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The Effect of Egg-White Gradient and Incubation Time on Sex Separation for Semen Quality in Dorper Rams

(*PENGGUNAAN GRADIEN PUTIH TELUR DAN LAMA INKUBASI PADA SEPARASI SEKS TERHADAP KUALITAS SEMEN DOMBA DORPER*)

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ABSTRACT

Improving Dorper sheep meat production efficiency through sperm sex separation (sexing) technology using egg white gradients is an economical alternative, yet it is often hindered by the decline in post-freezing semen quality due to cold shock. This study was aimed to evaluate the effectiveness of combining egg white medium concentration gradients and incubation times on the quality of liquid and frozen-thawed semen. Using fresh semen from two Dorper rams (aged 24 months), this study employed a 2x3 Factorial Randomized Block Design (RBD) with four replications. Treatments consisted of two gradient levels (15:30% and 20:35%) and three incubation times (30, 45 and 60 minutes). The obtaimed data were analyzed using Analysis of variane and continued with Duncan's multiple range test if there were significantly differences betrweem treatments. Results showed that incubation time significantly affected ($P<0.05$) motility, viability and concentration. Specifically, the 15:30% gradient with 45 minutes of incubation proved to be the most optimal, yielding 51.25% post-sexing motility and 53.88% viability. This superiority persisted through the post-thawing stage, where this treatment recorded the highest motility (12.50%) and viability (14.25%). However, frozen semen values were generally low. Conversely, 60-minute incubation significantly reduced quality due to metabolic exhaustion, while the 20:35% gradient had excessive viscosity that hindered sperm penetration, drastically lowering the final concentration to 4.75×10^7 cells/mL. Although fluctuations occurred, abnormality rates across all treatments remained below the 20% threshold. It is concluded that freezing techniques require evaluation, using a 15:30% egg white gradient with 45 minutes of incubation at 37°C is the best protocol in this study recommended for Dorper sheep sex separation, as it maximally maintains membrane integrity and sperm survival in both liquid and frozen phases.

Keywords: sexing; Dorper rams; egg white; concentration gradient; incubation time

ABSTRAK

Peningkatan efisiensi produksi daging domba dorper melalui teknologi separasi seks spermatozoa (*sexing*) menggunakan gradien putih telur merupakan alternatif ekonomis, namun seringkali terkendala oleh penurunan kualitas semen pascapembekuan akibat kejutan beku atau *cold shock*. Penelitian ini bertujuan mengkaji efektivitas kombinasi gradien konsentrasi medium putih telur dan lama waktu inkubasi terhadap kualitas semen cair dan semen beku (*post-thawing*). Menggunakan semen segar dari dua ekor kambing pejantan dorper (usia 24 bulan), penelitian ini menggunakan Rancangan Acak Kelompok (RAK) pola faktorial 2x3 dengan empat ulangan. Perlakuan terdiri atas dua taraf gradien (15:30% dan 20:35%) serta tiga waktu inkubasi (30, 45 dan 60 menit). Data yang diperoleh dianalisis menggunakan uji sidik ragam dan pada perlakuan yang berbeda nyata dilannjutkan dengan uji jarak berhanda Duncan. Hasil penelitian menunjukkan bahwa lama inkubasi berpengaruh nyata ($P<0,05$) terhadap motilitas, viabilitas dan konsentrasi. Secara spesifik, gradien 15:30% dengan inkubasi 45 menit terbukti paling optimal, menghasilkan motilitas ascaseksing 51,25% dan viabilitas 53,88%. Keunggulan tersebut bertahan hingga tahap *post-thawing*, dan perlakuan tersebut menghasilkan motilitas (12,50%) dan viabilitas (14,25%) tertinggi. Namun, nilai semen beku secara umum tergolong rendah. Sebaliknya, inkubasi 60 menit menurunkan kualitas sperma secara signifikan akibat kelelahan metabolisme (*metabolic exhaustion*), sementara gradien 20:35% memiliki viskositas terlalu tinggi yang menghambat penetrasi sperma, menurunkan konsentrasi akhir secara drastis menjadi $4,75 \times 10^7$ sel/mL. Meskipun terjadi fluktuasi, tingkat abnormalitas pada seluruh perlakuan tetap terjaga di bawah ambang batas 20%. Disimpulkan bahwa teknik pembekuan perlu dievaluasi, namun penggunaan gradien putih telur 15:30% dengan inkubasi 45 menit pada suhu 37°C merupakan protokol terbaik pada penelitian ini yang direkomendasikan untuk separasi seks domba dorper, karena mampu mempertahankan integritas membran dan daya hidup spermatozoa secara maksimal baik pada fase cair maupun beku.

