (Sukmawati et al., 2014; Indriastuti et al., 2020; Sophian et al., 2024). Artificial insemination has not been as widely used in sheep breeding as in other species, mainly due to the difficulties in achieving consistent and satisfactory fertility results (Alvarez et al., 2019). Many factors, such as the complex anatomy of the ovine cervix, semen preservation, animal management, husbandry system, health, physiological status of the sheep, semen collection technique, environmental factors, human factors, sperm concentration, or semen composition, influence the successful use of AI in the ovine species (Spanner et al., 2024).

Artificial insemination and semen cryopreservation technologies are paramount in large-scale farming operations. Artificial insemination is an essential tool in the official genetic improvement program for the local Indonesian breed (Darmawan et al. 2023). Semen cryopreservation is a widely used reproductive technology in animal husbandry, especially in cattle, and is a key component of AI technology.

Semen cryopreservation allows AI to overcome time and distance limitations, use semen from high-quality breeding rams, reduce breeding costs, and increase reproductive efficiency. In addition, semen cryopreservation can help reduce the spread of disease and has important implications for the conservation of endangered species (Zhang et al., 2024). However, changes in sperm quality during this process have been reported to reduce fertility with increasing storage time. Furthermore, the technique is rendered less effective because livestock fertility is subject to variation, the economic returns are low, and the quality of frozen and stored sperm is subject to deterioration (Bollwein & Malama, 2023). Semen cryopreservation technology, involving semen extenders and freezing methods, is critical as it can directly affect the cryopreservation efficacy of semen (Ugur et al., 2019). During the cooling, freezing, and thawing process, the extender provides energy to the sperm, reduces freezing damage, and increases semen volume (Zhang et al., 2024).

There are two categories of semen diluents that are currently used in artificial insemination centres in Indonesia (Arif et al 2022): homemade and commercial extender. Commercial having different lecitin sourse, such as extenders based on animal lipoproteins, extenders based on vegetable lipoproteins and liposome (Riwu et al., 2023). The animal source is mainly egg yolk, while the vegetable source is soya lecithin. In addition, the use of whole or skimmed milk for semen preservation has been documented (Miguel-Jimenez et al., 2020). The introduction of plant protein sources as a replacement for animal lipoproteins for semen cryopreservation has the dual benefit of

avoiding the potential diseases that may result from the use of animal products and maintaining the biosecurity benefits associated with preventing the spread of transboundary diseases (Bustani & Baiee, 2021). Considering the variety of commercial diluents on the market and the lack of research on their use for freezing sheep semen, this study is justified. It used several commercial diluents, each with different lecithin sources, all offered at similar prices. This study aims to compare the efficacy of different commercial extenders on the quality of frozen Indonesian local sheep semen.

MATERIALS AND METHODS

Animal Ethics Approval

Ethical approval was not required to conduct this study as the collection of sheep semen was carried out according to standard procedures without harming the animals, and there was no treatment of the sheep.

Time and Place

This study was conducted from January to April 2024 at the Reproductive Rehabilitation Unit Laboratory, Division of Reproduction and Obstetrics, School of Veterinary Medicine, and Biomedical Sciences (SKHB) IPB University, Bogor.

Semen source

The semen donors were three Indonesian local rams from the Reproductive Rehabilitation Unit Laboratory, Division of Reproduction and Obstetrics, School of Veterinary Medicine, and Biomedical Sciences (SKHB), IPB University. They were between two and three years old, with a body weight of 25 and 30 kg and a scrotal circumference of 15 and 20 cm.

Preparation of extenders

The present study used a commercial extender, namely Andromed, Steridyl, and OptiXcell. These three extenders were then diluted using distilled water according to the procedure stated on the packaging. The Andromed extender was diluted at a ratio of 1:4, the Steridyl extender was diluted at a ratio of 1:1.5, and the OptiXcell extender was diluted at a ratio of 1:4. At semen collection time, a portion of each extender was maintained on water bath at 35 ◦C (Arifiantini et al., 2024)

Semen Collection and Evaluation

Semen was collected using an artificial vagina once a week. The tube containing the semen was then kept in a water bath at 35°C. Semen variables, including semen volume, sperm motility, sperm concentration (10^6/ml), sperm viability, and plasma membrane integrity, were estimated according to the methodology proposed by Arifiantini et al. (2024). Semen volume (ml) was determined by measuring the graduated collection vessel directly.

