Quality and Proportion of Spermatozoa in the Percoll Density Gradient Centrifugation Sexing Method at Different Gradients using AndroMed® Diluent

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Abstract. The utilization of sexing technology is an effort made to improve the efficiency of livestock farming, which was created to predict the sex of the calves born so that it can be adjusted to the objectives of the farm. This study aims to analyze the quality and proportion of sexed spermatozoa using the percoll density centrifugation method at different gradients with AndroMed® diluent. The research material used was fresh semen from Belgian Blue Bull aged six years with a body weight of 600 kg and fresh semen quality motility ≥70%. This research is a laboratory experiment with two treatments and ten replicates. The treatments in the study were T0: Ten Gradient density percoll + AndroMed® and T1: Five Gradient density percoll + AndroMed®. The research analysis used a dependent t-test. The statistical analysis showed a significant difference ($P<0.05$) in treating ten gradients with five gradients in each layer regarding individual motility, viability, abnormality, and total motile spermatozoa. At the same time, in concentration, there was no difference ($P>0.05$) in the treatment of 10 gradients with five gradients. The average results on individual motility, abnormality, concentration, and total motile spermatozoa showed treatment of 10 gradients better than treatment of 5 gradients in each layer. At the same time, the variable viability showed that the gradient treatment is better than the five-gradient treatment. Proportion of spermatozoa in the Upper Layer T0 X (23.4%), Y (76.8%) and T1 X (16.9%), Y (83.1%). The proportion of Spermatozoa in the Bottom Layer was T0 X (84.1%), Y (15.9%), and T1 X (83%), Y (17%), respectively. In conclusion, sexing spermatozoa with the Percoll 10 and 5 Gradient Density Centrifugation method can separate X and Y spermatozoa.

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1 Introduction

The population of Indonesian beef cattle between 2018 and 2022 exhibited a stagnation trend. In 2018, the country's beef cattle population stood at 16,432,945 head. It showed a positive increase over the years, reaching 17,977,214 heads in 2021 and further growing to 18,610,148 heads in 2022. Despite these numbers, local breeders have struggled to meet domestic demand for beef, leading to substantial imports. In 2021 Indonesia imported 214,658.11 tons of beef to bridge the supply gap [1].

Additionally, the nation faced challenges in fulfilling its domestic milk requirements, importing 541,897.06 tons of milk in 2021 [1]. Biotechnological advancements, particularly in livestock reproduction, offer promising solutions to address these issues and enhance livestock productivity. One such innovation is spermatozoa sexing technology, which enables the prediction of offspring gender, aiding targeted animal husbandry practices.

The Percoll density gradient centrifugation method is highly effective in sexing spermatozoa, separating X and Y sperm based on differences in weight and head size [2]. However, it is crucial to note that this method can impact sperm quality. The sexing process can cause membrane damage, leading to changes in sperm motility and viability. Moreover, recent research by [3] indicates that this method reduces motility and viability and decreases sperm concentration. This reduction occurs due to the intricate process involved: separation, gradient levels, centrifugation, dilution, and freezing.

In separating spermatozoa, utilizing non-toxic separating agents and diluents is imperative. AndroMed®, a diluent medium by Minitube, offers a safe solution for freezing bovine semen, devoid of egg yolk and fortified with a potent antibiotic formula, ensuring the viability of spermatozoa while maintaining high motility. Notably, AndroMed® guarantees impeccable biosecurity as it lacks ingredients sourced from animals. The current study aims to evaluate the quality and ratio of X and Y spermatozoa post-sexing, employing the Percoll density gradient centrifugation method with two different gradients, ten and five gradients.

2 Materials and Methods

2.1 Collection of Semen and Treatments

Semen was collected from a healthy six-year-old Belgian Blue Bull weighing 600 kg using an artificial vagina, ensuring a standard quality of individual semen samples of ≥70%. The collected semen was subjected to sex sorting using the Percoll density gradient centrifugation method. The samples were divided into four treatments: T0, employing ten densities per coll (ranging from 65% to 20% density, highest to lowest); and T1, utilizing five densities per coll (ranging from 60% to 20% density, highest to lowest). These methods were adapted from the work of Susilawati [4] with modifications. AndroMed® diluent mixed with aquabidest at a ratio of 1:4 was used as the extender, as per the methodology outlined by [4]