Kata-kata kunci: separasi seks; domba dorper; putih telur; gradient konsentrasi; waktu inkubasi

INTRODUCTION

The continuously increasing demand for meat necessitates a corresponding increase in livestock populations to serve as a source of meat production. One such livestock is the sheep. The Dorper sheep, a crossbreed between the Dorset Horn from Southwest England and the Blackhead Persian from Iran, is an excellent meat-producing breed, capable of reaching a weight of up to 36 kg at 3.5 to 4 months of age (Sholikhah *et al., 2021*). Dorper sheep exhibit high adaptability and can thrive in hot climates like that of Indonesia. They are characterized by a round body and a black head.

In South Africa, the Dorper is specialized for meat production due to its ease of maintenance and breeding. To effectively utilize Dorper sheep for producing either breeding stock or meat-producing males, it is necessary to obtain the desired sex, females as potential dams and males for meat production.

Natural mating in livestock results in offspring with a 50:50 male-to-female sex ratio. This may not align with the breeder's expectations for a specific sex. Reproductive technologies, such as sexing or the separation of male and female spermatozoa, can be employed to produce offspring of a desired sex. Sexing is a biotechnological technique used to increase the population of a specific gender in animals by enhancing the ratio of sperm carrying either the X or Y chromo-

some (Solihati, 2017). The sexing technique can also be performed using egg white albumen. This method is based on the motility difference between X and Y spermatozoa, which arises from differences in mass and size; Y-sperm are smaller than X-sperm. Consequently, Y-sperm exhibit faster movement and higher penetration capability into solutions like egg albumen. Egg white, or albumen, is an egg component that acts as an antibacterial agent and a buffer to maintain the physical and chemical characteristics of the egg. The albumen used for sex separation is rich in albumin and has a composition of approximately 87% water. Its main solid component is protein at about 12%, supplemented by small amounts of glucose (0.4%), fat (0.3%) and mineral salts (0.3%). Furthermore, egg white contains various other functional proteins, enzyme inhibitors, antibacterial compounds and various vitamins and minerals in a bound form (Susilawati, 2014). Takdir *et al*. (2017) explained that egg white can be easily prepared in various concentrations to meet the principles of the sexing method, making it a viable alternative medium for separating X and Y spermatozoa. The high protein content in egg white also serves as an energy source for spermatozoa during the separation process.

However, current egg-white sexing protocols often result in variable success rates. A significant research gap remains regarding the optimal balance between gradient density and incubation time specifically for Dorper rams. Previous studies have reported limitations, such as low sperm recovery rates when gradients are too dense, or reduced viability when incubation is prolonged due to metabolic depletion (Anwar *et al.*, 2019). Furthermore, the interaction between these separation stressors and subsequent freezing (post-thaw survival) is critical, as sexed sperm are more susceptible to cold shock and oxidative stress. This study specifically examines gradient levels of 15:30% versus 20:35% and incubation times of 30-60 minutes. These gradients were chosen to test whether a slightly denser medium (20:35%) improves separation puri-ty without compromising motility compared to standard lower-density gradients. The incubation window of 30-60 minutes was selected to identify the critical point where separation yield is maximized before meta-bolic exhaustion occurs. Post-sexing semen quality can decline, leading to mortality and reduced motility. The longer the semen is treated, the more its quality will decrease. The novelty of this study lies in optimizing these parameters specifically for Dorper rams to mitigate the severe decline in quality typically seen during cryopreservation. Differences in the components and characteristics of spermatozoa among individuals may also affect the quality of semen produced after sexing and freezing. This study was aimed to evaluate the quality of the lower fraction (Y) of Dorper ram semen after sexing and freezing at different incubation times.