The mass movement of spermatozoa was examined by dripping 10 µl of fresh semen onto a slide and then examined under a microscope at 10x magnification. The assessment criteria encompassed the following categories: fast-moving semen, characterized by numerous mass waves and a dark and thick appearance (+++); slow-moving semen, marked by a few thin mass waves (++); and thin mass waves (+), indicating a lack of movement. The absence of mass waves was categorized as (0) (Arifiantini, 2012)

The motility of sperm was evaluated subjectively by observing the progressive movement of sperm. Four µl of semen was dripped onto a slide, followed by the addition of 40 µl of saline solution. The cover slips were then placed over the semen. Observations were conducted under a microscope, utilizing a magnification of 10 x 40. The assessment criteria encompassed the progressive movement of sperm, which was evaluated subjectively. The range of values is from 0 to 100%.

The sperm concentration ($\times 10^6$ sperm/ml) was determined using a photometer (SDM 6, Minitube, Germany). Sperm viability and morphology are evaluated concurrently. A semen sample, diluted with eosin nigrosine dye at a ratio of 1:10, was prepared as a smear and placed on a heating table to facilitate desiccation. Observations were made in 10 fields of view or until the number of cells reached 200 cells. The viability of sperm was determined by their ability to absorb the eosin nigrosine dye, with live sperm exhibiting transparent heads and

dead cells demonstrating a dark coloration. The sperm were enumerated by dividing the number of viable sperm by the total sperm count, multiplied by 100%.

The intact plasma membrane of sperm was tested using a hypo-osmotic swelling test (HOS test) solution. The HOS solution is a mixture of 7.35 g of sodium citrate and 13.52 g of fructose dissolved in 1000 ml of distilled water, yielding an osmolarity of 150 mOsm. A semen sample of 10 µl was transferred into a micro-tube containing 1 ml of HOS solution, which was then incubated in a water bath at 37°C for 30 minutes. Four µl of the solution was then deposited onto a slide glass and covered with a cover slips. The sperm that reacted and those that did not respond to the HOS solution were counted in 10 fields of view, totaling 200 cells. The sperm was calculated by dividing the number of sperm that reacted with the total sperm count by 100%.

Semen freezing process.

In this study, only semen samples exhibiting motility levels greater than 75% were considered for analysis. Each ejaculate was divided into three aliquots of equal volume, and each one was diluted using one step with a different commercial extender. The diluted semen was then loaded into 0.25-ml mini straws, which were then arranged on a freezing rack and equilibrated at a temperature of 5°C for three hours. After this equilibration, the semen was frozen in a conventional manner using a polystyrene box with the following dimensions: height x length x width (37.5 × 25.5 × 20.5 cm). The distance between the liquid nitrogen and the straw was 6 cm, and the freezing process was conducted in liquid nitrogen vapour for 10 minutes. The frozen semen was subsequently stored in liquid nitrogen containers for further testing.

Evaluation of Post-thawed semen quality

Following a 24-hour s period, the frozen straws were thawed for 30 seconds at 37° C in a water bath before undergoing subsequent analyses. The present study examined the quality of frozen semen samples using a method that focused on sperm motility, sperm viability, and sperm abnormalities. The testing procedure was almost identical to that of fresh semen, with a few modifications. Firstly, sperm motility was not diluted. Secondly, the ratio of eosin nigrosine for sperm viability and morphology was one-part semen to two-part dye. Finally, the scoring and counting method was the same as for fresh semen.

Statistical Analysis

The data were analysed using SPSS (25.0 version, IBM, Armonk, NY, USA, 2017) statistical software. One-way ANOVA was used to evaluate extenders and individual factors as treatment combinations and their interaction effect on sperm quality. When ANOVA revealed a significant effect, the values were compared using Duncan's multiple-range test. Statistically significant differences were defined as those with a p-value less than 0.05. The alterations observed in sperm were expressed as a recovery rate (post-thaw sperm cells/fresh sperm cells ×100, as described by Arifiantini et al., 2010). All variables were expressed as mean ± SEM.

RESULTS AND DISCUSSION

The statistical test results indicated that no statistically significant differences were observed in the quality of fresh semen among the rams in all the test variables. The semen volume was 0.94 – 1.12 ml, mass activity 3, with sperm concentration of 2,697.20±360.19 to 3,350.90±459.59 $x10^6$. This value is in the range of ram semen characteristics (Carvajal-Serna et al., 2018; Zaher et al., 2020; Marin et al., 2023).

This study demonstrated significant differences in the percentage of sperm motility and the recovery rate of Indonesian local sheep in different extender groups. Sperm frozen in Sterydil and OptiXcell extenders exhibited superior sperm motility and recovery rate to Andromed (Table 1). These results were consistent across all rams utilised in this study.