2.2 Sexing Procedure using Percoll Density Gradient Centrifugation Method

The sexing procedure utilizing the Percoll Density Gradient Centrifugation method, as outlined by [4], involves the creation of a Percoll density gradient using AndroMed® T0 diluent with ten gradients (ranging from 65% to 20%) and T1 diluent with five gradients (ranging from 60% to 20%). Each treatment consists of tubes with ten and five gradients, arranged from highest to lowest density, with each density level containing 0.5 ml for 10 and 1 ml for five gradients. Following the formation of the gradient, 1 ml of high-quality semen is added to the tubes for both ten and five gradients. The tubes are centrifuged at 2250 rpm

for 5 minutes, resulting in six layers. The top layer, containing seminal plasma, is removed, and the second layer, rich in Y spermatozoa, is identified. The bottom layer, abundant in X spermatozoa, is collected and transferred to a tube containing 3 ml of AndroMed®. This mixture is centrifuged at 1500 rpm for 5 minutes, and the supernatant is discarded, leaving behind 1-2 ml of sediment containing sorted spermatozoa.

2.3 Assessment of Sperm Quality After Sexing

The evaluation of sperm motility was conducted using a light microscope at 400X magnification. Viability and abnormality assessments were performed by placing a drop of semen on a warm glass slide and adding one drop of dual-staining eosin nigrosin solution. After gentle mixing, the slide was observed under the microscope at 400X magnification. Dead sperm cells absorbed the stain, while live sperm cells did not, and 200 sperm were examined in each sample, following the method outlined by [5]. Spermatozoa concentration was calculated using the Neubauer counting chamber, as described by [5] and [3]. The concentration was calculated using the formula provided by [3] as follows.

The number of sperm/ml = N x 5 x FP x 10.000

Where:

N : Average number of spermatozoa in chambers A and B
5 : Correction factor because only 5 out of 25 squares are counted
FP : Dilution factor (1:100)
10.000 : Depth of the Neubauer chamber (0.0001 ml/Neubauer chamber)

The total motile spermatozoa can be calculated by multiplying the percentage of individual sperm motility by the sperm concentration and the semen volume [7].

2.4 The Proportion between X and Y Spermatozoa

Spermatozoa morphometric measurements, including the length and width of the sperm head, were conducted using LC-Micro software. A total of 1000 spermatozoa in fresh semen samples were observed to establish the natural proportion (50:50). For each specific treatment, 100 spermatozoa were examined in each sample. Identification of X and Y spermatozoa was based on head size, where spermatozoa with heads larger than the average control size were identified as X spermatozoa. In comparison, those with smaller heads were classified as Y spermatozoa, following the methodology outlined by [8].

2.5 Data Analyses

In this study, two treatments were administered, each repeated ten times. Following the sexing process, semen quality data, encompassing parameters such as Individual Motility, Viability, Abnormality, Concentration, and Total Motile Spermatozoa, were subjected to statistical analysis using the paired t-test method.

3 Results and Discussion

3.1 Semen Quality Post Sexing

The effectiveness of sexing X and Y spermatozoa is evident in the sustained high quality of spermatozoa post-sexing, as well as the significant separation of spermatozoa ($P<0.05$) in terms of individual motility, viability, abnormalities, and total motile spermatozoa using both ten gradients and five gradients in each layer. Interestingly, no significant differences were

observed in the spermatozoa concentration variable (P>0.05). On average, results indicate that individual motility, abnormalities, concentration, and total motile spermatozoa using ten gradients are superior to those using five gradients. However, the average viability of spermatozoa using five gradients is higher than that of ten gradients, highlighting the nuanced differences in the quality of sexed spermatozoa based on gradient density.

Table 1. Semen quality post sexing

T	Layer	Motility (%)	Viability (%)	Abnormality (%)	Concentration (10^6)	Count of motile spermatozoa
10 Gradient	Top	71.7 ±4.8	86.58 ±1.8	4.01±1.7	228 ±58.4	160.2375 ±36.5
	Bottom	70.8 ±5.2	86.05 ±2.5	4.45±1.2	225.75 ±98.4	179.8125 ±68.2
5 Gradient	Top	45.98 ±14.5	92.96 ±8.8	8.13±3.2	219 ±104.6	96.799 ±47.1
	Bottom	55.9 ±9.6	93.26 ±7.9	8.45±14.5	217 ±130,1	122.045 ±730