RESEARCH METHODS

The materials used in this study were semen from two 2-year-old Dorper rams with a body weight of 50 kg, located at the experimental farm of the Faculty of Animal Science, Jenderal Soedirman University. All procedures involving animals in this study were conducted in accordance with ethical standards for animal welfare. The first treatment factor was the egg white gradient as a sexing medium with different concentrations (15:30% and 20:35%), while the second treatment factor was the effect of incub-ation time (30, 45 and 60 minutes). This experimental study used a 2 x 3 factorial Randomized Block Design (RBD), data were collected from four replications based on collection frequencies. Semen samples were processed individually from each ram without pooling; each replication represented a single collection from an individual ram to account for individual variability in sperm characteristics.

The experimental treatments consisted of six combinations derived from two egg white concentration gradients (15:30% and 20:35%) and three incubation durations (30, 45 and 60 minutes). Specifically, the 15:30% gradient was subjected to incubation

times of 30 minutes (g_1t_1), 45 minutes (g_1t_2), and 60 minutes (g_1t_3). Similarly, the 20:35% gradient was tested with incubation times of 30 minutes (g_2t_1), 45 minutes (g_2t_2), and 60 minutes (g_2t_3).

Extender Preparation

The Tris-egg yolk extender was prepared by mixing 1.6 g of Tris-aminomethane, 0.9 mL of citric acid, 1.4 g of lactose and 2.5 g of trehalose in an Erlenmeyer flask. Then, 80 mL of distilled water was added and stirred until homogeneous. Antibiotics (0.25 mg penicillin and 1 mg streptomycin) were added while stirring, followed by 20 mL of egg yolk, and stirred until homogeneous. The final osmolality of the extender was adjusted to approximately 310 mOsm/kg

Sex Separation Procedure

Egg white gradients were prepared by diluting egg white with Phosphate Buffered Saline (PBS). The specific gradient levels (15:30% and 20:35%) were selected based on preliminary trials and literature suggesting that gradients below 10% do not filter effectively, while those above 40% severely impede yield (Kusumawati *et al.*, 2019). The lower fraction collected after incubation is assumed to be enriched with Y-bearing spermatozoa, as validated by previous studies demonstrating that smaller, faster Y-sperm penetrate albumin gradients more rapidly than X-sperm (Susilawati, 2014; Takdir *et al.*, 2017).

The egg white solution as a separation medium was prepared by mixing PBS as a diluent at proportions adjusted to the required concentrations: 15% = 0.9 mL egg white + 5.1 mL PBS; 20% = 1.2 mL egg white + 4.8 mL PBS; 30% = 1.8 mL egg white + 4.2 mL PBS; 35% = 2.1 mL egg white + 3.9 mL PBS. The sex separation medium was prepared by layering the egg white solutions of different concentrations (2 mL each) into a centrifuge tube according to the treatment, starting with the highest concentration. One mL of semen was placed on top of the egg white gradient. The centrifuge tubes containing semen and egg white solution were incubated in a water bath at 37°C for 30, 45 and 60 minutes. The upper and lower fractions of the egg white solution were separated into different centrifuge tubes, taking the upper fraction first to avoid mixing. The separated egg white solutions were centrifuged at 1500 rpm for five minutes. The resulting pellet was collected using a micropipette and diluted with 1 mL of Tris-egg yolk extender for each treatment. The diluted lower fraction (Y) spermatozoa were observed for motility and concentration using a microscope. Abnormality and viability were observed by preparing a smear with 2% eosin solution and examining it under a light microscope.