Table 1. Percentage of sperm motility and recovery rates of Indonesian local rams with different extenders groups (mean ± SEM)

Ram number	Extenders	Sperm Motility (%)		Recovery rate (%)
		Fresh semen	Post thawing	
1	Andromed	76±2.45	42.45±1.18[b]	54.94±1.45[b]
	Sterydil		50.69±0.85[a]	66.25±1.11[a]
	OptiXcell		49.07±1.04[a]	63.24±1.46[a]
2	Andromed	81.5±2.18	43.50±1.15[b]	53.37±1.4[b]
	Sterydil		52.53±1.37[a]	64.46±1.68[a]
	OptiXcell		51.77±1.11[a]	63.53±1.36[a]
3	Andromed	80.0±1.58	43.14±0.88[b]	53.93±1.10[b]
	Sterydil		48.95±0.81	61.19±1.02[a]
	OptiXcell		47.80±1.34	59.75±1.68[a]

Note: The data are presented as the mean ± standard error mean (SE) of 5 ejaculations and five straws each ram (n =25), and Means in the same row with different superscripts differ significantly ($p<0.05$)

The present study's findings demonstrated that the outcomes of the evaluation of sperm viability and the recovery rate post-thawing exhibited results analogous to those observed for sperm motility. Sperm diluted with Sterydil and OptiXcell exhibited higher levels of viability than those diluted with Andromed (Table 2).

Table 2. Percentage of sperm viability and recovery rates of Indonesian local rams with different extenders groups (mean ± SEM)

Ram number	Extenders	Sperm viability (%)		Recovery rate (%)
		Fresh semen	Post thawing	
1	Andromed	83.00	48.47±1.90[b]	58.40±2.29[b]
	Sterydil		59.62±1.25[a]	71.83±1.51[a]
	OptiXcell		58.69±1.77[a]	70.71±2.14[a]
2	Andromed	84.67	43.50±1.15[b]	53.37±1.4[b]
	Sterydil		52.53±1.37[a]	64.46±1.68[a]
	OptiXcell		51.77±1.11[a]	63.53±1.36[a]
3	Andromed	88.34	52.65±1.80[b]	59.59±2.04[b]
	Sterydil		60.68±1.47[a]	68.69±1.67[a]
	OptiXcell		64.86±1.34[a]	73.42±2.84[a]

Note: The data are presented as the mean ± standard error mean (SE) of 5 ejaculations and five straws each ram (n =25), and Means in the same row with different superscripts differ significantly ($p<0.05$)

The study demonstrated no interaction between individual rams and the extender employed (Tabel 3). This finding can be interpreted to indicate that all rams exhibited optimal semen quality, as determined by the quality of their fresh semen. The rams utilised in the study were young and demonstrated high productivity. They were raised under optimal feeding and husbandry management, which indicates their status as 'good' rams due to their young age and superior semen quality.

Table 3. Interaction of individual rams with extenders

Variable	Interactions value
Sperm motilities (%)	0.69
Sperm viabilities (%)	0.68
Sperm abnormalities (%)	0.37

Note: The data from three rams, five ejaculations and five straws from each collection

The statistical analysis findings demonstrated that the type of extender utilised influenced the percentage of sperm motility and viability. However, sperm abnormality was unaffected by the type of extender employed (Table 4).

Table 4. Effect of extender on the quality of frozen Indonesian local rams' semen

Variable	Andromed	Sterydil	OptiXcell	P value
Sperm motility (%)	42.40±0.59[b]	50.29±0.59[a]	49.32±0.68[a]	0.00
Sperm viability (%)	50.17±1.09[b]	61.09±0.95[a]	62.29±1.31[a]	0.00
Sperm abnormality (%)	3.78±2.64	5.49±0.84	5.95±0.49	0.61

Note: The data from three rams, five ejaculations and five straws from each collection

The findings indicated that individual ram had a substantial impact on sperm viability. However, sperm motility and the abnormality of frozen semen were not influenced by individual factors (Table 5).

Table 5. Effect of individual rams on the quality of frozen semen

Variable	Number of Rams			P Value
	1	2	3	
Sperm motility (%)	46.72±0.62	48.56±0.62	46.73±0.62	0.06
Sperm viability (%)	55.59±1.19b	59.89±1.19a	58.06±1.19ab	0.03
Sperm abnormality (%)	4.19±1.62	6.98±1.62	4.04±1.62	0.35

Note: The data from 5 ejaculations and five straws in each collection

DISCUSSION

Tables 1 and 2 show sperm motility and viability decrease by approximately 30-35% from fresh to thawed semen. This decrease is due to dilution, equilibration, and thawing. Damage to the sperm membrane and extensive shrinkage or swelling may occur due to changes in osmotic pressure resulting from the addition of extender. It is recommended that freezing of sheep semen be undertaken using glycerol below 6%. It is detrimental to sperm upon thawing to add glycerol above 6% (Graham et al. 1978). A drop in temperature from room temperature to equilibration temperature, freezing and thawing cause stress to the sperm. The stress that occurs will undoubtedly affect the plasma membrane, causing reactive oxygen species to be produced, and will, of course, directly affect the cells.