The method of separating spermatozoa using Percoll density gradient centrifugation relies on the disparity in mass and size between X and Y spermatozoa [2]. Differences in the DNA mass of X and Y spermatozoa lead to variations in their weight and density [9]. Due to these differences, during centrifugation, X spermatozoa settle in the bottom layer, while Y spermatozoa remain in the top layer [3]. However, individual motility in both T0 and T1 decreased compared to the motility of fresh semen, suggesting that centrifugation impacts sperm motility negatively. Research by [10] indicated that centrifugation during the separation of X and Y spermatozoa can damage the cell membrane, affecting sperm function in fertilizing egg cells. Damaged membranes can harm internal cell structures, such as mitochondria, crucial for cellular respiration and energy production. Disrupted metabolic processes due to damaged cell membranes can impede sperm movement [11]. Notably, in this study, individual motility at T0 (Top 71.7±4.8%; Bottom 70.8±5.2%) was higher compared to T1 (Top 45.98±14.5%; Bottom 55.9±9.6%). This difference might be attributed to the thicker viscosity of the gradients in the Percoll density gradient centrifugation method using five gradients. This increased viscosity may require more energy for spermatozoa to penetrate the gradient, resulting in lower individual motility.

The viability of semen post-sexing at T0 (Top 86.58±1.8%; Bottom 86.05±2.5%) is notably lower in comparison to T1 (Top 92.96±8.8%; Bottom 93.26±7.9%). The average viability correlates with individual motility; however, T1 results indicate a higher viability than T0. Both upper and lower T1 layers are suspected to contain live spermatozoa that lack progressive movement. This aligns with [12] assertion that live spermatozoa may not necessarily be motile, whereas motile spermatozoa are undoubtedly alive. The abundance of live but non-motile or non-progressively moving spermatozoa results in a higher percentage of live spermatozoa than individual motility. Semen abnormalities following sexing at T0 (Top 4.01±1.7%; Bottom 4.45±1.2%) are superior in comparison to T1 (Top 8.13±3.2%; Bottom 8.45±4.5%). This disparity arises because spermatozoa in the upper and lower layers, subjected to sexing with five gradients, suffer membrane damage. [3] have added that the rotation during the centrifugation process causes internal spermatozoa collisions with tube walls and friction with the medium, leading to spermatozoa membrane damage. Such damage results in increased spermatozoa abnormalities [13].

The average spermatozoa concentration post-sexing at T0 reveals that in the lower layer, it is higher than the upper layer, measuring 228 ± 58.4x10^6/mL and 255.75 ± 98.4x10^6/mL, respectively. Conversely, at T1, the spermatozoa concentration in the upper layer is slightly higher than in the lower layer, registering 219±104.6x106/mL and 217±130.1x10^6/mL, respectively. The count of motile spermatozoa at T0 (Top 160.2375±36.5x10^6/mL; Bottom 179.8125±68.2x10^6/mL) is more significant compared to T1 (96.799±47.1x10^6/mL; Bottom 122.045±73.0x10^6/mL). This discrepancy is due to T1's low individual motility; [3] noted that higher individual motility and spermatozoa concentration increase total spermatozoa count. This finding aligns with [14] assertion that spermatozoa concentration and individual motility significantly influence the number of motile spermatozoa obtained.

3.2 Proportion of X and Y Spermatozoa after Sexing

Table 2. X and Y Spermatozoa Proportion

Treatment	Layer	X and Y spermatozoa proportion (%)	
		X	Y
10 Gradient	Top	23.4	76.8
	Bottom	84.1	15.9
5 Gradient	Top	16.9	83.1
	Bottom	83	17

The success of the sexing method can be seen from the proportion of X and Y spermatozoa produced. The proportion of X and Y spermatozoa after sexing can be seen in Table 2. The percentage of Y spermatozoa (Top) using five gradients is higher than that of ten gradients, 83.1% and 76.8%, respectively. Conversely, the percentage of X (Bottom) spermatozoa using ten gradients was higher than that of five gradients at 84.1% and 83%, respectively. Separation of X and Y spermatozoa by percoll density gradient centrifugation method using five gradients showed a better proportion of spermatozoa than ten gradients.

4 Conclusion

In summary, the findings of this study indicate that the quality of spermatozoa, encompassing individual motility, abnormalities, concentration, and total motile spermatozoa, is superior in the Percoll density gradient centrifugation sexing method employing ten gradients compared to five gradients. Both ten and five gradients were equally effective in separating X and Y spermatozoa proportions.

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