Semen Freezing Procedure

After dilution, the semen was placed in a sealed centrifuge tube and put into a beaker of water. The initial water temperature for cooling was 37°C, matching the fresh semen incubation temperature, and then placed in a refrigerator until it reached 5°C. After a four hour equilibration period, the tube containing semen was moved into a styrofoam box containing liquid nitrogen (N_2) at -110°C. The temperature was gradually decreased from 5°C to -110°C to -120°C by placing the tube 9 cm above the N_2 liquid surface at -110°C to -120°C for nine minutes. The tube was then placed approximately 2 cm above the liquid nitrogen surface for 9-10 minutes until the tube reached -140°C. The semen was then immersed in liquid N_2 at -196°C and stored.

Data Analysis

Data from each variable were tabulated and analyzed using Analysis of Variance. If the treatment had a significant effect on the measured variable ($P<0.05$), the analysis was continued with Duncan's Multiple Range Test using software application SPSS.

RESULT AND DISCUSSION

The sex separation process began with the examination of fresh semen to assess its suitability for further processing. The macroscopic and microscopic evaluation results of fresh semen were presented in Table 1.

Tabel 1. Macroscopic and microscopic evaluation of fresh semen

No	Evaluasi	Rata-rata
1	Volume (mL)	1,55 ± 0,11
2	Consistency	Thick
3	Color	Yellowish white
4	Odor	Characteristis
5	Ph	6,71 ± 0,05
6	Concentration (10^7/mL)	352 ± 22,85
7	Mass activity	2,5 ± 0,5
8	Motility (%)	82,50 ± 0,5
9	Viability (%)	85,25 ± 2,28
10	Abnormality (%)	5,25 ± 0,83

The macroscopic examination of Dorper ram semen showed a volume of 1.55 mL, thick consistency, yellowish-white color, characterristic odor and a pH of 6.71. The semen volume was lower than that reported by Sumaryadi *et al*. (2025) (2 mL), but the pH, consistency, odor and color were similar. The pH value was within the normal range for sheep, which is 6.4–7.8 (Garner and Hafez, 2000).

The microscopic examination revealed a concentration of 352 x 10^7 cells/mL. This concentration is higher than that reported by Nubatonis *et al*. (2022) in thin-tailed sheep (294 x 10^7/mL) and Sumaryadi *et al*. (2025) in Dorper sheep (249 x 10^7/mL). According to Sari *et al*. (2020), the average fresh semen concentration in Garut sheep is 2–3 billion cells/mL, while Susilawati (2014) states that the normal concentration for sheep semen is 3.5–6,0 billion cells/mL. The mass activity and individual motility were 2.5 and 82.5%, respectively. According to the Indonesian National Standard (SNI, 2014), the minimum motility requirement for fresh goat and sheep semen for freezing is above 70%. The fresh semen motility was considered good. The abnormality and viability were 5.25% and 85.25%, respectively. According to Garner and Hafez (2000), the maximum acceptable abnormality for good quality sheep semen is under 20%. The fresh semen abnormality was low, making the semen suitable for the next stage.

Post-Sexing Semen Quality

Statistical analysis showed that incubation time had a significant effect ($P<0.05$) on motility, abnormality, viability and concentration. The concentration gradient had a significant effect ($P<0.05$) on sperm concentration, as presented in Table 2.

Motility. Sex separation using an egg white gradient reduced sperm motility. The average post-sexing motility varied across treatments. This difference is likely due to sperm capacitation, a series of final biochemical changes spermatozoa undergo to become capable of fertilization. This process occurs when spermatozoa are in the medium. In this study, the 30 minute incubation period is thought to be the initial adaptation phase where most sperm are still motile but have not reached peak metabolic activity. The 45-minute incubation is considered the optimal time, where spermatozoa reach their metabolic peak and have successfully achieved a capacitated state. The concentration gradient did not significantly affect motility, but numerically, the 15:30% gradient (g_1) performed better than the 20:35% gradient (g_2). This suggests that a steeper concentration gradient requires more energy for spermatozoa to traverse, leading to a greater decrease in motility. Kusumawati *et al*. (2019) reported 50% motility using three concentrations of egg white sedimentation gradient (10%, 30%, 50%) with a 20-minute incubation time.