The recovery rates of the motility range from 53 to 66%, lower than in a previous study reported by Anel et al. (2003), who reported in Churra ram sperm post-thaw sperm motility of 64% with recovery rates of 76%. The freezing technique influences the value of sperm motility and recovery rates. This study uses a one-step conventional freezing technique, and the result is comparable with Jha et al. (2019). Furthermore, Jha et al. (2019) reported that the post-thaw and recovery rates are higher if two or three freezing are employed.

This study also found no interaction between the extender and individual factors (Table 3). The only influencing factor was the extender used. Andromed extender showed the lowest sperm motility and viability values ($P<0.05$) compared to Sterydil and OptiXcell (Table 4). Sperm abnormalities were not affected by the type of extender used ($P>0.05$). Andomed extender contains soya lecithin, whereas Sterydil contains egg yolk lecithin. Egg yolk has been a customary ingredient in semen extenders to protect spermatozoa against cold shock and seminal plasma proteins. In addition, it protects the processes of freezing and thawing. The low-density lipoproteins (LDL) contained within egg yolk act at the level of the cell membrane (Moustacas et al., 2011). Egg yolks in Steridyl's are pre-sterilised, making them safer, whereas egg yolks used in homemade extenders are feared to contain unwanted microbes. Optixcell contains liposomes that work similarly to lecithin and are more stable than soy lecithin.

The study found that the quality of frozen semen in soya lecithin was inferior to that in Steridyl. This difference can be attributed to the fact that both egg yolk's low-density lipoproteins and soya lecithin, which is recognized for safeguarding the sperm plasma membrane, are primarily made up of phospholipids. Nonetheless, their modes of action differ. Soya lecithin is composed almost entirely of phospholipids, while the low-density phospholipids in egg yolk include both phospholipids and proteins (Nguyen et al. 2019).

Table 5 shows that this study found that there was a variation in sperm viability between rams. Rams number two has a sperm viability higher than Rams number one. However, no variation was found in sperm motility and sperm abnormalities. Breed variation, individual variation previously reported by several researchers (Sukmawati et al. 2014; Indriastuti et al. 2020; Sophian et al. 2024). The present study is limited because it was conducted using a sample size of only three males. In contrast, other researchers have utilised larger sample sizes, potentially obscuring individual variations in the sheep population under study.

A notable finding in the present study was the observation of sperm abnormality, which exhibited no significant differences in fresh semen, was unaffected by extender, and was not influenced by individual factors. A 1.15% increase in the number of abnormal sperm was recorded, both in fresh semen and following freezing. This increase is considered negligible and can be attributed to the efficacy of the extender, freezing, and thawing techniques employed.

Cryopreservation of semen has several advantages apart from long-term conservation, such as cost reduction, easy storage, the prevention of genetic drift, and the promotion of genetic resource exchange and transportation worldwide (Fernandez et al., 2023). Although cryopreservation has many positive aspects, the cooling process also involves fast structural changes in sperm, which can promote different cell damages, such as reducing the membrane fluidity or altering lipid and protein organisation (Ugur et al., 2019).

However, the current trend to reduce substances of animal origin has encouraged the search for alternatives to replace egg yolk as a cryoprotectant. The use of egg yolk as a cryoprotectant has been questioned due to its potential contamination, particularly with bacteria. One of these alternatives is the evaluation of substances. One of these alternatives is the evaluation of substances of plant origin as potential replacements for egg yolk and non-permeable cryoprotectants in freezing media (Murphy et al., 2018). Soya lecithin is one of the alternatives to this compound, which has a mechanism of action like that of LDLs (Vidal et al., 2013) but with greater biosafety and lower cytotoxicity (Bousseau et al., 1998).

However, the results of replacing egg yolk with soya lecithin in the freezing of bull semen are contradictory. Sperm motility is greater after thawing than when egg yolk is used (Aires et al., 2003); soya lecithin is more effective than egg yolk (Crespilho et al., 2012). Another study demonstrated that an extender containing egg yolk is better than soy lecithin (Maleki et al., 2023).

The utilisation of liposomes for ram semen freezing remains restricted. Nevertheless, the present study demonstrates that OptiXcell is efficacious in this regard. The results of this study support previous reports using extenders based on liposomes are more efficient in protecting sperm viability and membrane integrity than those based on egg yolk (Luna-Orozco et al., (2019) and enhanced acrosomal and plasma membrane integrity (Miguel-Jimenez et al., 2020)

CONCLUSION

The study concluded that both OptiXcell and Steridyl extenders are effective for freezing local sheep semen, as evidenced by superior motility, viability, and sperm recovery rate after thawing in comparison to Andromed.

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