Abnormality. Post-sexing abnormality increased in all treatments compared to the fresh semen abnormality of 5.25%. Sex separation using an egg white gradient increased sperm abnormality.

Table 2. Quality evaluation of lower fraction (y) spermatozoa post-sexing

Variable	Gradient Concentration	Incubation Time			Mean
		30 minutes	45 minutes	60 minutes	
Motility (%)	15:30%	50.00±3.54	51.25±2.17	43.75±7.40	48.33±16.07[a]
	20:35%	47.50±2.17	48.75±3.54	40.00±3.54	45.42±18.92[a]
	Mean	**48.75±1.25[b]**	**50.00±1.25[b]**	**41.88±1.88[a]**	**46.87±2.06**
Abnormality (%)	15:30%	8.50±1.12	9.38±1.29	10.13±2.01	9.34±0.66[a]
	20:35%	8.63±0.96	9.50±1.22	10.75±1.03	9.63±0.87[a]
	Mean	**8.57±0.06[a]**	**9.44±0.06[ab]**	**10.44±0.31[b]**	**9.48±0.20**
Viability (%)	15:30%	54.13±3.97	53.88±2.41	44.88±6.86	50.96±5.26[a]
	20:35%	52.13±3.65	49.63±3.38	40.75±2.93	47.50±5.97[a]
	Mean	**53.12±4.21[b]**	**51.75±3.87[b]**	**42.81±6.05[a]**	**49.23±2.44**
Concentration (10^7/sel/mL)	15:30%	22.75±1.48	26.63±4.35	32.63±4.13	27.33±5.64[b]
	20:35%	13.25±1.48	18.75±5.03	25.63±2.46	19.20±6.63[a]
	Mean	**18.00±5.31[a]**	**22.68±6.55[b]**	**29.12±5.21[c]**	**23.26±6.01**

Note: Superscripts [a,b,c] in the same column indicate a significant difference ($P \le 0.05$)

Tabel 3. Quality evaluation of lower fraction (y) spermatozoa post-thawing

Variable	Gradient Concentration	Incubation Time			Mean
		30 minutes	45 minutes	60 minutes	
Motility (%)	15:30%	7.50±2.50	12.50±2.50	5.00±3.54	8.33±3.12[b]
	20:35%	6.25±2.17	8.75±2.17	3.75±2.17	6.25±2.04[a]
	Mean	**6.88±2.5[b]**	**10.63±7.5[c]**	**4.38±2.5[a]**	**7.29±1.47**
Abnormality (%)	15:30%	12.13±0.86	11.56±1.18	12.69±2.22	12.13±0.46[a]
	20:35%	12.18±1.51	12.93±1.41	14.53±1.48	13.21±0.98[a]
	Mean	**12.16±0.02[a]**	**12.25±0.68[a]**	**13.61±0.92[a]**	**12.6±0.76**
Viability (%)	15:30%	9.00±2.26	14.25±2.14	6.25±2.88	9.83±4.30[b]
	20:35%	7.00±1.84	10.13±1.56	4.63±1.56	7.25±2.98[a]
	Mean	**8.00±2.44[b]**	**12.18±3.08[c]**	**5.43±3.85[a]**	**8.54±1.82**
Concentration (10^7/sel/mL)	15:30%	4.80±0.25	5.40±0.53	6.10±0.74	5.43±0.79[a]
	20:35%	3.28±0.93	4.98±0.93	6.00±0.64	4.75±1.46[a]
	Mean	**4.03±1.09[a]**	**5.18±0.84[b]**	**6.05±0.74[c]**	**5.09±0.48**

Note: Superscripts [a,b,c] in the same column indicate a significant difference ($P \le 0.05$)

According to Sudarma *et al.* (2014), the albumin medium does not affect sperm morphology; the increase in abnormality is likely due to handling from collection to post-sexing evaluation. The abnormalities found were generally secondary, such as detached and bent tails. According to Ama *et al.* (2017), increased abnormality post-sexing can be caused by the spermatozoa being outside the body and separated from seminal plasma, as well as friction between the medium and the sperm membrane. Centrifugation during the post-sexing washing step can also contribute to increased abnormality. The abnormality rates in all treatments in this study were under 20%, making the semen suitable for further use. Abnormality values increased with longer incubation times.

Viability. Sperm viability was assessed using eosin staining, where dead sperm absorb the stain and live sperm do not (Sudarma *et al.*, 2014). The results were lower than those reported by Susilawati (2014) for sexed Etawah (Jamnapari) crossbreed goat semen, which was 70.39 ± 10.92% for Y spermatozoa. Viability is positively correlated with motility, and the percentage of viable sperm is always higher than motile sperm because viability assessment does not consider movement. Egg white contains lysozyme, an enzyme with strong antibacterial properties. Lysozyme may help select against dead or weak spermatozoa, indirectly increasing the percentage of desired spermatozoa in the fraction (Susilawati, 2014). The sexing process reduced sperm viability. Viability was similar at 30 and 45 minutes of incubation but decreased at 60 minutes, indicating that longer incubation affects sperm survival. The ideal incubation time for viability in this study was 30–45 minutes. Prolonged incubation can cause spermatozoa to deplete their energy reserves, reducing survival, whereas a too-short incubation time may not allow for optimal sperm concentration to be achieved. Viability at 30 minutes was slightly higher than at 45 minutes, in contrast to motility, which was higher at 45 minutes. This suggests that at 30 minutes, oxidative damage was still low, but the live sperm had not yet reached peak activation. In contrast, at 45 minutes, although viability was slightly lower, the live sperm had reached peak activation, resulting in better motility.

Concentration. Post-sexing concentration decreased in all treatments compared to fresh semen. This reduction is due to the separation process, which alters the natural 50:50 ratio of spermatozoa as they move from one medium to another. The results show that longer incubation times increased sperm concentration. The principle of progressive motility allows motile sperm sufficient time to move to the lower, desired fraction during longer incubation. However, concentration was negatively correlated with motility, which decreased with longer incubation due to energy depletion and reduced survival. The concentration gradient factor (g) significantly affected sperm concentration. The 15:30% gradient yielded better results than the 20:35% gradient. A steeper gradient makes it more difficult for spermatozoa to pass through, requiring more time. The 20:35% gradient was deemed less effecttive as it yielded fewer spermatozoa. According to Madrigali *et al.* (2021), a concentration of 200 million sperm per dose is used for Artificial Insemination (AI) in sheep with fresh semen. The g_2t_1 and g_2t_2 treat-ments had concentrations under 200 million, but concentration is not the sole criterion for AI; other factors like motility and abnor-mality are also important.

Post-Thawing Semen Quality

Statistical analysis showed that both incubation time and concentration gradient had a significant effect ($P<0.05$) on post-thaw motility, viability and concentration of the sperm, as presented in Table 3.

Motility. Post-thaw motility showed a significant decrease across all treatments compared to post-sexing motility. The resulting motility was very low and not suitable for AI. According to the Indonesian National Standard (SNI, 2014), the minimum post-thaw motility for AI is 40%. According to

Janur *et al*. (2015), sudden temperature changes can cause sperm death. During freezing and thawing, sperm cells experience stress from osmotic changes in their surroundings. This stress can damage the lipid and protein structure of the sperm's outer layer, destabilizing it and making it difficult for the cell to maintain internal osmotic balance. The suboptimal freezing process is a suspected cause of the low motility. The use of a styrofoam box for freezing and the regulation of height and evaporation time are likely contributing factors. Pre-freeze motility is a key determinant of post-thaw motility; lower pre-freeze motility will result in even lower post-thaw motility. The incubation time during sexing plays a crucial role. At 30 minutes, spermatozoa may not have reached optimal capacitation, leading to incomplete interaction with the extender. At 45 minutes, spermatozoa reached an optimal point for interacting with the extender. It is also suspected that at 45 minutes, the spermatozoa had not yet experienced fragility or adenosine triphosphate (ATP) depletion, as seen by the decline in motility at 60 minutes, both post-sexing and post-thawing. A steeper concentration gradient required more energy, as reflected in the post-sexing motility results for the 20:35% gradient. According to Susilawati (2014), motility reduction can occur because separated spermatozoa have undergone treatments that require significant energy to restore physiological conditions, ultimately causing motility to drop or leading to cell death.

Abnormality. Post-thaw sperm abnormality increased compared to post-sexing levels in all treatments. The highest abnormality was in the 20:35% gradient with a 60-minute incubation (g_2t_3) at 14.53 ± 1.48%. The post-thaw abnormality in all treatments was under 20%, making it acceptable for AI. The highest abnormality rates were observed with the 20:35% medium (g_2) (13.21 ± 0.98%) and the 60-minute incubation (t_3) (13.61 ± 0.92%). Increased abnormality can be caused by factors such as the cryopreservation process, thawing, the cryoprotectant used and cold shock. According to Hughes and Da Silva (2023), rapid temperature changes during freezing can cause irreversible damage to the sperm membrane, a phenomenon known as cold shock.

Viability. Post-thaw sperm viability significantly decreased compared to post-sexing levels. The freezing process can cause cold shock, osmotic stress and ice crystal formation. Pre-freeze viability is a determining factor for post-thaw viability. Longer incubation times during sexing resulted in lower pre-freeze viability. The 45-minute incubation had higher viability than the 30-minute incubation post-thawing. This suggests that at 45 minutes, the sperm membrane had undergone reorganization or capacitation, making the membrane more permeable and able to interact optimally with the extender. According to Aires (2021), Low-Density Lipoprotein (LDL) in egg yolk provides lipids to maintain and enhance sperm membrane elasticity during drastic temperature changes. Anwar *et al.* (2019) stated that excessively long incubation times can lead to increased damage to sperm cells, thus lowering their quality. A steeper concentration gradient required more energy, as shown by the post-sexing viability results for the 20:35% gradient.

Concentration. Post-thaw spermatozoa are those that have been sex-separated and then packaged into 0.25 mL straws for AI. According to SNI (2014), one AI dose requires above 50 million sperm. In this study, treatments g_1t_2, g_1t_3 and g_2t_3 met this concentration requirement. Factors affecting post-thaw sperm count include insufficient semen volume loaded into the straw and excessive extender addition during processsing. Semen with concentrations below the SNI standard can still be used for AI by administering a double dose to meet the required sperm count. The 60-minute incubation yielded the highest concentration, but it should be noted that concentration is negatively correlated with the desired percentage of Y spermatozoa. Therefore, the 45-minute incubation is considered more ideal for sexing when considering motility, viability, abnormality and the expected percent-

tage of Y sperm. The 20:35% concentration gradient consistently yielded lower sperm concentrations across all incubation times compared to the 15:30% gradient. This suggests that Dorper ram semen requires a concentration gradient of under 35% and an incubation time of under 60 minutes to achieve an ideal concentration while maintaining other quality parameters.

CONCLUSION

Simultaneously, the egg white medium concentration gradient and incubation time affect the quality of the lower fraction (Y) spermatozoa, including motility, abnormality, viability and concentration, both post-sexing and post-thawing. The best motility and viability for sexed Dorper ram semen, in both liquid and frozen-thawed states, were achieved with a 15:30% egg white medium concentration and a 45-minute incubation time at 37°C.

SUGGESTION

Environmental conditions and temperature must be carefully managed during each treatment stage, including sex separation, incubation, equilibration and post-thawing. The best method to produce high-quality Y spermatozoa is to perform sex separation using a 15:30% egg white medium gradient incubated for 45 minutes. In cases of low sperm count, a double insemination dose can be administered to achieve the desired lambing targets.

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